The Design and Application of a Small-Scale Corn Degerming Process for the Recovery of Transgenic Products from Corn Seeds

Thesis submitted for the degree of
Doctor of Engineering
in
Biochemical Engineering

by
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November 2004

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Abstract

This thesis presents the issues involved in designing a small-scale corn seed degerming process based on large-scale principals, its characterisation and optimisation using the method of statistical experimental design and analysis, and its application to the processing of different types of corn seed.

During the early stages of process development for the extraction and purification of recombinant proteins produced in genetically modified crops, scarce quantities of material limit the extent of process investigations at pilot-scale. The small-scale degerming process was therefore designed for the development of a process suitable for handling small quantities of transgenic corn. This process consisted of a small-scale degerming device, followed by a separation process consisting of roller-milling and sieving, capable of processing 50 seeds (approximately 12g). Successful degerming of transgenic corn seed separated oil- and protein-rich germ (waste stream) from the product-containing endosperm fraction (product stream) of the seed. Oil content and mass of the product and waste streams were used to assess and compare degerming quality between all seed types.

The degermer and separation process design were based on the principal operating factors identified in the large-scale operations used in corn dry milling. Both operating factors and material properties affected the quality of degerming. The factors which had the largest impact on degerming were the degermer disk clearance, roller-milling and seed moisture content. Maximum degerming and separation, consisting of product stream containing 32.5% seed oil in 90.2% seed mass, occurred at a seed moisture content of 21%, (w/w), a disc clearance of 9mm, and three stages of roller-milling.

Variation in quality between seed types was shown to affect degerming quality when operating the degermer under constant conditions. Five different types of seed were processed through the small-scale degermer. Two were transgenic, and three were non-transgenic, and one of these was additionally processed through a pilot-scale

Beall degermer. The small-scale corn degerming process was shown to separate seed fractions from all varieties of seeds processed. The greatest difference in quality of small-scale degerming between these different seed types was between the 'high quality' non-transgenic hybrid and either of the two transgenic seed types. Better degerming was achieved using the high-quality seed hybrid (detailed above) than the transgenic seed (84.2% seed mass, 46.8% seed oil) when processed using the optimised operating conditions.

The quality of degerming that could be achieved using the pilot-scale Beall and small-scale degermers was compared using the 'low quality' non-transgenic seeds, which were physically the most similar to the transgenic seeds. The small-scale degerming process was shown to separate a greater proportion of germ from the endosperm, represented by the high mass of lower oil content product stream (81.5% seed mass, 29.3% seed oil) than that seed which had been degermed using the pilot-scale Beall degermer (92.5% seed mass, 64.3% seed oil).

Acknowledgements

There are many people who have generously offered their help and support within university, during my industrial placement, and otherwise. I am grateful to Eli Keshavarz-Moore, my supervisor, for her guidance and encouragement throughout the project, and to Parviz Shamlou, my advisor, for his technical advice and his contagious enthusiasm. Members of the engineering workshop generously gave their time and knowledge for the manufacture of the equipment. In particular, I would like to thank Martin Town, Alan Craig and John Graham, whose patience was forgiving, and whose humour and company greatly enjoyed.

Through the collaboration between Epicyte Pharmaceutical and Lonza Biologics, liaisons with Scott Borneman, Julian Bonnerjea and John Birch were fruitful, and I am grateful to them for having created this research project and for generously offering help and advice whenever it was sought. During my placement at Epicyte, Michael Pauly, my advisor, and Tom Darlington, my host, were very supportive and helpful, and excellent company both within the workplace and otherwise. I would also like to express my gratitude to Von Kaster and Paul Bullock, of Garst Seed Company, for their interest in, and dedication to this project. The financial support of the Engineering and Physical Sciences Research Council was greatly appreciated, without which none of this would have been possible.

Last of all, but by no means least, I would like to express my sincerest gratitude to my family and friends, without whom, my sanity would have been lost. Through their practical interest, bountiful encouragement and unswerving belief, my parents have been the cornerstone in my education, and to whom I owe every success.

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Glossary

Beall

The name given to the one of the most commonly used industrial degerming devices, manufactured by the Beall Degerminator Company

DEF

Degerming Evaluation Factor

Degermer

The device used to mechanically remove germ from corn seed

Degerming

The process of germ removal from corn seed

Degermination

The old fashioned term for the act of removing germ from corn seed

Endosperm

The largest component of the seed, composed mostly of starch, and constituting approximately 85% by mass

Germ

The second major component of the seed, the embryo, composed of a high proportion of seed oils and protein, and constitutes as little as 10% by seed mass.

Hull

The outer layer of the seed, covering most of the seed surface. It has a high wax content, which prevents moisture transfer across the seed boundary.

Hybrid

The product of the crossing of two inbred crop varieties

Product stream

High-mass, low-oil content endosperm-rich output stream, consisting mostly of endosperm

Seed - high quality

These seeds produce amongst the highest yields per harvested acre, are approximately equally sized (0.24g) and shaped seeds, and well suited to Beall degerming

Seed - low quality

Plants produced by these seeds are generally weak, yield a fraction of the quantity of seeds per harvested acre compared to high quality seeds, are irregularly shaped and can be of a much wider range of sizes, making them unsuitable for Beall degerming.

Tempering

The procedure by which seed moisture content is raised by the addition of water. Sometimes called moisture conditioning.

Tip-cap

A part of the seed, which connects the seed to the cob, and the only point through which water passes to and from the seed

Transgene

A segment of recombinant DNA that is integrated stably into the germline of its host plant, and is transmissible to subsequent generations

Transgenic plant

A plant that is stably altered by the introduction of recombinant DNA through human intervention

Waste stream

Low-mass, high oil-content, germ-rich output stream consisting mostly of germ

Chapter 1 - Introduction

Many biopharmaceuticals are produced in recombinant systems, including cultured mammalian cells, bacteria, and fungi, and the demand for existing biopharmaceuticals, and for new therapeutic proteins discovered through genomic efforts, is expected to rise considerably. Alternative production methods are continuously sought which could cost-effectively ensure future availability of safe recombinant proteins. Transgenic plants provide an attractive expression vehicle, and the potential of these 'bioreactors' has been apparent for over a decade. With practical, economic and safety advantages over conventional systems, transgenic plant production systems have rapidly advanced and are likely to gain wider acceptance.

In 1989, Hiatt et al. was the first to report antibody expression in tobacco, which demonstrated that plants could assemble complex functional glycoproteins with several subunits. Since then, a remarkable diversity of recombinant proteins, including fully functional secretory antibodies (Ma et al., 1995), have been expressed in several important agronomic species of plant, including tobacco, tomato, corn, potato, banana, canola, soybeans and alfalfa. Plants which have traditionally been grown on a large-scale are particularly advantageous when large volumes of recombinant protein are required. In combination with the economically favourable scales of production, transgenic plants provide a means of production which might address the increasing demand for recombinant proteins.

Successful development of transgenic plants for the large-scale production of recombinant proteins depends upon the fusion of the existing, large-scale infrastructure, practices and processes, with the traditional methods of downstream processing. Whereas certain farming practices and standard protein purification techniques may be directly applicable to the processing of transgenic crops, some changes may be necessary at the point of transfer from agricultural processing to downstream processing. This thesis presents research into the application of existing agricultural processing methods for the production of a high quality feed for downstream purification.

1.1 Production of recombinant proteins

1.1.1 World production capacity

In a review of current mammalian cell capacities, and the number of therapeutic proteins in the production pipeline, Hood et al. predicted that demand would greatly exceed capacity by 2007 (Hood et al., 2002). At the time when this commenced, the rate of approval of new biopharmaceuticals was increasing at an astonishing rate, with 22 in 1999 and 32 in 2000. This increase was believed to be a result of the huge success of existing antibodies developed using recombinant DNA technologies (Larrick et al., 2002), in conjunction with the large increases in high-throughput systems and robotic screening of drugs.

Global capacity is distributed between contract manufacturers and pharmaceutical company in-house production. While contract manufacturers are close to capacity, the capacity of some pharmaceutical companies is split between that currently used for manufacture, and that held in reserve awaiting new approvals. Other pharmaceutical companies are waiting for approval of their drugs before building facilities. However, at costs of $200-$400 million per facility and 4-5 years to come online, it is unlikely that mammalian production facilities will meet the impending demands for manufacture (Hood et al., 2002).

Possible solutions to the predicted shortfall in capacity might lie in building entirely new facilities, expanding current production facilities, or in developing an entirely new production system. The construction of new mammalian cell processing facilities takes 4 to 5 years, requiring massive investment which very few companies may be willing to risk. Current processes are limited in scaling up production. Extra space would be required, which in some cases simply is not available. Since current processes are mostly running at maximum capacity, the products of an additional bioreactor would require extra capacity downstream. This extra processing would effectively be a form of scale-up involving multiple processes on the same site. Building into the existing process to incorporate the utilities would involve

considerable plant down time, which, if not made impossible through long term contractual obligations, would be an additional expense through lost revenue. Finally, and perhaps the most important aspect of production using traditional hybridoma systems, the high production costs and instability of murine hybridomas in long term cultures compounded to the previously widespread belief of the poor suitability of this system to current production requirements. More recently, however, significantly higher titres, in the range of 1-2g/L, have been achieved in optimised mammalian cell culture (Chadd and Chamow, 2001), thus reducing the advantage margin previously held by production in transgenic plants. Transgenic plants remain the most likely option for the large-scale production of the more complex proteins required in vast quantities, such as secretory monoclonal antibodies. This is because this production system enjoys favourable economies of scale without the high capital costs involved in the manufacture of an upstream processing plant constructed out of steel and cement. As a result, large volumes of complex proteins can be produced in plants at a fraction of the cost involved in culturing mammalian cells.

1.1.2 Antibody production in transgenic plants

Transgenic plants can be manufactured for the economical production of a wide range of recombinant proteins. Perhaps one the most successful applications of transgenic plants would be for the production of therapeutic proteins required in high doses, in order to fully capitalise on the favourable economies of scale. Therapeutic proteins, in particular, monoclonal antibodies, are required in greater abundance. These antibodies are of wide interest to the biotechnology community, since they are used as research agents in the diagnosis and therapy of important human diseases, for immunoaffinity purification, and as abzymes. Traditionally, these have been produced using hybridoma or microbial fermentation systems (Smith and Glick, 2000). However, the extent of their use has been severely impeded by poor hybridoma stability, limited yield and high costs. Using plants as a production system has the potential to cost-effectively produce vast quantities of monoclonal antibodies, which has been a major objective since the first antibodies were produced in plants.

Passive immunotherapy is one particularly intriguing and attractive application of plant produced antibodies, sometimes called plantibodies, in which secretory antibodies are applied to the mucosal surfaces of animals and humans (Ma and Hein, 1995). Secretory antibodies are the most abundant form of antibody in the human body, and their use in passive immunisation of the mucosal sites could be effective against bacteria, fungi and viruses (Giddings et al., 2000). Examples of such antibodies include those designed to fight cystic fibrosis and sexually transmitted diseases (Zeitlin et al., 1998), to prevent conception (Potera, 1999) and to prevent the formation of dental carries (Ma and Hein, 1995). Repeated large doses of antibody are required for topical passive immunotherapy, in order to overcome the losses inherent to the mucosal surfaces. Finally, as a result of each of the above conditions affecting large numbers of people, the market demand for these products is huge. Currently, it is not possible to manufacture these highly valuable proteins on the size of scale, and for the low costs, required. It is possible that transgenic plants might fulfil this requirement.

1.2 Transgenic plants

Molecular farming and molecular breeding are two different approaches to the production of transgenic plants with different applications. Whereas molecular farming utilises plants or cultured plant cells as bioreactors to produce a protein of interest for purification and further use (*'ex situ'* application), molecular breeding exploits transgenic protein expression that renders the plant of interest resistant against herbicides, insect and microbial attack (*'in situ'* application) (Franken et al., 1997). The focus of this study is on the *ex situ* application for the production of recombinant proteins using plants.

1.2.1 Pros and cons of plant based protein production

Transgenic plants are potentially one of the most economical systems for the large-scale production of recombinant proteins for industrial and pharmaceutical use. The production of recombinant proteins in plants has many practical and economical

advantages for generating diagnostic and medicinal recombinant proteins compared with recombinant microbes, transfected animal cell lines or transgenic animals. These include low cost of large scale production of biomass (section 1.2.10 and section 9.2.2), ease of scale-up, by increasing planted acreage, the availability of natural protein storage organs in seeds and tubers, and the use of established infrastructure, practices and processes for their efficient harvesting, transporting, storing and, to some extent, processing (Whitelam et al., 1993). Other advantages include the stable inheritance of the transgenic trait by self-fertilisation of the transgenic crop, the potential to compartmentalise recombinant proteins into different organelles for improved stability and a reduction in purification requirements (Goddijn and Pen, 1995), the possibility of oral administration of the product in food, and that plants do not serve as hosts for human pathogens, such as HIV, BSE and prions (Zhong et al., 1999).

The perceived disadvantages of producing recombinant proteins in plants are based on insufficient information on post-translational events, relatively low accumulation levels, lengthy production timelines, regulatory hurdles, and the lack of data on downstream processing (Kusnadi et al., 1998b). There is a large variation in each of these disadvantages, depending on a number of factors, such as the transformation technique and technology used, the selected target host system, complexity of product and the purity required. Some of these issues, and their impact on the success of transgenic plants as production systems for recombinant protein production, are tackled primarily from the perspective of producing recombinant proteins in corn, and are discussed in the following sections.

1.2.2 Choice of crop

Individual economic and species-specific factors play a large role in crop selection (Stoger et al., 2001). Crop selection is affected largely by the organ which will be harvested as the raw material source. Seeds offer many advantages over green and fleshy fruit tissues, such as the lower processing costs and not being subjected to the numerous restrictions imposed by production in a highly perishable material (section 1.2.2.a) (Delaney, 2002). Other important issues to consider include the ease of plant

transformation, proper post-translational processing, yield of product per unit biomass and the required product volumes and purity, host system properties, such as biomass yield, growth cycle and the option of product targeting, the ease of scale-up, and the availability and feasibility of applying exiting agricultural practices (Kusnadi et al., 1997). Traditionally, plant properties have been critical to the development timeline, since research and development had to wait for the products of each harvest. However, these timelines are constantly being challenged and shortened, not least by the use of transient expression systems (section 1.2.8.a) (Hood et al., 2002; Russell, 1999). Each plant must be considered on its individual merits. Previously, there was no consensus regarding the best species or tissue for commercial production of recombinant proteins (Daniell et al., 2001). However, maize (corn) has become the main commercial production crop for recombinant proteins, and is used for the production of recombinant antibodies and other technical and pharmaceutical enzymes (section 1.2.2.b) (Hood et al., 2002; Ma et al., 2003). Using the correct promoters, it is possible to target production to specific plant components (e.g. seeds or leaves), to specific organelles e.g endoplasmic reticulum (Russell, 1999), oil bodies (Moloney, 2002), or even to species-specific components such as the tobacco root hairs or leaves, or germ or endosperm of corn seeds. The facility for product targeting can have huge implications on the crop selected for production based.

1.2.2.a Production in green tissue

The major advantage of green tissue, such as tobacco and alfalfa, is the sheer productivity, with the possibility of supporting several crops, in the form of cuttings, per year. The annual biomass yields greatly exceed that available when using wheat, rice, or corn. Other advantages of tobacco include its relative ease of genetic manipulation, the potential for rapid scale-up owing to prolific seed production, and in the impending need to find alternate uses for this hazardous crop. There is great potential in protein manufacture in other green tissues, such as alfalfa, which, as a perennial crop, can become a stable, eternal source of recombinant molecules (Khoudi et al., 1998). However, just as it is important to ensure the stability of seed banks, it is equally important to ensure plant stability and that the same product is obtained consistently (section 10.2) (Miele, 1997).

The major disadvantages of production in leaves and fleshy fruits are the high perishable nature of these tissues and the complexity of the media from which the product is to be extracted. Leaves and fleshy fruits are dynamic structures where proteins are synthesised and degraded with a high turnover rate. Due to the likely instability of the protein in the harvested crop during senescence and dry down, processing costs are expected to be much greater than for seeds. These high costs are a result of the need for infrastructure to be developed for the immediate freezing, drying or processing of harvested leaves (Conrad and Fiedler, 1998; Russell, 1999). Although the processing facility might have to be located near to the growing fields minimise product losses after harvesting, it also allows for considerable flexibility in matching downstream processing with the harvesting season (Larrick et al., 2002).

The extraction and purification of recombinant products from green tissues, such as tobacco, is likely to be inefficient and expensive, due to the presence of chlorophyll, thylakoid membranes, and phenolic compounds, which may dramatically and irreversibly interact with proteins and alter their structure, and secondary metabolites such as neonicotine (anabasine) and nicotine (Giddings et al., 2000; Larrick et al., 2002). Although the high alklaoid content of tobacco has been reported to allow harvesting and transport without significant recombinant protein losses (Larrick et al., 2002), it is a toxic compound which must be separated from the product. Methods to eradicate these unwanted compounds include the identification and breeding of low-alkaloid varieties (Ma et al., 2003), for example, and in the application of membrane processes, such as tangential flow ultrafiltration and diafiltration (Larrick et al., 2004).

1.2.2.b Production in seeds

Using seeds for the production of therapeutic proteins has the potential to capture the full value of a plant-production system, including scaling, culturing, shipping, storage and processing. It is not surprising, therefore, that a cereal crop such as corn (maize) is now the main commercial production crop for recombinant proteins (Ma et al., 2003). Corn seed can be inventoried for year-round production (Nikolov and Hammes, 2002), offering a flexibility not seen with many other systems, such as

increasing output to suit market demands, and a freedom in the proximity of the growing fields to the processing site.

Soybean holds similar advantages as a production system for recombinant proteins over green tissues. Compared to corn, soybean could be regarded as a better production system as a result of the cheaper production costs of the same quantity of protein, and the benefits, from a regulatory standpoint, of the reduced risk of contamination through pollen flow since the plant is largely self-pollinating. However, corn still compares favourably by the production of the same quantity of recombinant protein as soybean in less area, it is easier to work with genetically, and faces fewer patent barriers to the commercial use than soybean (Delaney, 2002). Unlike tobacco, both soybean and corn, as food crops, face significant opposition due to concerns based on the potential for the transgenes to spread into crops grown for food purposes. The regulatory issues involved in the safe production of recombinant proteins in plants are discussed in Chapter 10.

From a crop handling point of view, seeds are one of the more appealing options for protein production. The possibility of applying the existing, extensive agricultural infrastructure, practices and processes directly to the processing of transgenic material (Hood et al., 1997; Stoger et al., 2002; Whitelam et al., 1993) holds many practical and economical advantages, as discussed in section 1.2.6 and section 9.3.2. Of these, large-scale corn processing, for the fractionation of the product rich component, is particularly interesting, and has not yet fully been applied to the processing of transgenic corn for recombinant protein recovery (section 1.3).

The major drawback of producing proteins in seeds is the lengthy development time scales required for the production of a transformed superior line. Lines differ greatly in their transformation efficiency. Often, the transformation of superior lines is too inefficient, and so low quality varieties are selected for transformation followed by breeding the transgene into the superior lines which hold greater agronomic value (Russell, 1999). A more detailed discussion of these timelines is given in section 1.2.7.

Whereas in tobacco, both phenolic and alkaloid compounds are expected to reduce downstream processing efficiency, the same is predicted in cereal grains resulting from the presence of high levels of lectins (Stoger et al., 2001). Also, the presence of starch and oil can adversely affect the processing efficiency of cereal crops, such as by increasing process stream viscosity thus raising pumping requirements, but have been reported to have no negative effect on ion-exchange or hydrophobic interaction chromatography (Kusnadi et al., 1997). The type, number and cost of chromatographic separations vary with the biochemical nature of the products and extract media from which they are separated. It is possible that different extraction media might affect these columns differently. Therefore, methods used to improve the quality of feed to the chromatography columns, and the reduction in the number of chromatographic operations, are both likely methods to successfully reduce downstream processing costs (Daniell et al., 2001).

It is possible to achieve high levels of purification using membrane separations, but these operations are highly susceptible to fouling, particularly by oils. Therefore efforts to reduce the oil content in the feed to membranes, present in ground whole seed extract, will at least reduce the rate of membrane fouling and thus further increase process efficiency, and at best present membranes as a viable alternative operation to chromatography in product purification. Methods to lower the processing stream oil content include the selection of a crop or a variety of crop which produces seed with low oil content, or to target accumulation to seed components which contain minimal quantities of oil, and separate this component mechanically prior to extraction. This latter method forms the basis of this research study.

1.2.3 Genetic modification of the host crop

Several methods exist for the transformation of plants for the stable introduction of transgenes into plants. Each method has its advantages and disadvantages, and the choice of transformation depends both local expertise and the responses and requirements of plant tissues of different species to basic elements of the transformation process (Lindsey, 1996). Transformation techniques include vector-mediated gene transfer systems, such as *Agrobacterium* and viral vectors, and vector-

free gene transfer, consisting of chemical, electrical and physical methods. The two most commonly used methods of transformation are *Agrobacterium* mediated transfection and particle bombardment, in which DNA-coated microprojectiles are accelerated into plant tissue. *Agrobacterium*-mediated transformation is possibly the most widely used and successful method (Franken et al., 1997). However, as a result of Intellectual Property Ownership (IPO), access to this technology is severely restricted.

Other methods, such as whiskering, electroporation and protoplast transformation have not so far successfully been used for molecular farming applications (Ma et al., 2003). Garst Seed Company (Slater, IA, USA), with whom Epicyte were collaborating for the production of transgenic seed, used whiskering technology for the transformation of corn (section 2.1.2.a). The transformation of plants using whiskering technology consists of the introduction of DNA into cells following the high speed penetration of long silicon carbide, needle-like crystals (whiskers), by vortexing the solution containing whiskers, plant cells and the recombinant DNA (Lindsey, 1996).

1.2.4 Engineering high levels of correctly assembled proteins

Product quality is measured in terms of stability, efficacy and safety. Plant stability, as well as product stability, is important for the large-scale production, in order to avoid large variations in antibody production in successive generations caused by segregation of genes during sexual reproduction. Transgenic plants use the same pathway as mammalian cells for the correct assembly and folding of stable, functional antibodies and other foreign proteins (Franken et al., 1997). Different glycosylation patterns between mammalian systems and plants could potentially result in the manufacture of immunogenic or even allergenic plant-produced proteins (Cabanes-Macheteau et al., 1999). Although this has been a major constraint for applications in human healthcare, methods have been proposed which could circumvent the problem, such as the removal of the glycosylation site(s) from the antibody molecule or by removing the glycan itself from the plant (Ma and Hein, 1995). More recently, developments in the regulation of genes to obtain typical 'human' glycosylation

patterns have been reported as a potential solution to this problem (Bakker et al., 2001; Larrick and Thomas, 2001), and have already been proven successful (Hood et al., 2002). Antibodies have been produced in plants which have been demonstrated to be equally stable, safe and effective as those produced using cultured mammalian cells (Zeitlin et al., 1998).

Using metabolic engineering, it is possible to increase product expression levels by the manipulation of the correct metabolic pathways. Incorporation of the correct promoters in the gene construct can target product accumulation to the storage organs of the plant, where they are not affected by plant metabolism and do not negatively affect plant growth. Many different factors of metabolic engineering may lead to unremarkable product expression levels, such as not using appropriate regulatory elements in the expression construct (Stoger et al., 2001), and not using codon-optimised constructs. However, it is beyond the scope of this research project to discuss the mechanics of achieving high-level expressing transgenic plants.

1.2.5 Expression levels

Previously, it was believed that the expression level of recombinant proteins in transgenic plants was directly related to the content of native proteins. Therefore, plants with a naturally high protein content would be more cost effective for the production of recombinant proteins, due to the reduction in the required quantity of crop grown and harvested, and in the reduction in processing volumes (Kusnadi et al., 1997). Under this assumption, it was believed that the production of antibodies in plants could be between 10-100 times cheaper than production using traditional systems. However, using the same method of transformation, Stoger *et al.* did not observe any dramatic differences in the amount of functional scFv in different plant systems (Stoger et al., 2001). Although more complex molecules, such as multi sub-unit proteins or proteins requiring extensive folding, may behave differently, these results show that high protein expression levels are more dependent upon the selection of the proper modification technique and metabolic engineering methods, rather than the protein content of the host system. Under these circumstances, the economics of production would shift away from high protein expressing plants, which could

potentially increase the complexity of the purification process. Instead, a more favourable production system, in terms of the product purity in protein extract, would consist of a system either low in native proteins, or one in which protein accumulation could be targeted to tissues relatively low in native proteins, and could also be separated relatively easily prior to protein extraction (section 1.2.6).

Other methods used for increasing protein expression levels include the selection of certain hybrid lines which can be transformed more efficiently (Delaney, 2002), utilising transient expression systems during transgenic seed development (section 1.2.8.a), using tissue-specific promoters (Conrad and Fiedler, 1998), and by controlling gene silencing (De Wilde et al., 2000). Some of these methods are briefly discussed in the sections to follow. More importantly, however, is the variability in expression levels achieved between plants and the modification techniques used. Large variations in the expression levels of recombinant proteins have been reported by different groups of researchers, ranging from 4.0% total soluble protein in tobacco seeds (Conrad and Fiedler, 1998) to 10% in the tobacco leaves, (Cramer et al., 1999), (6.8%) for corn seeds (Kusnadi et al., 1998b), and even higher (25% of total soluble protein) in the production of transplastomic tobacco plants (Maliga, 2002). Typically, the minimum product expression level believed to be suitable for processing is 1% of the total soluble protein{Epicyte Pharaceutical, Inc.).

1.2.6 Targeting accumulation

By targeting protein production to specific compartments or tissues of the plant, the product can be sequestered from rapidly degrading (Kusnadi et al., 1997) before and after harvesting. This is a particularly strong advantage of accumulation in the available natural protein storage organs such as seeds or tubers, in which the product can be stored relatively cheaply over a period of 1.5 years without degradation (Ramirez et al., 2000). Targeting is also beneficial to foreign protein production by obviating the potentially negative effects of the product caused by interference with plant metabolism, particularly when the product is toxic to the cell, resulting in adverse effects on plant physiology (Kusnadi et al., 1998a). This, in turn, may not only limit the product expression levels, but also reduce the range and quantity of

products that can be synthesised (Goddijn and Pen, 1995; Smith, 1996). From a processing perspective, benefits of targeting include separation of the product-rich component from components void of product, which serves to both separate large quantities of endogenous plant proteins from the product stream, which might reduce downstream processing complexity, and potentially provide revenue from waste material to defray processing costs (Kusnadi et al., 1998a; Nikolov and Hammes, 2002). Separation of the unwanted component increases the product concentration, reduces the process volume, pumping requirements, vessel sizes, and utility requirements, and could potentially reduce the number of the required downstream purification operations.

Product expression levels can vary enormously depending on the plant tissues or compartments into which production is targeted. Certain cellular compartments of the selected host system are more suitable to expressing foreign proteins than others. Expression systems that target antibodies to the endoplasmic reticulum (ER) (Artsaenko et al., 1995) and secretory pathway (Firek et al., 1993) afford some degree of protection from post-translational degradation, and thus higher accumulation levels of active antibody. Retention of scFv's in the ER, for example, resulted in maximal yields, from 1% to 6.8% total soluble protein, which was equivalent to an increase by 10- to 20-fold (Fiedler et al., 1997) and 100-fold (Schouten et al., 1996), respectively. Epicyte Pharmaceutical did not determine the localisation of antibody deposition. No ER retention signal was attached to the antibody, thus the presumption was that the antibody accumulated extracellularly. Full secretion of antibody into the extracelular space, however, has been shown to have a major detrimental effect on antibody yield. This decrease in yield was thought to have been a result of the increased exposure of the antibody to the principal sites of protease activity (Sharp and Doran, 2001).

The methods used to target production also affect the levels of accumulation. Some promoters have been shown to be more successful than others in increasing product expression levels. For example, Hood *et. al.* reported that using a seed specific promoter could lead to a four-fold increase in expression, but using the constitutive ubiquitin promoter increased product expression by 10-20 fold (Hood et al., 1997).

Therefore, it is crucially important to ensure that the correct promoter is used to target accumulation into the most suitable compartment of the selected host system.

Using corn as the host production system, production in the seeds is of paramount importance for the direct application of certain large-scale agricultural practises. Further product targeting to specific components of the seed, such as germ (embryo) or endosperm, provides for the added advantages discussed previously, by the application of existing agricultural processes, such as dry-milling for seed fractionation, which successfully separate the product-rich components from the rest of the seed. This concept is discussed in greater detail in section 1.3 and section 1.4.

1.2.7 Production timelines

Whereas cultured mammalian cells can quickly produce gram quantities of protein for characterisation and clinical trials, in some plant systems it can take at least a year to produce milligram quantities of protein for utilisation in animal studies. However, these timelines are constantly being challenged and shortened by, for example, the use of transient systems in the form of tobacco cell culture or transgenic leaf material for recombinant protein production in as little as 3-4 months (Hood et al., 2002). Even without transient production systems, 1-10mg of corn-produced protein can be produced in 8-9 months, with scale up to kilogram quantities in less than two years (Hood and Nikolov, 2002). In the selection of an appropriate host system, it is important to consider the investment required for initial set up, the ease and time involved in plant modification to reach desired expression levels, and the ease of large-scale plant production. These factors can widely differ between different transformation systems, due to variations arising from the time needed for selection and regeneration (Stoger et al., 2001).

1.2.8 Seed development

The general belief in the pharmaceutical industry is that it will take approximately one year to produce milligram quantities of protein from transgenic seeds for use in animal studies. Scale-up to kilogram quantities has been reported to take approximately 2 years (Hood et al., 2002; Stoger et al., 2002). Transgenic plant

technology is still in its infancy and is constantly improving, thus these timelines are expected to be reduced considerably as plant production companies work with pharmaceutical companies to develop mutually compatible timelines.

For the development of seed-based protein production in transgenic plants displaying good agronomic traits i.e. high-quality plants so called because they can produce equal quantities of biomass per acre as their commercially produced, non-transgenic counterparts, there may be no escaping long development time scales. These time scales are a result of several factors. First, the natural 3-month life cycle of corn from callus or seed germination to seed production is one factor which cannot be changed. Second, the selected modification technique used might not yield plants with acceptable expression levels. Breeding these plants with plants expressing higher levels of product is an approach commonly adopted to raise seed expression levels, but this method is very time consuming, since it relies on the plant growth cycle. Thirdly, certain modification techniques are only successful in achieving high expression levels by the transformation of low quality hybrids of seed. A hybrid of seed is produced only by the crossing of two inbred varieties of the crop. In order to improve the agronomic value of the low quality transformed crops, they must be cross pollinated with superior, high-quality plants e.g. inbred UU01 (section 2.1.1), which again is reliant on crop growth cycles.

1.2.8.a Transient expression systems

Transient expression systems can be valuable for improvements in expression levels, by accelerating gene design using transient assays (Voinnet, 2003), and by the manufacture of recombinant proteins within a short time frame, enabling the characterisation of the recombinant protein before the time-consuming manufacture of transgenic plants for large-scale, long-term, protein production (Fischer et al., 1999). Transient genes can be introduced into plants using biolistic methods, agroinfiltration or by the use of viral vectors. The proteins produced using these methods include complex heterologous proteins, which can be induced at an advanced developmental stage, thus avoiding the potentially negative effects of the product on the developing plant and can optimise the yield of the desired product. This also avoids the time-

consuming breeding programs to generate the complete proteins in agronomically valuable crops. Previously, agrobacterium-mediated infection was limited in its applications because of low expression levels and its transient nature, such that expression often disappeared after less than 5 days after inoculation. Recent advances in the suppression of gene silencing, however, have raised expression of a commercially relevant IgA by more than 50-fold, which have also persisted for much longer periods (up to 12 days) (Voinnet, 2003), thus adding to the advantages of this production system in the reduction of development timelines.

1.2.9 Production scale-up

The ease with which the production of recombinant proteins in transgenic plants can be scaled up is a major advantage of this production system. Favourable economies of scale are important in offsetting the initial start-up costs, and in producing recombinant proteins which are far cheaper than conventional production methods (Mison and Curling, 2000). The expenses involved in increasing the planted acreage of transgenic crop, additional to the low cost agricultural practices, result from the requirements of the regulatory agencies responsible for the production area (e.g. the Food and Drugs Administration (FDA) in the United States of America), to ensure safe and proper crop production. The regulatory issues involved in the large scale production of pharmaceutical transgenic crops are discussed in Chapter 10.

1.2.10 Cost reduction

Once minimum expression levels of 1.0mg protein per gram of tissue are obtained in plants exhibiting the high-quality agronomic traits of their non-transgenic counterparts, Mison and Curling demonstrated how unit costs of recombinant protein would continue to fall with increased production, from US$49.4/g for an annual output of 0.1 tons/year, to US$4.5/g for 100 tons/year (Mison and Curling, 2000). These cost savings are a result of the reduced significance of the major production costs at smaller-scales i.e. capital-related and labour costs, and an increase in the proportion of costs resulting from the large-scale production of low-cost biomass. A more detailed discussion of the economics of recombinant protein production in corn is provided in results Chapter 9.

1.2.11 Regulatory considerations and biosafety

Commercially produced transgenic plants have expressed input traits in commodity crops grown on a large-scale, and until recently, have only been approved for feed and food uses prior to commercialisation. New brands of transgenic crops have been modified for the production of high-value products not intended for food and feed uses. These crops containing foreign proteins must be treated differently to the modified commodity crops grown for food and feed uses. The myriad of established and emerging regulatory considerations are fundamentally different, leading to different methods of growing, handling, managing and processing (Chapter 10) (Emlay, 2002).

Transgenic crops are grown in much the same way as non-transgenic crops, but with a strong element of control imposed by the appropriate regulatory agency to the country in which they are grown, to prevent cross-pollination, contamination, and the introduction of transgenes into the food chain. Due to the great diversity in genetically modified crops and their uses, regulations are species and product specific, and therefore must be evaluated on a case by case basis. In addition to the technological and regulatory challenges of producing recombinant protein in plants to the required standards, advances in the widespread use of transgenic crops are hindered by opposition from environmentalists, and face unprecedented levels of public scrutiny. Plant biotechnology industry must now demonstrate to the wider audiences their complete commitment to, and ability to achieve, 100% containment and safe production of transgenic crops.

1.3 Corn Processing

Little is known about the adaptability of existing large-scale agricultural processes to the processing of transgenic material. In principal, the farming of transgenic corn, including planting, harvesting, transporting and storing, is expected to be very similar to the farming of non-transgenic seeds. In practice, however, it will be much more

carefully contained and monitored in order to meet regulatory requirements (Chapter 10). It is also believed that standard extraction and purification processes can be successfully applied for the recovery of recombinant proteins from transgenic plants. Fusion of existing, low cost agricultural or food processes into the processing of transgenic crops, for the preparation of the harvested transgenic material to provide the highest quality feed material to the extraction and purification processes, would maximise the cost saving potential available using this method of protein production.

To our knowledge, large-scale corn processes have not yet been applied to the processing of transgenic corn seed grown for pharmaceutical applications. Several studies have quantified the laboratory-scale extraction and purification of recombinant proteins produced in transgenic corn. Although there is a general consensus that fractionation of product compartmentalised seed could facilitate its processing, it has not been widely studied. One study reported the use of a small-scale degerming operation for the processing of transgenic corn seed, but scarce information was provided regarding this operation. Details of these studies using transgenic corn seed are provided in section 1.4.3 and section 1.4.4. It was therefore necessary to analyse the methods of corn seed fractionation used on the large scale, in order to manufacture a small-scale process which could achieve a similar output quality.

Several different corn processes exist, some of which are designed to manufacture very different products from corn seed. The degerming processes separate germ from endosperm, whereas the non-degerming processes grind seeds whole. For the processing of transgenic seed, in which the recombinant protein is targeted to one of the seed components, such as the endosperm, a degerming system is required. Two different types exist. These are wet-milling and dry-milling. Dry milling is the more suitable of the two for processing transgenic seed for several reasons. These are based on the advantages inherent to dry-processing and include a reduction in utility requirements and a simplification of the transportation of the process stream. The degerming operation within this process was of particular interest, since this is the first operation in the process which separates germ and endosperm, and is responsible for the separation of the greatest quantity of germ in one single step. Consequently, this operation was selected as the focus of research in this study.

It was important to keep the value of the corn processing operation within the context of its use, since the aims for transgenic seed processing are quite different to the large scale processing of regular corn seed. Whereas the major products of corn dry-millers are large fragments of endosperm (grits) for the production of, for example, corn flakes, transgenic corn dry-millers generally would not be concerned with the size of the separated endosperm fragments. At a point in the process after degerming, these endosperm fragments would be ground in preparation for product extraction. However, insofar as the production of an endosperm stream low in oil and native protein content is a requirement of both seed types, there is great value in transferring corn seed processing information to the processing of transgenic seed.

1.3.1 Selection of a corn degerming process

Of all of the corn processes, dry-milling with degerming is the most suitable for the milling of transgenic material. Having been developed for the production of a low-fat endosperm fraction and an oil-rich germ fraction, the benefits of fractionation of a transgenic seed with product targeted to the endosperm are immediately apparent. Approximately 80% of endosperm fragments produced using the Beall degermer in industrial dry-milling contain less than <0.8% (w/w) oil (Brekke, 1970). It is much more preferred than wet-milling corn seed. Wet-milling also successfully separates corn germ, but is likely to involve considerable product losses and damage during the steeping process, which involves soaking the seeds in acidic water for 22-50 hours at 52°C (Shandera et al., 1995). Also, wet-processing transgenic seed would incur significant additional utility costs, in the form of the vast quantities of water and power requirements for heating and pumping these extremely large volumes. Therefore, wet-milling fractionation methods were discarded in favour of the benefits of dry-milling.

It is important to consider not only the degree of success of corn seed degerming, but also how this is measured in terms of the target products. Milling objectives differ between processes, and just as the products of wet- and dry- milling are not identical, neither is it intended to achieve products identical to either of these methods of

degerming in the degerming of transgenic seed. Different types of degermer exist for the dry-milling degerming of corn seed, and the ones selected for large-scale processing depend upon the type of product sought e.g. large grits or whole germ. Therefore, the design of the small-scale degerming device involved the identification and selection of the most pertinent design features of these large-scale degermers, and their incorporation into the design of the small-scale degerming device for transgenic seed processing.

1.3.2 Corn milling objectives

Corn millers face the challenge of producing separated seed fragments of a consistently high quality from irregularly sized and shaped seeds from the same ears of corn, and from commercial seed types of different compositions (Brekke, 1970; Earle et al., 1946). The product quality of dry-milling degerming cannot be accurately predicted when processing a new type of seed, due to the large number of material and process variable factors involved. There is no fixed set of conditions known to successfully degerm all types of seed. Extensive variations between processes, such as in the use of different unit operations e.g. Beall and Entoleter degermers, and in the use of different operating conditions of same equipments, result in large overall variations between processes designed ultimately to achieve similar goals.

Modern degerming systems remove from corn seed practically all hull, germ and tip cap in the production of what is collectively known as prime products i.e. the grits, meal and flour, categorised according to particle size. The degerming devices could therefore more accurately be called degerming / dehulling devices, but for simplicity will be called degerming devices or degermers. A cross-section view of corn seed, illustrated in Figure 1.1, illustrates the relative positions of the major components within the seed. These components were distinguishable by colour. Floury endosperm was white, vitreous endosperm yellow, germ was cream, the tip cap was white on the inside and black on the outside, and the hull was a pale yellow. Details of the large number of product streams manufactured from the milling of large volumes of seed are reported elsewhere (Watson and Ramstad, 1987).

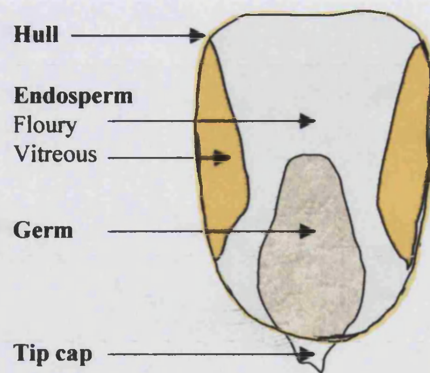


Figure 1.1 Basic corn seed structure

Grits and meal are generally low in fat content, consist mostly of the vitreous part of the endosperm, and are the most valuable products of the degerming process. Sometimes called cereal flaking grits or puffing grits, these endosperm fragments are more valuable when larger, which has enormous implications on the degerming process and selected operating conditions. Whereas increased abrasion and scouring may improve germ separation, for example, it does so at the expense of reducing grit particle size. Flour mostly consists of floury endosperm, and contains more fat and fibre than the grits and meal. The fractionation of oil-rich and fibrous material from endosperm results in products with a longer shelf-life, a greater variety of uses, and thus higher revenues than non-degermed products.

1.3.3 Process summary

The degerming dry-milling process is more properly called the tempering-degerming (TD) process, due to the controlled addition of moisture to corn seed prior to degerming. The major operations of the TD system are summarised in Figure 1.2. These process operations often vary between millers, and have been described elsewhere (Brekke, 1970). Since much of the dry-milling process is beyond the scope of this research, the major operations are briefly summarised in this section, in order to appreciate the role and value of the degerming operation.

Typically, seeds are stored at 12% moisture content to prevent spoilage. Prior to degerming, the seed is cleaned and moisture content is raised from 12% to

approximately 18-21%, by the addition of a controlled amount of liquid to a known mass of seed mixed in a rotary drum mixer. The final level of moisture achieved, and the method of moisture addition, can have a large impact on the quality of degerming. The methods and mechanics of moisture conditioning are discussed in greater detail in section 1.3.4.

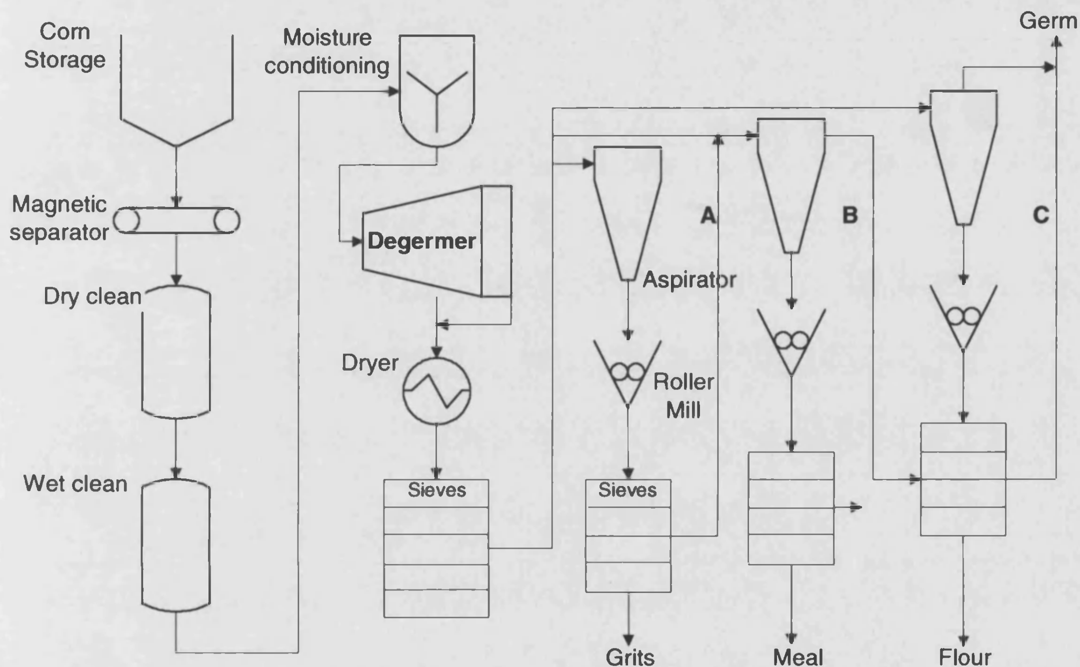


Figure 1.2 Simplified dry-milling process schematic

The best of the commercially available degermers alone cannot achieve complete separation of seed components, and thus follows a complex process of scrubbing and separation through operations including aspiration, roller-milling and sieving (Brekke, 1970). Conventional separation techniques such as gravity tables have mostly been replaced with more sophisticated operations, such as aspirators. However, the principles on which the separations are based, and quantity and quality of products, remain much the same.

Degermer output is often combined and treated by cooling and drying for the control of moisture content, and sized through a series of sieves of the following apertures: 5.6mm, 4.0mm, 2.8mm, 1.7mm, 0.71mm, and 0.0mm (fines). The upper sieves

contain mostly large grits and degermer recycle material, and the lower sieves contain mostly germ and hull and degermer fines. Separation of germ and endosperm retained in these sized fractions is achieved via capitalisation of the size and density differences, and of the different toughness and workability between the components. Due to the high oil content of germ, it is less dense than endosperm and more pliable, which helps it to withstand abrasion in both the degermer and roller-mill. The roller-mill has two functions. The first is to remove attached germ via a scraping action, created by the speed differential between rollers. The second is to roll flat the freed germ into larger particles. In this way, the larger germ particles can be separated from endosperm either by further sieving or by manipulation of the differences in density and aerodynamic properties.

Three different processing routes for degermed seed (A, B and C) are illustrated in the processing schematic in Figure 1.2. It represents the processing steps, recycle complexity, and the tendency toward separation of germ as the lighter fraction, separation of grits with just a small amount of processing, and flour with more extensive processing. Case A represents the recycle of some roller-milled fractions back into the aspirator for further germ-endosperm separation. Case B illustrates the combining of the light fraction of the aspirator, with other sieved fractions of corn seed for further processing. Case C represents the immediate separation of germ fragments via aspiration alone, and by processing without extensive recycling. Whereas some streams remain separate after just a small amount of processing, and consist of relatively pure endosperm fragments i.e. large grits or germ, a number of the fractions produced consist of less pure fractions, and these are often blended together according to protein, fat, starch and fibre content of the required products such as animal feed.

1.3.4 Moisture conditioning

Moisture conditioning, or tempering, as it is known in the industry, is regarded as a critical step in facilitating endosperm isolation from the other components of the corn seed. Numerous studies using the Beall degermer have indicated that the moisture content of corn before the degerming step has a greater effect on the output quality

than any variation in the operating conditions of the degermer itself. Furthermore, the moisture content of the degermer output has been shown to have a considerable impact on the final product quality. However, the important factor in this study was the degree of separation of oil from the endosperm fragments, whereas in large-scale Beall degerming it is often primarily the production of large grits of low oil content. Therefore, although seed moisture content was also an important parameter in small-scale degerming, the data provided by the research based on Beall degerming was limited in value, since the intentions of processing were quite different.

Effective moisture conditioning capitalises on the swelling stresses induced by the differential rate of water uptake between the different corn components. Water is added to the seeds, often held in stirring bins or rotary drums, either be spraying or soaking. Raising germ moisture increases its elasticity and pliability, which helps to resist breakage inside the degermer and enables flattening of the germ, without breakage, into a platelet during the roller-milling operation of subsequent processing (Brekke, 1970). Additionally, a short holding time with rapid moisture uptake maximises the germ swelling stresses (Shelef and Mohsenin, 1966), which facilitate effective germ separation (Mehra and Eckhoff, 1997). Rapid moisture uptake is dependent on the quantity of water readily available for the seeds to absorb through the tip cap (Henderson, 1952; Shelef and Mohsenin, 1966). Excessive holding time may allow for moisture equilibrium to be established within the seed, thus nullifying any initial benefit gained from germ swelling. Therefore, as now widely used in the large-scale dry-milling industry (Kent Rausch, University of Illinois at Urbana-Champaign, USA, personal communication), a short-duration tempering operation would be tested in the small-scale degerming process investigation, additional to the more conventional, long-duration tempering procedure.

A wide range of methods have been investigated and utilised in the moisture conditioning of corn to pre-determined moisture contents (Brekke, 1965; Brekke, 1966; Brekke, 1970; Hood et al., 1997; Mehra and Eckhoff, 1997; Peplinski et al., 1984). Some results suggest that a 24-hour, 3-stage procedure produces the greatest quantity and highest quality of the prime products (Brekke, 1970), whereas others have found that a reduced temper time to less than 30 minutes can achieve similar

results by making better use of the germ swelling stresses, thus enabling better separation of the corn components (Mehra and Eckhoff, 1997; Peplinski et al., 1984). Of these methods, the one favoured for transgenic seed degerming is that which facilitates good separation of the corn components with minimal time and expenditure. Since prime products, in the form of large grits, are not required in transgenic seed processing, degerming seeds of lower moisture content was the preferred option for several reasons. These include obviating the chance of product losses through leaching into the temper water, more rapid processing, reduced water requirements, and the potential decrease in drier load in subsequent dry-milling processes.

1.3.5 Degerming

Several different types of degermer, additional to the Beall degermer, have been used commercially in the tempering-degerming process. Amongst these are the Satake VBF degermer, Entoleter, granulator, disk mill and roller-mill. Two dry-degerming systems have also been developed, called the Miag process and the Ocrim process. However, these are less commonly used and more complex operations than some of the aforementioned methods used for the processing of tempered seed, and consequently will not be discussed any further

1.3.5.a Beall Degermer

The Beall degermer is the one of the most, if not the most widely used degerming device in the world (Kent Rausch, University of Illinois at Urbana-Champaign, personal communication). A large number of material and operating factors affect the quality of the degermer output, some of which change to suit the type of corn being processed. Variables include seed quality, moisture content, method of moisture addition, degermer feed head, rotor speed, rotor-stator clearance, screen size and tailgate back-pressure (Bess, 1971; Brekke et al., 1963; Brekke, 1965; Brekke, 1966; Henderson, 1952; Kirleis and Stroshine, 1990; Peplinski et al., 1984; Shelef and Mohsenin, 1966; Wichser, 1961).

The exact mechanism of germ-endosperm separation within the Beall degermer, consisting of germ removal and grit polishing, is not known, although it is believed to be the combination of several factors. It has been speculated to be primarily the result of seed-seed interaction (Brekke, 1970). Other contributory factors include the interaction between the rotor and seeds, and the shearing and scraping of grits, which removes hull and germ, during passage through the screen perforations. Based on the high-quality germ-endosperm separation capabilities, the extensive base of research and its popularity amongst dry-millers, the operating characteristics of the Beall degermer were analysed most closely for the identification of the major operating factors for incorporation into the design of the small-scale degermer, in order to achieve similar qualities of degerming on the small-scale.

Design

The Beall Degerminator (Bess, 1971), manufactured by Beall Degerminator Company (Decatur, IL, USA) is essentially a conically shaped corn mill capable of degerming and dehulling corn seed. It consists of a horizontally mounted, rotating inner conical section (Figure 1.3), encased in a complementary shaped, stationary shell. The rotor is designed such that there is an auger section at the small end, at the point of feed inlet, consisting of helical corrugations which drive the seed into the attrition region. These corrugations blend into a helical arrangement of studs, approximately 10mm high, along much of its length. At the large end, a short cylinder with corrugations in the opposing direction retards the flow.

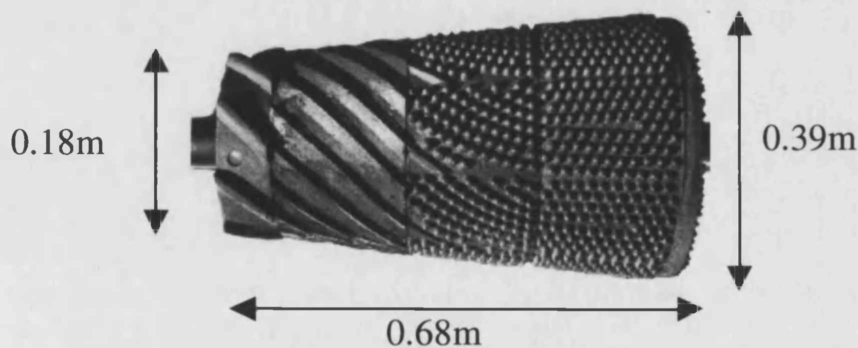


Figure 1.3 Beall Degermer cone

Between two and four perforated plates are fitted into the casing. These plates vary in perforation size (5.6mm – 7.1mm), depending on the size of the seed being processed and miller's preferences of products. The clearance between the rotor studs and these perforated screens is roughly 13mm, although this depends on the variable lateral position of the rotor. The remainder of the casing is covered with studs of the same size as those on the rotor. Traditionally made of cast-iron, some parts have been replaced with tougher chrome-nickel-steel alloys which have lowered maintenance costs (Brekke, 1970).

Only one size (size number 2) of Beall Degermer is currently commercially available, which processes corn seed at a rate of 3600kg/hour to 4500kg/hour. Few of the smaller degermers (size number 0), which are capable of processing approximately 680kg/hour, still exist. However, a pilot-scale Beall degermer, capable of processing as little as 50kg of seed, was used to compare the quality of degerming with that achieved using the small-scale degermer designed herein (Chapter 7).

Processing

Corn enters the degermer from the top, under a head of pressure, at the small end of the rotor, where the auger section drives seed into the attrition region of the mill. Detached germ and hulls are discharged through the perforated plates fixed into the stationary shell, and surrounding approximately 2/3 of the cone, while the grits are discharged at the rear of the device, at the tailgate. The production of the germ and hull "through-stock" in situ relieves the degermer load and reduces the damage done to the germ by allowing these fractions to pass from the degermer as soon as they are released. At the rear of the degermer, the loading of weights on the tailgate varies the head pressure of corn inside the device, which reduces throughput, increases residence time, work done on the seed, and the quality and yield of grits, or "tail stock" or "tails", and through-stock. Optimum tailgate opening provides maximum capacity and limited recycling.

The design and operating variables of the Beall degermer allow for the modification of a wide range of conditions in order to maximise degerming efficiency with any variety of corn. The design of the perforated screens is the major structural design variable. Once fixed, the operating variables are divided into two categories. These are the feed quality and degermer operation variables.

Feed quality

The Beall degermer is typically used for the processing of white or yellow corn, and there are two major factors relating to feed quality which can greatly affect the quality of degerming. These are seed size and moisture content. Seed is not sized prior to degerming. However, millers do have a certain amount of control in purchasing seed of the preferred average size, and this is important since smaller seeds are known to degerm to a lower quality than larger seeds in the Beall degermer.

The method of moisture conditioning and the final moisture content achieved have a large impact on the quality of degerming (section 1.3.4). Beall manufacturers believe that degerming is optimised using seed moisture conditioned to approximately 21% (w/w), but between individual millers, the moisture content and the methods used to reach the final moisture contents (tempering), vary considerably. Some treat the seeds with hot water on a conveyor belt, whilst others have found that simply hosing the seeds for a set period of time resulted in seed of a suitable quality for degerming. The resulting moisture contents can vary from 16% to 21% (w/w). Most often, the ideal seed moisture content is believed to be 21% (w/w). Any greater, and the produce appears 'gummy'. Any less, and the seeds are pulverised into fragments of an unsuitable size for the intended discharge and separation processes (Beall Degerminator Co., Decatur, IL, USA).

Operation variables

Degermer operation variables include the rotor variables i.e. the speed of cone rotation and the clearance between the rotor and casing, and seed pressure, maintained by the head of seed and the tailgate loading. The only operating guidelines, provided

by Beall Degerminator Company, recommended specific rotor speeds, seed moisture contents, temper times, and screen perforation sizes, for the production of a range of sizes of grits, extent of oil recovery and rate of throughput. Rotor-stator clearance and tailgate loading, variations of which can have a large impact on degerming by changing the intensity and duration of abrasion and scouring, are left to the discretion of the miller.

The recommended rotor speed range of the large-scale units is 700-900rpm, providing a tip-speed range of approximately $14.3\text{-}18.4 m.s^{-1}$ (section 3.2.4.c). Variations in the size of perforations in the plates in the degermer casing affect the quantity and quality of the through stock. Rotor-stator clearances typically used, measured between rotor stud tips and the perforated screens, are within the region of ½-inch, but the exact position depends largely upon the seed being milled and the products required by the individual miller. Changes in clearance and tailgate loading are frequently implemented *in situ* by adjusting the rotor lever, which slides the rotor horizontally into position, or adding or removing tailgate weights, respectively. Together, they have a large impact on seed pressure and residence time, and thus work done on the seed and the quality of degermer products. The level of work is typically sufficient to create a temperature differential across of the device of approximately $10^{o}C$ (Brekke, 1970).

Variations in through stock quality correspond to the quantity of whole germ, endosperm fragments and fines. The correct perforation size for the type of seed processed would minimise the production of fines and endosperm content of the through stock, and maximise the content of whole germ, hull and tip cap. Seed factors influencing this decision include seed size and density, which vary between crop variety and harvests, and the selected conditions of processing.

1.3.5.b Alternative degerming devices

Entoleter

The Entoleter is an impact mill, consisting of a horizontal, disk-type rotor with stationary housing. The rotating disk usually has two rows of concentrically arranged vertical pins, which intermesh with downward-facing pins mounted on overhead stationary disk. Feed enters from above onto the rotor operating at moderate speed, and is subjected to impact as it travels through the rows of pins and thrown against the stationary liner surrounding rotor.

Granulator

A granulator consists of a cylinder in which eight knives, or blades, are mounted on a horizontal shaft. Approximately three quarters of the cylinder's surface consists of a perforated screen, through which all corn must pass. Variations in this operation include the possible addition of two stationary knives, and the position of the edges of the knives. Some millers operate with the knife edge leading, whilst others prefer to have them trailing. The latter situation results in more of an extrusion effect, similarly to the effect of seed pressure inside the Beall degermer forcing seed fragments through the perforated plates.

Disk mill

Disk mills are composed of two facing, horizontally or vertically mounted grinding disks, one of which rotates whilst the other is held stationary. The major design and operating variables include the design of the facing disks and the spacing between them. The design may be a series of concentric, interrupted corrugations or ridges, and variations in the spacing between the disks affects the pressure and work done on the seeds.

Roller-mill

Roller-milling is the original commercial method for degerming corn. Good germ release was dependent on the proper adjustment of roller corrugations, spacing, and

seed tempering. The most common target product for dry milling using roller-mills was grits. Maximum extraction of this fraction occurred at the expense of separating whole germ, which was broken into small particles. Roller-milling is no longer used as an independent process for degerming corn seed. It remains a valuable operation downstream of degerming, since combined with sieving, germ and grits in the through stock and tail stock can effectively be separated using this method.

1.3.5.c Comparison of products between the different degermers

The aim for the small-scale degermer design was to incorporate those design features of the large-scale degermers which were the most important in the effective separation of germ and endosperm. The Beall degermer encompassed all of the degerming mechanisms i.e. high speeds of rotation, seed pressure, and extrusion through perforations, observed in each of the other degermers. However, by avoiding the reproduction of certain aspects of the Beall degerming mechanism, such as seed-seed interaction when degerming using the Entoleter, it was possible to reduce quantities of oil rich fines and small germ fragments produced. This was an attractive option for the small-scale degerming of corn seeds, in which it was predicted to be difficult to reproduce the interactions between corn seeds, and to separate small fragments of germ and endosperm contained in the fines. The factors which were investigated are detailed in section 2.3.2.

1.3.6 Evaluating milling quality

A number of studies have been carried out to assess performance of dry-mills, based on several seed characteristics and processing techniques, in terms of feed and product quality. Of particular interest was one study which combined the feed and product data into a Milling Evaluation Factor (MEF) (Kirleis and Stroshine, 1990). The seed properties in question were density, hardness, test weight, and susceptibility to cracking and breakage. Test weight is a concept used by the grain trade to account for the deviation in seed densities from the standard test weight assigned to the particular type of seed. Seed density and test weight were the two most important factors affecting the MEF, and larger grits were produced by milling hard seeds, with a high density and test weight. Other seed-related processing conditions required for

the production of large grits include the use of high drying temperatures with controlled moisture loss after drying (Kirleis and Stroshine, 1990), and elevated temperatures during a shortened tempering stage (Brekke, 1965; Brekke, 1968; Mehra and Eckhoff, 1997; Peplinski et al., 1984).

The study by Kirleis and Stroshine identified the important seed properties and required treatments for maximising the quality of degerming, but did not investigate the effect of varying the operating conditions of the horizontal drum type degermer on the quality of the products generated. For the processing of transgenic corn seed, in which there was no possibility of selecting different qualities of seed, it was the latter of these situations which was required i.e. the determination of the effect of the different degermer operating conditions on the one type of seed. In order to achieve this, an evaluation factor similar to the MEF was required in order to account for the effect of the different operating conditions of the small-scale degerming process on the mass and oil content of the product and waste streams tested. More details of this evaluation factor are provided in section 5.2 and section 5.4.

1.4 Application of traditional corn processing methods to the processing of transgenic corn seed

To our knowledge, large-scale corn processes have not yet been applied to the processing of transgenic corn seed for the production of recombinant proteins. It is not known whether existing corn TD processing will work as effectively on transgenic seeds. Even if it were possible to assume that to a large extent it would work as effectively, the optimum processing conditions currently could not be predicted. Based on the different processing requirements of similar non-transgenic seeds and the trial and error experimentation required for in-process optimisation, it was most probable that the same methods would have to be applied to transgenic seed processing.

It is neither economically nor practically feasible to build up a transgenic seed bank of sufficient size to allow for the direct application of large-scale seed processes without

prior knowledge of the output quality. Product quality varied with the *in situ* modification of the degermer operating conditions for optimisation of the degermer output. It would not be possible to apply the same optimisation methods, or the operation itself, to the degerming of transgenic seed, particularly during the early stages of production, due to restricted seed availability, massive costs of seed production and regulatory concerns regarding product quality. Ultimately, it is quite likely that large-scale degerming would be required, to improve the quality of the feed stream to the most costly aspect of plant-based protein production, that is, the extraction and purification process (Nikolov and Hammes, 2002). Therefore, the issues involved in the application of large-scale corn processes to the processing of transgenic seeds, and research based on the small-scale processing of transgenic seeds, are discussed in this section.

1.4.1 Application of non-transgenic seed degerming processes

The industrial fractionation of corn seed into components typically requires a throughput in too great excess for the quantity of seeds available during the early developmental stages of transgenic corn seed processing. The Beall degermer, for example, operates with a flow rate of approximately 3500kg/hour. Even the pilot scale Beall degermer (no longer in production) requires a minimum of 50kg of seed for processing, which was too great to apply to the processing of transgenic seed. From reports by industrial millers on the different qualities of degerming achieved with different types of corn, it is not possible to predict the quality of degerming on a new type of corn seed, based on the quality of degerming of other varieties of seed. It might not even be possible to degerm transgenic seed at all using these existing processes. Therefore, the availability of smaller degermers was investigated in order to establish that nothing was commercially available on the scale required, and to acquire information pertinent to the successful small-scale degerming of corn seed.

Corn-millers have scaled down the milling process for research purposes in wet-milling (Eckhoff et al., 1993; Singh et al., 1997), and tested a range of material and processing conditions using smaller models of large-scale degerming devices in dry-milling (Brekke, 1965). Furthermore, there has been research into reducing the

number of stages and processing time (Mehra and Eckhoff, 1997; Peplinski et al., 1984). Samples as small as 100g have been used in wet-milling research to determine corn milling characteristics (Eckhoff et al., 1996), and these results were compared to other laboratory, and pilot-plant, scale studies (Eckhoff et al., 1993; Singh et al., 1997). Although wet-milling was believed to be unsuitable to processing transgenic seed, this information on small-scale processing was useful on two accounts. Firstly, it showed that it was possible to degerm small samples of seed. Secondly, it provided information on the major issues involved in small-scale processing, and the requirements of small-scale processing in order to draw reliable comparisons between scales. Dry-millers, however, generally have not required, and therefore not extensively explored scaling down the degerming process. A variety of small degermers have been used for research purposes (Hood et al., 1997; Kirleis and Stroshine, 1990; Peplinski et al., 1984), in addition to the pilot scale Beall degermer. None of these operations have been used to degerm fewer than 0.5kg of seeds, none were described in detail, and the conditions of operation to maximise germ-endosperm separation were not reported.

1.4.2 Large-scale degermer suitability to transgenic seed processing

The single intention of degerming transgenic seed was to maximise the yield of a low-oil content endosperm fraction. The size of the endosperm particles was unimportant. However, a pure germ fraction was also required insofar as endosperm, and therefore product, lost to the waste stream was minimised. None of the degermers available were designed to achieve both of these criteria.

The structure of corn seed, with germ embedded into the seed, prevents easy separation of its components. Whereas on the one hand it may be possible to achieve an endosperm fraction very low in oil content, this could not be achieved without considerable abrasion and the production of fines. These fines would consist of germ and endosperm, which although would alleviate the need for degermer recycle, it would also necessitate a separate process to recover the product entrained in the fines. On the other hand, reduced abrasion would reduce the level of fines, and therefore reduce the complexity of the process to recover endosperm from germ-rich fines, but

this would be at the expense of a reduced quality of the endosperm fraction i.e. higher oil content. One condition could not be achieved without forfeiting the other, and so a compromise between the two conditions had to be reached. It was inevitable that the target quality of product could not be achieved without lengthy post-degerming processing. The best, realistic small-scale degerming system would produce an endosperm stream with minimal and acceptable levels of contamination, and a waste stream with minimal and acceptable quantities of product.

Whether it was a result of interactions between seeds or interactions between seeds and the high speed devices, impact and abrasion were the prerequisites for the successful separation of germ and endosperm in any of the degerming devices. For the processing of small quantities of seed, in which interaction between corn seeds was likely to be significantly lower, there was a greater reliance on the interaction between seeds and rotating parts of the degermer. Consequently, on the basis of whole germ release and sufficient, but not excessive, scrubbing of endosperm to produce a good, low-oil product stream, elements of the Beall, Entoleter and disk mill degermers were incorporated into the design of the small-scale degerming device.

1.4.3 Whole ground seed processing

Most studies reporting the extraction and purification of recombinant proteins from transgenic corn seeds involved grinding whole seeds for extraction from corn flour. Such proteins include aprotinin, avidin and β-glucuronidase (rGUS), (Azzoni et al., 2002; Kusnadi et al., 1998a; Kusnadi et al., 1998b; Zhong et al., 1999). Grinding whole seeds is a quick and simple method to reduce particle size for improved extraction efficiency. However, in terms of large-scale production, the quantity and quality of the feed material to the extraction and purification process, with regard to the quantities of native proteins, starches and oils, becomes much more significant in the overall process economics. The fractionation of corn seed could potentially vastly improve the quality of this feed.

1.4.4 Fractionation of transgenic seed

Based on the predicted majority costs of downstream processing in the production of recombinant proteins using transgenic plants, it is possible that improving the feed material might affect the entire feasibility of plant-based recombinant protein manufacture. Transgenic corn seeds have been manually and mechanically fractioned prior to the purification of several transgenic recombinant proteins, including avidin, aprotinin and rGUS (Hood et al., 1997; Kusnadi et al., 1998b; Zhong et al., 1999). Mechanical fractionation was achieved using a custom-made dehuller / degermer, which was designed for the fractionation of corn seed for the recovery of product contained in corn germ (Hood et al., 1997). In this study, 77% of the total germ in a feed of 500g, with a purity of ca. 35%, was separated by the fractionation process consisting of dehulling / degerming, followed by sieving and aspiration of the selected, germ-rich sieves. The high purity germ, required for the extraction and purification of avidin, was obtained by hand-picking the free germ.

Details of the small-scale degermer / dehuller design, its operation, and possible capacity range were not reported. Process variables were not tested to maximise germ-endosperm separation, and the results were not compared to those achieved by large-scale degerming. The physical characteristics of the seed were also not reported, and so it was not known if the seeds processed were of a suitable quality for large-scale degerming. Finally, the sample sizes (500g) required for use in this degermer / dehuller, which was the smallest of the reported small-scale degermers, were too large for the quantities of transgenic seed available for use in this study.

1.4.5 Application of corn seed fractionation processes

In one study, in which product accumulation was targeted to the germ, approximately 10 times more rGUS per gram of solids was extracted from ground germ, separated using the same degermer / dehuller discussed previously, than from ground whole seed samples (Kusnadi et al., 1998a). Although the presence of germ oil had no effect on the extraction efficiency, its removal was believed to hold several advantages, such as reducing the viscous properties of the process stream and the fouling potential of expensive purification systems. In terms of the process efficiency, the choice of

starting material i.e. whether to degerm or not, would depend largely on the economics of the entire process (Evangelista et al., 1998; Kusnadi et al., 2001). Corn seed degerming has been shown to be of value, but the extent of this value would remain specific to the product, the quality of the seed being processed, and to the quality of degerming that could be achieved on a large scale. These parameters currently cannot be determined unless the large-scale processes are applied to the transgenic seed.

Few studies have investigated the application of existing food processes at the point of transfer from agricultural processing to product purification (Hughes et al., 2000; Mott et al., 2000). Mott *et al.* discovered that the quality of rapeseed processing differed in the level of solvent oil extraction between transgenic and non-transgenic rapeseed (Mott et al., 2000), and this was proposed to be a result of the size of the plastic particulate (polyhydroxybutyrate) being extracted, and the different morphology and composition between transgenic and non-transgenic seeds. Although the large-scale production of corn-produced recombinant proteins has already been achieved (Kusnadi et al., 1998b), the utilisation of a large-scale degerming process has not been reported. It was therefore not known whether transgenic corn seed would fractionate in the large-scale degerming process to the same and known quality as non-transgenic seed.

1.4.6 Seed fractionation quality assessment

On the basis of the recombinant protein being largely concentrated in one seed component, its concentration in the fractionated corn seed streams would be the best indicator of degerming quality. Mass balancing would provide important information on the extent of recovery and loss of this product across the fractionation process. However, in the absence of transgenic proteins for which there are specific and highly sensitive assays, such as when there are insufficient supplies for process development, alternative means of process development and fractionation assessment are required. Seeds of a suitable quality for processing were selected (section 2.1.1), and the differences in the mass and oil content between the seed components were the major parameters for the assessment of fractionation quality.

1.5 Epicyte Pharmaceutical Inc.

1.5.1 About Epicyte

Epicyte Pharmaceuticals Inc. (no longer trading), our collaborating company, was a small biopharmaceutical company created for the production of human antibodies in plants. Epicyte's strategy was to target common inflammatory and infectious diseases which remain widely untreated because traditional technology is unable to produce sufficient quantities of antibodies. It had a pipeline of monoclonal antibodies focused on new options for treating major healthcare problems, including Herpes Simplex Virus (HSV), Human Papilloma Virus (HPC), Human Immunodeficiency Virus (HIV), *Clostridium difficile*, Alzheimer's disease, ulcerative colitis and hepatitis viruses. Its major product was an antibody designed to treat Respiratory Syncitial Virus (RSV). RSV is an illness which hospitalizes 90,000 infants every year and may be second only to influenza as the cause of wintertime respiratory ailments among the elderly. Corn was selected as the host production system, and Epicyte had reached in-field production before it finally closed due to business and technology difficulties at the start of 2004.

1.5.2 Limited seed quantities

At Epicyte Pharmaceutical Inc., the gene sequence encoding the target product was designed, and corn cells were modified by the collaborating companies, using these gene sequences and their licensed technologies, for the production of plants expressing the secretory immunoglobulin A (SIgA) protein. The plants cultivated in subsequent generations were selected on the basis of expression levels to second generation (T2) plants (section 2.1.2), which each yielded approximately 300 seeds. The low quantities of seeds produced were mostly retained for further seed production, leaving very few available for process development (section 2.1.2.c).

1.5.3 Product accumulation in the endosperm

Epicyte produced transgenic corn seed in which production of the antibody was targeted to the endosperm fraction of the seed. The endosperm was reported to

contain very little protein other than the product, whereas the germ contained very little product and the bulk of the seed proteins and oils. Therefore, separation of the germ and endosperm would separate a large proportion of native seed proteins and oils from the product (section 1.2.6), thus improving the feed quality at entry into the separation process (section 1.4.5). Epicyte realised the potential benefits of separating of this component from the rest of the seed. However, since scarce quantities of transgenic seed were produced during the early stages of crop development, it was not possible to allocate the necessary large quantities of seed for process development using existing processes. Corn processes of a suitable capacity for transgenic seed processing were not available on the required scale.

1.5.4 Small-scale degerming

The fractionation of corn seed components, mainly germ and endosperm, is a well-established practice in the corn processing industry (section 1.3). Where existing processes can be applied to transgenic seed without damage to the recombinant protein, the overall process economics are further improved by the application of relatively cheap food processing machinery for the improvement of a biopharmaceutical feed quality to downstream processing. The available degermers could not process the small quantities of seed and it was neither wise, for reasons discussed previously (section 1.4.1), nor feasible, due to time pressures, to wait until seed production had increased sufficiently to allow for processing through the large-scale degerming units.

Mechanically fractionated seed was required for the development of a purification process during the ramping up phase of seed production. In the absence of commercially available small-scale degermers, Epicyte created this research project for the implementation of a small-scale process to produce high quality fractionated corn streams using the small quantities of seed available. The benefits of small-scale corn processing were several fold, and these are discussed in the following section.

1.6 Aims

An attractive opportunity to reduce the costs of processing transgenic crops for the production of recombinant proteins lies at the point of transfer from agricultural processing to protein purification. Agricultural practices have successfully been applied for the large-scale cultivation of transgenic plants. Standard downstream processing techniques have been applied for the recovery and purification of plant-produced recombinant proteins. However, the extent to which large-scale agricultural processing can be applied to the processing of transgenic corn seed, in preparation of material for downstream processing, remains unknown.

At the time of publication, no system was available for the small-scale degerming of corn seed. Ideally, a small-scale process would exist which could reliably degerm very small quantities of transgenic seed. This has never been required before, since non-modified corn seeds have been available in great abundance (Brekke, 1965; Kirleis and Stroshine, 1990). The aim of this study was, therefore, to incorporate the important design features of the large-scale degerming process into the design of a small-scale degerming process, consisting of a novel degerming device and germ-endosperm separation process, for the reliable degerming of small quantities transgenic corn seeds.

Using this small-scale degerming process, it was intended to test whether corn seed could successfully be degermed on the small-scale. Secondly, the processes would be tested in order to establish the extent of influence of seed physical characteristics and operating conditions on the quality of degerming. Thirdly, it would produce a low fat, product-rich endosperm stream from small quantities of seed, of a similar quality to that which would be achieved on a large-scale, for the development of a laboratory extraction and purification process. Fourthly, simple and rapid experimental protocols would be designed and tested, using minimal quantities of seed, for the generation of the required information necessary to optimise the processing conditions to suit the seed being processed. Fifth, it could be used in the manufacture of sufficient quantities of product for pre-clinical testing and clinical trials. Sixth, it might be used for the assessment of seed suitability to large-scale degerming, based

on the extent of fractionation achieved using the small-scale process. Finally, it would have the capacity to provide important processing information either for scaling up transgenic seed processing, or to predict large scale degerming performance based on the quality of degerming achieved on the small-scale.

Chapter 2 - Materials and Methods

In order to design a simple and effective small-scale degerming process, knowledge of the large-scale process and of the material (i.e. corn seed) properties was an essential prerequisite. The major factors of large-scale degerming which were essential to the successful degerming of corn seed were incorporated, where possible, into the design of the small-scale device. Material properties included the size and density of whole seed and seed components, and their protein and oil content. Information on seed properties facilitated the identification of unit operations suitable to the processing of small quantities of seed, and provided a means to rapidly analyse the quality of degerming, which was measured in terms of the mass and oil content of the different fractions produced. The degerming process was fully characterised and optimised prior to processing transgenic seed, which was evaluated in terms of mass, oil and antibody content of the waste and product streams.

2.1 Seed properties

Several different types of seeds were processed using the small-scale degermer. Differences in the quality of degerming were expected between different non-transgenic seed varieties, as observed by large-scale dry-millers, and also between non-transgenic and transgenic seeds, due to the differences between the hybrids and their progeny involved in the manufacture of transgenic seed, and the non-transgenic seed. The different seeds processed, and their major physical and chemical characteristics, are detailed in this section. The most important seed characteristics were average seed mass and oil content, and the mass and oil content of seed fractions. Additional characteristics which were measured included seed mass distribution, and whole seed and seed component density and protein content. These latter characteristics were useful for comparisons between the different seed varieties, but were not used in degerming analysis.

2.1.1 Non-modified seed

In the absence of the genetically modified material available for processing, two high quality hybrids (section 1.2.8) of a yellow dent corn were processed: hybrid 8366 (cross of inbreds BQ30 and UP25) and hybrid 8342GLS/IT (cross of inbreds BD68 and UD70). Hybrid 8366 seeds were used throughout the design, characterisation and optimisation of the process, and hybrid 8342GLS/IT seeds were processed at the optimised conditions for the comparison of the quality of degerming that could be achieved between two high quality seed hybrids . Whereas hybrid 8366 seeds were selected for use due to their close resemblance to the elite inbred UU01, hybrid 8342GLS/IT seeds were the progeny of two inbreds, BD68 and UD70. Inbred UD70 was a version of UU01, the inbred into which it was intended to breed the transgenes. It was envisioned that the seed production system would make use of the 8342GLS/IT hybrid, hence its use as a high quality hybrid, yet one which bridged the seed quality void between hybrid 8366 and the transgenic seed.

All seeds were supplied by Garst Seed Company. In the event of seed size strongly affecting degerming quality, the seeds were sized prior to processing. They were classified medium flat medium (MFM), meaning that they were of a medium size and categorised as more flat than round. Throughout the design phase of the study, seed size was an important parameter to control, in order to fairly assess the effect of all other factors upon a selected response parameter, without any impact from the potentially large variations in seed sizes caused by natural diversity. Although not genetically related to the transgenic seed, its structural similarities to the high-quality inbred seed into which the transgene was introgressed (UU01), made it a valuable starting point in the design process.

A low quality non-modified seed inbred, named B73, one of the parent seeds used in plant transformation, was available in abundance. In this case, the low-quality description is a reference to the type of seed most suitable to large-scale degerming (Kirleis and Stroshine, 1990), and this trait i.e. the lower quality, was identifiable by their smaller sizes and more irregular shapes. This parent seed was selected for processing on the basis of its physical similarity to the transgenic seed, and was used

to predict the performance of a pilot-scale degerming device on transgenic seed. This prediction used the data generated in a comparison study, which investigated the quality of degerming that could be achieved by processing B73 seed on both small- and pilot- scales.

2.1.2 Transgenic seed

Prior to the processing of transgenic seed, it was necessary to establish whether seed size affected the quality of degerming. The seed used in this study was transgenic 8366Bt seed (cross of inbreds BQ30 and UW18), which Garst Seed Company had substituted for 8366. The parent seeds, or inbreds, used in the production of these two seed varieties were not identical. 8366Bt was produced using the same female inbred parent as hybrid 8366 seed (BQ30), but UW18 as the male. Inbred UW18 was the same as UP25, but with the commercial Bt transgene bred into it, for the expression of the Bt (bacterial) protein which gives the plant resistance to caterpillars. The transgenic and non-transgenic hybrids of 8366 seeds were identical by observation, but different due to the expression of the Bt protein in the transgenic variety. Although transgenes can affect seed properties, this is not always the case, and any affect of the Bt transgene was expected to be minimal because the Bt line is a commercial line and was selected for this study on the basis of it not affecting other characteristics.

Two seed varieties were used in the production of a high-quality transgenic seed. One, a low-quality hybrid produced by crossing parent seeds B73 and A188, was required for its relative ease of genetic modification and regeneration from callus. Both parent seeds were low-quality varieties of seed. The transgenic plants produced from these seeds were also low quality with regards to the size, structure and the yield of seed.

In order to improve the physical characteristics of transgenic seed, the transgene was transferred into a more favourable genetic background by backcrossing with high-quality inbred lines, including UU01 or UE95 seed. This process of backcrossing, called introgression, results in the production of high-quality transgenic seeds

expressing the desirable seed characteristics of the high inbred varieties, and carrying the inserted gene sequence for expression of the antibody. After several generations of back-crossing with UU01, the genetic background of the transgenic seed would essentially be UU01, and the only genetic element of the original transgenic plant would remain that of the transgene.

The quality and degerming properties of two different varieties of transgenic seed, HVY2 and HVF1, were investigated. These seeds were produced by the genetic modification of a cell culture of the hybrid B73*A188, and generating plants (B73*A188) containing the GE10 transgene encoding the anti-RSV antibody, called EPI19. The transgenic plants were then twice cross-pollinated using UU01 plants (section 2.1.2.d), to produce the second generation (T2) seeds (HVY2 and HVF1) used in this study i.e. T2 UU01 outcross. Transgenic seed antibody content data was provided by Epicyte on HVY2 and HVF1 seeds, which consisted of seeds pooled from individual plants (Figure 6.4), and another variety of transgenic seed (HOY3) (Figure 6.5), in order to illustrate the range of antibody content between different seeds produced by one plant generated in a single transformation event.

2.1.2.a Transformation for the manufacture of transgenic seed

All transgenic seeds investigated were varieties of yellow dent corn, and were provided and owned by Garst Seed Company. Garst used whiskering technology (section 1.2.3) for the insertion of the gene sequence encoding the anti-respiratory syncitial virus F-glycoprotein (RSV) antibody (EPI19). Epicyte produced this gene sequence, and owned the antibody contained within these seeds.

2.1.2.b Variability

Genetically modified corn tended to vary in size considerably more than its non-transgenic counterparts. This was not unexpected during the early stages of its production for several reasons. In addition to the inherent genetic variability between seeds, which was thought to be no different to regular crops, the major cause of the large variation in seed size was due to incomplete fertilisation of the ovules. This was

caused by crops being grown in a contained environment, in which there was considerably less pollen available for fertilisation than in an open field filled with pollinating crops. The ensuing growth pattern of the seeds was less uniform, as the seeds tended to grow into the empty spaces occupied by unfertilised ovules. It was not possible to size the seeds using the same method as used for non-modified seed, due to the severely restricted quantities available. However, to a certain degree, the transgenic seeds were sized by the determination of the average seed size, and selecting enough seeds closest to this average until the batch size was complete.

2.1.2.c Availability

The time and seed quantities involved in the production of high-quality transgenic crops greatly reduce the quantity of material available for process development (section 1.5.2). The modification techniques used produced plants with a wide range of expression levels. All of the plants known to express antibody were cultivated for seed production. These seeds were tested for antibody content. The retention of seeds with high expression levels in the breeding programme greatly reduced the number of seeds made available for process development. These generations of seeds (T1 & T2) were typically of poor quality, both structurally i.e. size and shape, and in terms of the antibody content, and it was these which were made available for this research.

2.1.2.d Seed development

Distinguishing between the transgenic plants produced enabled the tracking and selection of those plants with the highest expression levels, in order to continue a successful breeding programme for the manufacture of high-expressing seeds. Unique three letter codes, e.g. HVY and HVF were used for the identification of plants produced from a single callus of a specific transformation event. The numbers following these three letter codes e.g. HVY2 and HVF1, represent the transgene inserted during that specific transformation event. The plants generated from each callus were then numbered e.g. HVY2_01, HVY2_02. These plants were cultivated and cross pollinated with the high-quality UU01, which produced first generation, or

T1 transgenic seed. The siblings of transgenic seed were then numbered e.g. HVY2_01/001, HVY2_01/002, cultivated and crossed once more with UU01 seeds. These T2 seeds contained the transgene, 25% of the genes of the cross between A188×B73 and 75% of UU01 genes. In this study, T2 seeds taken from the same transformation event were combined for analysis.

2.1.3 Manual seed fractionation

Seeds were manually fractionated for two purposes. These were to identify physical or chemical differences between the components which might enable the separation, and assessment of the extent of this separation. The manual degerming of seed produced two fractions, representing the waste (germ) and product (endosperm) streams as would be produced during ideal degermer operation. The waste stream consisted of germ and tip cap, the product stream contained the endosperm and hull. All types of seed were analysed for whole seed and seed component mass and oil content. Hybrid 8366 and hybrid 8342GLS/IT were also analysed for differences in density and protein content. Manual fraction provided the highest level of separation of the major seed components. Analysis of these components provided the "Gold Standards" of degerming, which were valuable in the assessment of the quality of degerming using the small-scale degerming process.

Manual germ removal from yellow dent corn at 12% (w/w) moisture content proved to be incredibly difficult due to seed hardness. In order to make germ removal feasible, seeds were soaked in water in order to raise the seed moisture content. Raising seed moisture content, as practised in industrial corn dry-mills, facilitates germ separation by both softening the seed and loosening the bonds holding the germ in the seed. Soaking the seeds for 30 minutes sufficiently softened the seed, enabling the germ to be cut out of the seed using a sharp knife. Two cuts either side of the germ, starting at the base of the seed, either side of the tip cap, were completed at the top of the germ at a single point. Once cut, germ was made to 'pop out' by pressing down on the tip cap and pulling the exposed top of the germ out of the seed. The methodology used for the determination of the gold standards for non-transgenic seed degerming was applied to transgenic seeds. However, as a precautionary measure

against product losses through leaching into the water, as experienced by Kusnadi *et al.* after soaking transgenic corn seeds in water (Kusnadi et al., 1998b), transgenic corn seed moisture content was raised to 18% by the addition of a calculated volume of water to seed held in a sealed container (section 2.1.8.c).

2.1.4 Different sizes of seed

A broad range of seed classifications exist based on size i.e. large, medium and small, and shape i.e. flat and round. The process and operating conditions had been determined using only one type of seed, which was of controlled size (MFM). It was possible that the quality of degerming would vary with different sizes of seed when processed under identical operating conditions through the small-scale degermer.

As a result of the very wide range of sizes of transgenic seeds within the small quantities available, it was not possible to screen the seeds according to size using the same methods as for the sizing of hybrid 8366 seed. However, a sufficient quantity of seed was available to allow for a small degree of sizing prior to processing. This sizing consisted of selecting seeds for processing on the basis of minimising the difference between their mass and the average mass of 100 seeds. This sizing prevented the selection of very small seeds which would have resulted in an atypical feed. Even though seed numbers were limited, the average mass of seeds was increased for processing by using this method of sizing, from 0.2029g to 0.2125g. However, the samples of sized transgenic seeds available for milling through the small-scale process still consisted of a wider range of seed sizes than those of sized, non-transgenic seed. Therefore, it was also necessary to investigate the extent to which the quality of the degermer output varied when processing different sizes and shapes of seed, in preparation for the processing of transgenic seed.

2.1.5 Sample size

Batches of 50 seeds were used for the determination of the gold standards of manually fractionated seeds. During process development, samples of 100g of seed were processed. Samples of this size were used to provide reliable processing information, and to establish a standard processing methodology in preparation for processing

transgenic seed. Sample sizes of this magnitude had to be reduced in order to meet the requirements of transgenic seed availability, whilst ensuring maintained process and analytical reliability.

The most important factors to consider in the reduction of the feed sample size were the frequency with which germ was released from seed inside the degermer, and that the process operating conditions were suitable for the production of large germ fragments. The frequency of germ release varied with different operating conditions. The extent of germ release and its size had a large impact on the mass collected on the top sieve (sieve A, 4.0mm) (section 6.2.1), and thus the extent of applicability of the analytical assay. Sieve A most often contained the smallest quantity of sample, and operation of the process under a range of high-attrition conditions sometimes did not yield measurable quantities of the different degermed fractions (section 5.2). Therefore, the feed size was reduced once optimised process operating conditions had been established. Reduction of the sample size fed into the degerming process was accomplished by measuring the mass and oil content of the different sizes of fractions produced, when the feed size was reduced to 50g, 25g, 12.5g and 10g of seeds (section 6.2.1). Analysis of each of the fractions produced generated information on the variation in product quality between replicate experiments using samples of the same size, and the variation in this quality between samples of different sizes. It was vitally important during the reduction of sample size that process data reliability was maintained.

2.1.6 Standard sample preparation

A standardised procedure for sample preparation was required to minimise or prevent external factors, such as seed moisture content and sample particle size, affecting the analysis of whole seed and seed fraction oil, protein and antibody content.

2.1.6.a Drying procedure

Inconsistencies of sample moisture content have been shown to affect the concentrations of recombinant proteins measured in transgenic corn (Kusnadi et al., 1998a). Different levels of moisture between samples would also affect the

measurement of mass and density, and oil and protein concentrations. Therefore, samples were dried prior to analysis. Initially, non-modified seed fractions produced through manual degerming were dried for 16 hours at 70°C to remove excess moisture absorbed through soaking. However, due to the increased risk of product damage with drying at higher temperatures (Kusnadi et al., 1998b), and to enable a direct comparison between non-modified and transgenic seed fraction mass and density, the drying temperature was reduced to 37°C. The overnight drying time remained the same.

2.1.6.b Particle size

The inconsistency of particle sizes, typical of the products of the degerming process, was the most likely factor to produce inaccurate information on sample content, as observed by Bai *et al.* in the extraction of rGUS from ground transgenic canola seeds (Bai and Nikolov, 2001). Therefore, to improve assay efficacy and reliability, the degermer products were ground and sieved through pores of 710μm diameter, which set an upper limit on the size of particles used for extraction of oil, protein and antibody.

Grinding of the hybrid 8366 seeds used throughout process design and development, and B73 seed in scale comparison testing, was accomplished using a household coffee grinder (Moulinex, Cedex, France). In the event that there was insufficient sample for complete grinding in the Moulinex mill, samples were ground using a mortar and pestle to ensure that there was an adequate sample size for analysis. All other varieties of seed, including transgenic seed, were ground using a 2000 Geno/Grinder (SPEX Certiprep, Metuchen, USA). The grinding mechanism in the Geno/Grinder was essentially that of a bead mill, in which a single steel ball crushed samples contained in a tube of approximately 10ml volume. It was possible to grind individual samples in up to 48 of these tubes at any one time, by rapidly shaking in the vertical plane at speeds of up to 1000 strokes per minute. The major advantage of using this device for grinding was that the sample size required for grinding, in order to obtain the required sample size for analysis, was significantly reduced due to the decrease in operation losses (section 6.2.2.a)

The minimum mass of sample needed for grinding, accounting for losses, was determined for the production of a sample size of sufficient mass for oil extraction. Using a coffee grinder to prepare the samples for oil analysis imposed severe sample size limitations, owing to the large volume within the grinder and the subsequent reduced frequency of contact between the blades and the particles. Additionally, the large range of particle sizes produced, including the agglomerated oil-rich germ particles, resulted in large fractions being lost in the subsequent sieving operation. The minimum mass of sample required for oil analysis, determined previously, did not account for any losses in the sample preparation, which were significant when using the coffee grinder. The use of the Geno/Grinder™ enabled the processing of much smaller samples with greatly reduced losses. The efficacy of these two methods of grinding was compared in terms of the extent of sample losses after grinding and sieving, and in terms of the extent of oil extraction. Sample preparation for oil analysis using Geno/Grinder involved grinding up to 0.40g of seed fractions for 1 minute at 650 strokes per minute. Although the mass required for oil analysis alone was reduced to 0.15g, the minimum mass which was to be collected on sieve A was 0.4g, in order to provide samples for analysis of transgenic seed antibody content (section 6.2.2).

2.1.7 Physical and chemical properties

2.1.7.a Mass and density

Whole seed and seed component mass and density were determined from samples of 50 seeds. Average seed mass was determined from these samples of seed containing 12% (w/w) moisture (as received). Seed density was calculated using the batch mass combined with the volume displaced by these seeds when submersed in 20ml ethanol contained in a 100ml measuring cylinder. This same batch of seed was then moisture conditioned to facilitate manual fractionation (section 2.1.3). These fractions were then dried, and the same methods of mass and density determination were applied as for whole seeds.

2.1.7.b Oil content

Corn seed components contain very different quantities of oil. Since the degerming process was designed to separate these fractions, oil content was recognised as a useful tool to analyse the efficacy of germ separation during equipment and process design. The observed difference in total oil content between corn germ and the rest of seed was sufficiently large to allow for the application of a hexane extraction procedure, which provided information to a satisfactory degree of accuracy.

Oil concentration of each fraction was determined using a modified method of hexane extraction (Junker et al., 1998), in which sample oil contents were determined to an accuracy of 4 decimal places, providing that the oil concentration in solution was no less than $5g.l^{-1}$. Based on data available in the literature, it was possible to calculate the minimal mass of sample needed to meet the assay requirements (Appendix A). The efficacy and reliability of the oil assay was verified by investigating the effect of mixing time, sample masses and hexane volumes on the quantity and concentration of oil extracted (section 3.3.1 and Appendix B). This was achieved by the application of a 2-level, full factorial design experiment (section 2.3). The variable factors investigated were sample mass, hexane volume and the mixing time. The effect of these factors was measured in terms of oil concentration in hexane and the calculated oil concentration in the sample. Whole ground corn was used for this experiment in order to establish the minimum sample mass for oil extraction, on the basis of the lower reliability of extraction due to the lower oil contents, compared to extraction from samples of oil-rich germ.

Based on the calculation of the minimum extraction mass, and the wide range of feasible extraction ratios available using the hexane extraction technique, the extraction ratio of 1:5, using 0.2g of sample and 1ml hexane, was selected for the determination of sample oil content. Oil concentration of each fraction was determined by vigorously mixing 0.200g (± 0.005g) of dried, ground sample with 1ml hexane for 1 minute, spinning in a Biofuge model 13 centrifuge (Hereaus Sepatech GmbH, Osterode, Germany) for 15 minutes at 5000rpm to remove any particulate matter, and measuring the mass of oil residue in 0.75ml of the hexane-oil solution dispensed into a pre-weighed aluminium cup, placed in a HG 53 Halogen Moisture

Analyzer (Mettler-Toledo Ltd., Leicester, UK), and heated at 70°C until a constant mass was obtained. Typical times for drying were 3 minutes in order to achieve a constant dry mass of the cup before extract addition, and 5 minutes afterwards.

The sample size used for oil extraction from processed transgenic seeds was reduced to 0.150g (±0.005g), by reducing the sample mass from 0.2g, by decrements of 0.05g, and measuring the oil content and concentration by extraction using 1ml hexane (section 6.2.2.b). In order to rapidly screen large numbers of samples, all of the aluminium cups for each experiment were pre-dried in an oven at 70°C for five minutes to determine the original cup mass, and then for a further 10 minutes to determine the quantity of oil deposited after burning off excess hexane from the oil-hexane mixture.

2.1.7.c Protein content

Seed component protein content provided an alternative means of assessing the quality of germ separation. The protein content of two seed hybrids, hybrid 8366 and hybrid 8342GLS/IT, and their fractionated components, was determined using the Bradford assay (Bradford, 1976). The sample size available for protein extraction was affected by the same seed availability limitations as for oil extraction.

Protein was extracted from 0.2g samples of dried and ground whole seed and seed components by vigorously mixing with 1ml of de-ionised water for 1 minute. The mixture was then centrifuged for 15 minutes, at 5000rpm and room temperature. After centrifugation, 50μl supernatant was carefully withdrawn and diluted prior to protein measurement. A range of sample dilutions were required in order for the light absorbance to fall within the linear range of the standard curve. Since germ was considerably richer in protein, germ extract was diluted by factors of 5, 20 and 50 in de-ionised water, whereas non-germ extract was tested without dilution, and with dilution factors of 5 and 20.

50 μl of diluted samples were added to 950μl Coomassie dye (Perbio Science Ltd., Cheshire, UK) contained in a cuvette, which was inverted several times to achieve

good mixing prior to 5 minutes incubation at room temperature. The reaction was recorded at an absorbance of 595nm using a Pharmacia Biotech spectrophotometer (Ultrospec 2000, Pharmacia Biotech, Cambridge, UK). The light transmission through a control (Coomassie dye mixed with 50μl RO water) was set to zero. Sample protein content was measured by observing the change in light transmission upon the addition of protein. All samples were measured in triplicate. Quantification of protein concentration was achieved using standard curves of absorbance at 595nm against known concentrations of albumin standards (Appendix C) (Perbio Science Ltd., Cheshire, UK).

2.1.7.d Anti-RSV Antibody Elisa

Sample antibody concentration was determined using the method of enzyme linked immuno sorbant assay (Elisa). A 96-well, Nunc Immuno Maxisorb (Nalge Nunc International, Rochester, NY, USA) plate was coated with 100μl per well of Goat Anti-Human IgG (gamma) (Southern Biotech, Birmingham, AL, USA) diluted 1:1000 in phosphate buffered saline (1XPBS) (Sigma-Aldrich Corp., St. Louis, MO, USA), and left to incubate overnight for 16 hours at 4°C. The plates were washed three times with PBS-T washing buffer (1XPBS + 0.1% Tween-20) (Sigma-Aldrich) and slapped dry, before adding 200μl of blocking buffer (1XPBS + 2% BSA + 0.02% Sodium Azide) (Sigma-Aldrich) to each well and incubating on a shaker for 1 hour. The blocking buffer was then removed, the plate washed 3 times with washing buffer and slapped dry, after which the plate was ready to receive samples and standards.

The standards were prepared by diluting 1 mg/ml of Synagis IgG1 (Medimmune, Maryland, USA) in blocking buffer to a final concentration of range of 1 to 25 ng/ml according to the regime in Appendix D.

Promoters targeted antibody production in the endosperm, resulting in a large variation of antibody concentrations between corn components, and across the different sieves of the degermer output. In order to reliably detect antibody over this range of concentrations, a substantial variation in dilutions was required (5 ng/ml to 10 μg/ml) for the detection of antibody content of seed fractions. Three different

dilution factors (5, 20 and 400) were used to cover this range, and duplicate measurements were taken at each dilution factor for reliability purposes. The dilution range was then reduced for the analysis of the degerming process output, reported below, based on the expected quantities of germ and endosperm in each sieve fraction of the process output. The extract from seed fractions collected on the upper two sieves were diluted by factors of 25 and 100, in order to detect the concentration levels in the region of 0.250ug/ml of germ extract, as determined by extraction from manually fractionated germ, and to cover the larger range of concentrations expected as a result of contamination by endosperm fragments. The extract from seed fractions collected on the lower three sieves were diluted by factors of 100 and 400, in order to detect the typical concentration levels in region of 1.150ug/ml of endosperm extract, determined by extraction from manually fractionated endosperm.

Positive controls consisted of Rituxan (IDEC, California, USA) and Bethyl Human-IgG (Bethyl Laboratories Inc., Maryland, USA), both diluted from 1 µg/ml, by a factor of 100, in blocking buffer, to achieve a final concentration of 10 ng/ml. The values required for the Bethyl positive control were between 3.5 – 5.0 ng/ml, and 6.5 – 8.0 ng/ml for Rituxan. 100 µl of standards, samples and controls were added to the plate and incubated for 1 hour on a plate shaker. After one hour, the plate was washed 6 times with wash buffer and slapped dry. Detection Antibody consisted of 2% Goat Serum (Sigma-Aldrich) and Goat Anti-Kappa (+HRP) (Southern Biotech, Birmingham, AL, USA), diluted 1:5000 in PBS. 100 µl was added to each of the wells for one hour agitated incubation at room temperature. After incubation, the plate was again washed 6 times with wash buffer. Assay development involved the addition of 100 µl 3, 3', 5, 5' tetramethylbenzidine (TMB) (Pierce Biotechnology Inc., Rockford, IL, USA), at room temperature, to each well, and immediately measuring light absorbance at 605nm, every 13 seconds over a 4 minute period, using a micro-well plate reader.

2.1.8 Moisture content

Seed moisture content was a critical parameter in the degerming of corn seed. Using correct treatment methods, corn seeds absorb water which creates stresses inside the

seed, facilitating the release of germ from the seed (section 1.3.4). Raising the germ moisture content also increased germ pliability, which reduced breakage inside the degermer and roller-mill.

Garst Seed Company provided seed at which had been air-dried in a greenhouse to 12% moisture content. Confirmation of this level of moisture was achieved by observing the reduction in mass of two samples of 10 seeds, resulting from seed moisture evaporation upon heating at 80°C and 100°C (Appendix E). A consistent level of moisture content in the supplied seed negated the need to dry samples for moisture content determination each time the moisture content was raised, since it was possible to calculate seed moisture content based on the initial and final sample masses over the moisture conditioning period. The methods and calculations involved in moisture conditioning were limited in precision due to the accuracy with which the initial seed moisture content could be measured.

Two methods were investigated for increasing seed moisture content, and for their effects on the quality of degerming. One tested the effect of short duration moisture conditioning in an excess of water. The other method involved the addition of a calculated volume of water required to raise moisture content to a pre-determined level.

2.1.8.a Seed moisture content

With confirmation of initial seed moisture at 12% (w/w), the dry mass of any batch of seed was determined according to the following equation (all equations in decimal):

$$DM = M1 \times (1 - M_C1)$$

Equation 2.1 Seed dry mass

In which:

DM = Seed dry mass (g)

M1 = Initial seed mass (g)

M_C1 = Initial seed moisture content

The seed qualities inherent to hybrid 8366 seed, i.e. size, treatments (e.g. drying) and moisture content, were typical of those widely accepted by industrial dry-millers, and from which moisture content is increased for industrial-scale processing. Similarly, the seeds provided by Garst Seed Company, having been prepared i.e. dried using standard industrial procedures, were treated according to their moisture content upon delivery, using the methods detailed below.

2.1.8.b Short duration moisture conditioning

A short-stage temper procedure was investigated in order to determine the change in seed moisture over a range of holding times in an excess of water. Samples of 100 seeds at 12% initial moisture content were immersed in an excess of tap water for up to 40 minutes, and then drained and blot dried before weighing. Longer soaking times in an excess of tap water were avoided for reasons detailed previously (section 2.1.3). The final seed moisture content after conditioning i.e. wet mass, was calculated based on seed dry mass:

$$M_C2 = \frac{M2 - DM}{M2}$$

Equation 2.2 Final seed moisture content

In which:

M_C2 = Final seed moisture content (%)

$M2$ = Seed wet mass (g)

2.1.8.c Long duration moisture conditioning

The second method of raising seed moisture content was based on the traditional, industrial scale process of adding calculated volumes of water to achieve specific moisture contents. Typically, water volumes were calculated based on the initial mass of seed of known moisture content, and added to this seed in order to achieve final

moisture contents in the range of 15% to 25%. This method was used for raising the moisture content of small samples of seeds, held in sealable containers, to pre-determined levels.

Batches of seed were pre-weighed in order to calculate seed dry mass, based on an initial seed moisture content of 12%, and to calculate the volume of water required for the conditioning process. The added water was dispersed around the seeds, held in a sealed container, by shaking the sample. It was then left for 16 hours at room temperature to absorb the moisture. After 16 hours of conditioning, seeds had absorbed all moisture and were immediately processed. Required water volumes were calculated as follows:

$$\Delta W = W2 - W1$$

Using: $W2 = M_C2 \times M2$

And be rearranging Equation 2.2:

$M2 = DM / (1 - M_C2)$

The required volume of water for addition was calculated using:

$$\Delta W = DM / (1/M_C2 - 1) - W1$$

Equation 2.3 Volume of water addition

In which

ΔW = Change in seed water (g)

W1 = Initial seed moisture (g)

W2 = Final seed moisture (g)

2.2 Small-Scale Degerming Process Design

The design of the small-scale degerming process consisted of an analysis of large-scale dry-milling systems, and the incorporation of the major operating factors responsible for the separation of corn seed germ and endosperm. It was not intended to replicate the large-scale operating conditions on the small scale.

2.2.1 Degermer design

Beall Degermer manufacturers and dry-millers alike recognise the need to adapt to different seeds, and that no one set of conditions would suit all seeds or the requirements of all millers. This wide scope of operability and uncertainty in the detailed mechanics of the point of germ removal provided a wide range of design requirements and options for investigation.

The design of this degermer was to accommodate the very small quantities of transgenic seed available, to produce fractionated seed of a consistent quality, and to be capable of processing seeds with large variations of physical properties within and between the different varieties. The requirement of small sample sizes presented limitations on the reproduction of certain operating conditions, such as the high pressures which facilitated interaction between seeds, as observed in the Beall and disk mill degermers. However, those parameters which were not limited by sample sizes were built into the design of the small-scale degermer.

The variable factors of the small-scale degermer which were tested include rotor speed, clearance between the rotating and stationary disks, recycle, and the design and arrangement of the studs on the facing disks (stud geometry) (section 3.2.4). These factors, in addition to the separation process variable of roller-milling (section 3.4.2) were tested on a range of responses, such as product and waste stream oil content, using the method of factorial experimental design (section 2.3). The results of these experiments are provided in Chapter 4 and Chapter 5.

2.2.2 Separation process design

Experiments testing the early designs of the small-scale degermer resulted in the production of seed fragments containing free germ, which were identified by visual analysis. However, a better system of analysis was required, one which did not reply on human input, to reliably determine the extent to which germ was released inside the degermer. A mechanised process was devised for this purpose. It eliminated the slow and potentially biased method of human assessment. Similarly to the design of the small-scale degermer, large scale industrial processes were studied in the search of suitable operations for the separation of small quantities of corn seed components.

The large-scale separation of germ from particles of seed is achieved by the manipulation of the differences in density and workability of the seed components. The long separation process consisting of multiple operations and recycle streams, detailed in section 1.3, is unsuitable for processing small quantities of seed, in which minimal losses were essential. However, a greatly simplified and reduced scale process of similar design was conceptualised with the potential to satisfy the processing requirements. This process consisted of a roller-milling operation followed by sieving (Figure 3.15). The roller-milling of degermer output would, ideally, flatten the germ into large particles, and crush the non-germ fragments into smaller particles, both of which could then be separated via sieving.

For the development of a continuous process, additional equipment was required for the transfer of materials and enclosure of the process. These included a feed hopper with a method of agitation for feed flow rate control, a degermer collection chamber, and a connection between the degermer and the roller-mill to transfer the processing material in a continuous process.

2.2.2.a Feeder

The degermer feed rate was expected to affect the quality of degerming by varying the extent of interaction between the seeds and seed fragments. In order to closely control the operating conditions, it was intended to limit the mechanism of degerming to stud-seed interaction, and not seed-seed interaction. This was achieved by

manually feeding seeds into the degermer individually, and controlling the feed rate to approximate the residence time, which was typically no more than one second. By both visual and audible observation, whole seeds were fed into the device once the last seed had been processed. The occasional seed-seed interaction was observed, but this was mostly at lower levels of disk speed and clearances. In these instances, one seed or a large seed fragment became lodged, and the second seed knocked it out of its lodged position.

Process mechanisation was important primarily for the eradication of human input for operating consistency purposes, and also in planning for the processing of larger quantities of seed as would be required for the production of clinical trial quantities of product. A feeder was designed for the controlled addition of seed into the degermer, and investigated by testing variations in the power input, hence the frequency of revolutions, the pipe diameter and the position of the agitator within the pipe. Seed flow rate was measured either by counting the number seeds per minute, or measuring the time taken to process a given mass of seed, and was converted in kg/hour by using the average seed mass (approximately 0.24g). Control of the feed rate was not intended to be more accurate than that already tested by manual addition of seed. The variable parameters tested included the size of the outlet from the hopper, the shape of the agitator, and the speed of agitation.

2.2.2.b Degermer enclosure

The combination of the large centrifugal forces driving the seed through the attrition region, and the high impact between studs and seeds, resulted in seed fragments rapidly discharging from the device. This resulted in considerable product losses and was a hazardous laboratory operation. The collection chamber designed to surround the degermer (Figure 3.6(a)) reduced the level of these losses and improved safety.

2.2.2.c Roller-milling

A standardised mechanism of degermer output quality assessment was required which did not rely on visual analysis of germ fragments. Roller-milling was introduced into

the degerming process for this purpose. Within the dry-milling industry, roller-milling is widely used for germ-endosperm separation, is effective and relatively simple (section 1.3.3). Use of this operation limited deviation from existing technology, which it was hoped would both facilitate scale comparisons, and would be readily available commercially.

A manually-operated prototype roller-mill was first tested and was valuable for 'proof-of-principle' processing. However, it was not only physically demanding and capable of very low material flow-rates, but was also inconsistent between experiments, with regards to the different number of passes and gap settings between the rollers. For these reasons, a mechanised roller-mill was introduced into the process. It was tested over a similar range of processing conditions as the manual roller-mill in order to confirm that a similar output quality could be achieved.

Manual roller-milling

A standard kitchen pasta machine was used to roller-mill the output from the small-scale degermer. This mill consisted of two parallel steel rollers, 25.4mm (1 inch) in diameter and 127mm (5 inches) in length, both manually driven at equal speeds, and the gap between the two could be controlled by 0.2mm increments from 0.2mm up to 2mm. The surface of these rollers was smooth, which prevented the seed fragments from being gripped and forced through the gap between the two rollers. To increase the friction, and thus the rate of processing through the rollers, the rollers were scored using a sanding disk fitted to a power drill.

A simple approach was adopted for the roller-milling of seed fragments. The gap between the rollers was set according to the size of the seed particles. It was reduced to the point where all fragments did not pass through immediately without turning the rolls, and thus at which some were crushed upon turning the rolls, and all were processed. When the gap was reduced too much for some particles, it was not physically possible to process them through the rollers, either due to the insufficient force which could be generated manually using this device, or because of the lack of friction on the surface of the rollers. Under these instances, the gap was increased

until the larger fragments would pass through, and then gradually reduced until all seed fragments were processed through the minimum gap setting. Germ was observed to be separable using this proof of principle method.

Mechanical roller-milling

One manufacturer (Crankandstein, Marietta, GA, USA) built roller-mills to a variety of specifications for the purpose of rolling wheat in preparation for brewing beer. These roller-mills were not designed for roller-milling corn seed, and had not been tested on material other than wheat. However, with the available designs and possibility of modifications, there was the opportunity to transfer several aspects of large-scale, and proof-of principal small-scale processing, onto a motorised small-scale roller-mill. The design, with modifications, and operation, are detailed in section 3.4.2.b.

2.2.2.d Product transfer

Joining the separate unit operations speeded up the processing, and reduced losses through the increased containment and the reduced manual transfer of feed between operations. The major link between operations was between the degermer and the roller-mill. This link consisted of a tangential feed outlet from the degermer chamber which redirected the flow of particles, using air flow and gravity, into the roller-mill situated beneath the degermer.

2.2.2.e Sieving

In the large-scale processing of seed, sieving is most commonly the first operation which separates seed fractions after degerming (section 1.3.3). A large range of sizes are separated, the largest of which are recycled into the degermer, whilst the smallest are separated not processed any further. The range of sieve sizes used in this first separation was selected for the separation of degermed and roller-milled seed generated in the small-scale process.

The time required for completion of the sieving operation depended on the quantity of seed being processed per batch, and the rate of oscillation, i.e. sieve shaking. The rate of change of mass on each sieve was investigated using 100g of degermed and roller-milled seed. The mass of products collected on each of the sieves was measured every 5 minutes for 25 minutes until a constant mass was achieved on each sieve. The time given for the sieving operation to complete was determined using the data obtained (section 3.4.1).

2.3 Factorial experimental design

Elements of several of the large-scale corn degermers had been integrated into the design of the rotating-disk, small-scale degermer. Pilot runs using hybrid 8366 seed demonstrated that seeds could be broken and degermed, but rigorous experimentation was required to finalise the degermer design, and for the characterisation and optimisation of the process. Conventional experimental techniques were unsuitable for this purpose, since they involve changing one factor individually, whilst maintaining the other factors constant. When there are a large number of factors to consider, such as feed quality and degermer operating variables, this method becomes unfeasibly time-consuming, requires large quantities of material for the greater number of experiments, and shows no interaction between any of the factors. Factorially designed experiments, however, provide a means by which multivariable experiments may be designed, and the results analysed statistically, in order to assess the impact of a set of individual factors, and their interactions, on a single response parameter. This approach greatly reduces the quantity of material required, and provides a more rapid means of identifying the major parameters affecting the process, and their subsequent optimisation. More details of this approach to experimentation can be found elsewhere (Montgomery, 2000; Mount et al., 2003).

Initially, particularly in the absence of prior knowledge about the extent of influence of each factor, the number of factors may be too high to allow for a full factorial experiment design. The experimental limitations might be imposed by material or time constraints. In addition, it is possible that such detail on each of the factors is not

necessary, since some factors have a much smaller impact on the response of interest, than others. In this situation, the first experiment would rapidly identify the major factors, and thus eliminate the less influential factors from further study. The design of the next experiments would typically expand into a more complete factorial design experiment, investigating in greater detail the effect of these major factors, with the levels of the less influential factors fixed.

2.3.1 Experimental design

Use of factorial experimental design for the design and characterisation of the small-scale degerming process began with establishing the range of the variable factors, which had been built into the design of the degerming device, to be tested experimentally. The extremes of the range of each of these factors were set as the high and low levels to be tested experimentally, and the mid-points between these two levels were also incorporated into the experiment design for improved data reliability. Since both numerical (degermer speed, degermer clearance and seed moisture content) and categorical factors (recycle and degermer stud geometry) were incorporated into the experiment design, the required use of centre points for each of the factors affected the experimental design, and the levels of the categorical factors investigated. For example, whereas speed was a numerical factor and it was possible to test degerming quality at a centre point between any two selected levels of the factor, it was not possible to operate at a mid point between one and two recycles through the degermer. Therefore, the range of the categorical factors was established to enable processing at a mid point between the extremes. The high and low levels of recycle through both the small-scale degermer and the roller-mill were set to three and one passes, respectively, which enabled processing at the mid-point, or 2 passes. Also, to overcome the categorical nature of the degermer stud geometry, different levels of intermeshing between the studs were established so that the design of the geometry could be tested at a point which approximated the median of the two extremes (section 3.2.4.d).

During the design of the small-scale degermer and investigations into the important parameters which affected degerming quality, a range of factorial experimental

designs were implemented. Only one full factorial design experiment was applied to the testing of the small-scale degermer once its design had been finalised. Other experiments included a ½-fraction factorial design for the testing of different arrangements of studs inside the degermer, and ¼-fraction factorial designs, which were implemented once the degermer design had been finalised and the mechanised roller-milling operation had been incorporated into the degerming process. There was little difference between the design efficiency and data reliability between the qualities of the data generated by the ½-fraction and the ¼-fraction experiments. Additionally, by minimising the number of experiments required to determine the optimum operating characteristics for any given type of seed, it was possible that similar, low-fraction factorial experiments might be applied to the testing of transgenic seed, which was only available in scarce quantities.

2.3.2 Factors

The factors investigated in the design of the degermer were different to those tested in the characterisation and optimisation of the degerming process. The purpose of the first factorial design experiment, based on the small-scale degermer alone, was to establish the arrangement of different sizes of studs on the two opposing disks. This was the only experiment involved in the design of the degermer, and once the stud geometry was established, other factors tested in this experiment, detailed below, were carried forward into the next experiments involving the roller-milling germ-endosperm separation process.

2.3.2.a Degermer disk speed

Stud tip speeds in the small-scale device were required to reach 18.4m.s^{-1} to match the maximum speeds achieved in the Beall degermer (section 3.2.4.c). Selection of the minimum speed was based on there being sufficient power to break the seeds, for centrifugal forces to be sufficiently large to throw the seeds through the attrition region and out of the degermer, and for practical reasons, such as creating an air vortex to carry seeds through to the roller-mill.

2.3.2.b Clearance

The clearance range was selected such that it was not too large so that seeds would pass through unbroken, and not too small such that seeds would not enter the attrition region. Theoretically, based on the positions of centre radii (pcr) and the size of the studs, detailed below, the clearance could have ranged from 6mm to 8mm. At 6mm clearance, the studs would have intermeshed by 2mm, and at 8mm clearance, they would have been level:

Stud ring 3	**Stud ring 4**
Stud size = 3mm	Stud size = 5mm
PCR3 = 31mm	PCR4 = 39mm

In theory, the disk clearance of 6mm would have resulted in a distance of 10mm between the two outer rings of studs, measured from the centre of the studs on the surface of the disks. This distance was determined using Pythagoras' Theorem. Due to the sizes of studs used in these rings, this disk clearance would have resulted in a gap of 2mm between the studs. In practice, however, the gap between the studs was not large enough for operation without undesirable contact between the studs. This was due to the method of fixing the stationary disk in position on the clamp stand. The tightening of a screw into the clamp stand resulted in a slight twisting of the stationary disk in the horizontal plane. Since this twisting action occurred at the axis of the fixing mechanism, it was accentuated toward the outer edges of the degermer, near to the point where contact between studs was intended to be closest.

Increasing the disk gap to 7mm, resulting in studs intermeshing by 1mm, was sufficient in preventing contact between the studs. This clearance was set as the minimum between the disks, providing an intermeshing of 1mm. The converse of this situation was a gap between stud tips of 1mm in the horizontal plane, which was achieved with the disk clearance set at 9mm. It was predicted that further increasing this would have resulted in substantially larger numbers of whole seeds passing through the device unprocessed. The mid-point of the clearance range was the median of the former settings, in which disk clearance was set to 8mm and there was no intermeshing of the studs.

2.3.2.c Degermer recycle

The introduction of a recycle through the degermer was partly the result of observations of large scale processing, in which the largest particles in the output are recycled, and partly due to product quality observations when operating the small-scale degermer under certain conditions.

Within the large scale degermers, particularly the Beall and disk mill degermers, a large amount of work is done on the seed. The important details of this work are the high internal pressures which create significant seed interaction, and the longer residence time. When compared to the small-scale degermer, in which there was no internal pressure and the only type of interaction was that split second interaction between seed and seed particles, and the studs, considerably less work was done on the seeds. Using only small quantities of seed, it was possible to increase the level of work by increasing the residence time, either by creating a degermer with a larger disk diameter, or more preferably, by processing the output several times through the same device.

Introducing a recycling step into the operation of the degermer was also useful to overcome two issues observed with its operation under certain conditions. These were the incomplete processing of seed, given by the presence of whole seeds in the degermer outlet, and in the incomplete degerming of seed, identified by large fragments of seed consisting of whole germ attached to half of the rest of the seed.

Since not all operating conditions resulted in incomplete degerming, it was decided that recycle need not be extensively tested i.e. by testing large numbers of recycle. Therefore, to fit the requirements of factorial experimental design, three levels of recycle were tested. The minimum involved just one pass, the maximum three, and together with the mid-point value, these settings were considered adequate to indicate whether degerming was improved by increasing the recycle.

2.3.2.d Feed moisture content

There was a large variation in the reported ideal seed moisture contents and methods of moisture conditioning used in industrial degerming, discussed in section 1.3.4. Therefore, two methods of conditioning were tested. Both methods were reported to be suitable for achieving the target moisture contents required for good quality degerming.

The first method for raising seed moisture content involved soaking seeds in an excess of water. High and low levels of the time allocated to soaking were set at 34 minutes and 2 minutes respectively, which resulted in a mid-point value (18 minutes) approximating the median of the optimum soaking times reported for grit recovery (10 minutes), and germ and hull recovery (30 minutes) (Mehra and Eckhoff, 1997). The moisture contents attained approximated 13%, 16% and 18% for the low, middle and high levels of the factor, respectively.

Moisture conditioning for longer periods of time with controlled moisture addition was necessary for testing degerming quality of seeds conditioned to specific moisture contents. As widely reported, and supported by Beall manufacturers, optimum moisture content for degerming was 21% (w/w). Typically, the range of moisture contents varied from 18% to 22%. Therefore, the moisture content range over which the small-scale device was tested was 15% to 25% (w/w), which again resulted in the mid-point moisture content (20%) closely approximating the reported optimum moisture content.

2.3.2.e Roller-milling

The roller-milling variable, as described in section 3.4.2.b, was a combination of the two important characteristics of the operation, the gap between, and the number of recycles through the rollers. Although it was an important, independent operation in the degerming process, it was not necessary to investigate recycle with all of the other processing variables fixed. Unlike the degermer, once the seed had passed between the rollers operating with a fixed gap, very little additional grinding would have been achieved with recycling through the same gap.

The important mechanisms of the roller-mill were the reduction of particle size, which facilitated the release of attached germ, and the flattening of freed germ. Germ was less likely to be flattened without release from larger seed particles. Release of germ from larger seed particles was unlikely unless achieved during degerming, or during an earlier break through the rollers operating with a larger gap, as seen in large-scale grading processes. Therefore, three passes were tested with different gaps between the rollers, in order to provide this earlier break.

2.3.3 Responses

A means by which the degerming quality could be assessed was critical in the design and development of the small-scale degermer. Although a range of methods exist for the analysis of starch, oil and protein contents, which can also vary in complexity (Black et al., 1967; Junker et al., 1998), simplicity was paramount during the earlier stages of design, when the degermer and operating conditions were continually subject to improvements. Upon development of the separation process, more output streams were produced, and the quality each of these streams was important to the evaluation of degerming quality. The development of an appropriate method of product analysis is detailed in this section.

The products of the degerming process were analysed in terms of a range of different response. Without any prior knowledge of the potential success of degerming, the analysis of the output from the first sets of experiments included whole seeds, whole germ, germ mass and germ yield. Only once the germ-endosperm separation process was established did the responses change to the mass and oil contents of the different sizes of particles generated, and the combination of these responses in the form of the degerming evaluation factor.

2.3.3.a Counting whole seeds

The first batches of seed processed through the prototype degermers were degermed to varying degrees of success. It was not even known if it was possible to break open this seed, due to the hardness of seeds, and the very small grinding area through

which the seeds had to traverse. Therefore, the simplest indicator of seed degerming was to count the number of seeds which were broken, and the number which remained whole. Seeds would not be degermed if they remained intact. There was a slight grey area in this response, which was in the categorising of some seeds which were cracked, but remained mostly intact. For the purposes of degerming, these seeds were considered to be more intact than they were broken, and were therefore categorised as whole seeds. This method of broadly categorising simplified the analysis to benefit the identification of germ-releasing conditions (section 4.3.1).

2.3.3.b Counting whole germ

Once conditions had been identified in which most seeds were ground, visual identification of whole germ, and germ fragments separated from whole seeds, became the next best method of degerming assessment. Germ, when whole, could be identified by its oval shape. Broken germ fragments were identified either by their cream colour, or by the intensity of the pink colouration which was greatest on the germ than on the other seed components (Figure 3.16) (section 4.3.2). The pink colouration was used to identify seeds which had been chemically treated with fungicides and insecticides post harvesting (Captan 400 (fungicide), Allegiance (fungicide), Actellic (insecticide)).

Again, the identification of whole particles, this time the germ, was subjective in the identification of whole germ. Whole germs were very rarely identified. Instead, germ fragments were frequently broken off whole germ during processing. Therefore, the 'whole germ' category would more accurately be described as 'most of one germ', but 'whole germ' and 'germ fragment' were the two categories used in the assessment. The subjectivity lay in distinguishing between when a 'whole germ' had been sufficiently reduced in size to become a 'germ fragment'. To overcome this subjectivity, a method was required which would separate all fragments of germ, regardless of size, for the analysis of a germ mass. The first method tested was that of flotation.

2.3.3.c Germ separation by flotation

Germ-endosperm separation by density difference is extensively used in the corn processing industry. Differences were observed in the density differences between the components of seed used in process design (Table 3.1), and so separation of the components based on these differences was investigated on the smaller scale. Trial separations involved dropping degermed samples, containing free germ and germ fragments, into a sodium nitrate solution of 1.27 specific gravity (Peplinski et al., 1989; Wichser, 1961), produced by dissolving 93g $NaNO_3$ in 0.2L deionised water. The floating fragments, consisting mostly of germ were skimmed from the surface, and the solution was drained from those fragments collected at the bottom of the vessel. Both fractions were dried for 16 hours at 70°C (section 2.1.6.a), and their masses were determined. The mass of floating sample was taken as the mass of germ separated, and the seed fragments which sank were endosperm.

2.3.3.d Mass and oil content of fractions separated by rolling and sieving

A mechanical method of dry-separation of germ was much preferred to wet processing, due to the potential scale-up limitations and the loss of product to the liquid used for flotation. Roller-milling followed by sieving was the most likely combination of operations capable of relatively simply and successfully separating germ on the small scale. The design of this separation process is discussed previously (section 2.2.2.c).

A good and simple method of assessing germ separation achieved using the roller-milling and sieving process was in the mass of separated germ. Germ occupied approximately 12% by mass of the seed, and so the target of separation was to achieve this same proportion of the feed mass for the germ fraction. However, the purity of this fraction was also needed in order to establish the proportion of this mass which was germ, and not entrained endosperm. This purity was measured in terms of oil content. The large oil content of germ (85% of seed oil) provided the most definitive parameter available for the analysis of separation of degermed non-modified seed. The quantity and concentration of oil collected on each sieve in the output of the degerming process were used to determine which of the streams (i.e. sieves) were to be classed as waste streams, and which were product streams (Figure 3.14 and section

5.2). Additional assessment involved comparisons of the oil content of processed seed fractions with the gold standards achieved by the extraction from manually fractionated seed (section 2.1.3).

2.3.3.e Dimensionless analysis

Both mass and oil content of the various output fractions were important to the analysis of the degerming process. One of these parameters alone was insufficient to reliably establish the operating conditions required for successful degerming. Therefore, the two were combined in the form of a degerming evaluation factor (DEF). This factor was a dimensionless numerical index designed for use as a tool to characterise and optimise the processing conditions required for the production of a high oil content, low mass waste stream, and a high mass, low oil content product stream.

2.3.3.f Antibody content of degermed transgenic seed

Once the small-scale degerming device and germ-endosperm separation process had been optimised, transgenic seed was processed. Analysis of the degerming quality was carried out in terms of the mass, oil and antibody content of the different fractions produced. Antibody production and accumulation was targeted to seed endosperm. Since there was a greater difference in antibody content between the germ and endosperm components of transgenic seed than oil content, quantification of the antibody content in the degermed fractions provided a better means of analysis of the quality of germ separation than the analysis of oil content, by showing a more distinct difference between the waste and product streams. However, analysis of oil and antibody content facilitated the quantification of the extent of germ separation using the small-scale degerming process.

2.3.4 Analysis of data

Design-Expert ® (Stat-Ease Inc, Minneapolis, USA) software was used as a tool in the analysis of the experimental data and characterisation of the process. Statistical information was generated on the efficiency of the experiment design, the magnitude

of the impact of each of the factors and their interaction on the selected response parameter, the confidence in the model equation generated to fit the experimental data, and the prediction of the factor levels required in order to optimise the selected response parameter.

Results and Discussion

Chapter 3 - Design of the Small-Scale Degerming Process

3.1 Introduction

The design criteria for the small-scale degermer were based on the design of the large-scale degermers used in industrial dry-mills. It was intended to reproduce the conditions in the large-scale degermers which were essential for releasing germ from the corn seed, by the identification and integration of the operating factors which were pivotal to creating these conditions. Material factors, notably seed size, and the variation in seed sizes, were also important criteria in the design of a device which was to be capable of processing all seeds in a feed consisting of a wide range of seed types and sizes. Content of the seed components were analysed for the identification of a means to assess the quality of degerming, and potential methods of component separation, such as flotation or roller-milling. These two separation processes were tested on the basis of the different physical properties between corn components. One was tested to observe the separation that could be achieved based on density difference, whilst the other was tested to observe the degree of separation that could be achieved based on differences in component malleability and pliability.

3.2 Small-Scale Corn Degermer Design

Much of the small-scale design work was based on the principles of degerming using the Beall degermer. The design criterion was essentially to implement a high impact mill with the means to change the intensity of abrasion, and which would successfully work on every seed processed. Two prototypes were designed and built. Although the first was entirely unsuccessful, certain aspects of its operation highlighted the importance of several processing requirements, such as speed and clearance, and these were incorporated into the design of the second device. The second device was

initially slightly more successful than the first, but upon modification and identification of the favourable operating conditions, it was shown to successfully degerm corn seed.

3.2.1 Design criteria

For the recreation of process operating conditions at different scales, elements of an operation which are critical to its success must be identified and incorporated into the design of the replicated operation at the required scale. For the scale-up or scale-down of standard operations, such as mixing vessels used for purposes such as fermentation, precipitation or reactors, engineering equations exist for the replication of operating conditions. These are based on the geometry of the equipment and the design, position, and speed of the agitator to create the required fluid flow characteristics and equal rates of energy dissipation between the two scales (Boychyn et al., 2000; Neal et al., 2003).

The transfer of essential processing characteristics from large to small scales might involve additional operations for the recreation of conditions which are impossible to achieve on the small-scale in one single device. For example, for the separation of solids by centrifugation, smaller centrifuges cannot achieve the high levels of shear as experienced at the point of entry into the large centrifuges. In order to replicate processing conditions in a small-scale process, an additional operation was designed to subject the process stream to the same levels of shear as experienced in the large scale centrifuge (Boychyn et al., 2001). In terms of scaling down Beall degerming, a similar situation was expected since it was not possible to replicate all of the important operating conditions, such as seed pressure, seed interaction and rotor speed inside a single, small-scale device.

For the design of small-scale devices for the replication of processing conditions in unique large-scale operations such as the Beall degermer, there are no standard design equations. Additionally, the approach to select in the design is less apparent when the exact mechanism of operation is not known. Due to the uncertainty in the mechanism of degerming (section 1.3.5.a), all factors, from seed quality to the back

pressure exerted by the tailgate, were important contributory factors to the level of work done on the seed. It was considered that each seed would have been subjected to high levels of work, which was a function of power input, volume and residence time, resulting from substantial interaction with the rotor, other seeds, and the abrasion through the screen perforations and exit at the tailgate. The prototype degermer was designed with the intention of subjecting seeds to high levels of work, by crushing seeds either individually or in multiples, thus producing an additional crushing action by the interaction of seeds or seed particles, as they progressed through the small, annular degerming chamber (section 3.2.3).

Rotor speed was the major design criterion on which the second degermer design was based (section 3.2.4.c). It was intended to replicate the high levels of impact achieved in the Beall degermer by operating the rotating element at speeds which would approximate those achieved in the large scale degermer. In comparison with the design criteria for scaling up or down mixing vessels, of which the agitator speed, position and size ratios between the vessel and agitator are essential parameters for reproducing processing conditions on the small scale, it was not possible to closely replicate the geometry of the large-scale degermer on the small scale. This was due to the restrictions imposed by the categorical nature of the feed material i.e. numbers of whole seeds as opposed to an infinite range of liquid volumes, and the very small quantities available for processing. Moreover, it was not intended to replicate the conditions of the large scale Beall degermer, rather the production of a high quality degermed fraction was most important, regardless of the method used. However, insofar as studs in the Beall degermer were used, it was also intended to use studs in the small-scale design to mimic the point of impact between the rotor and the studs.

3.2.2 Mass and density of whole seeds and seed fractions

It was important to establish the range of material properties i.e. corn seed sizes, which had to be accommodated for in the design of the small-scale corn degerming device. It was also important to identify a reliable method of quantifying the success of their separation, including both processing methods for the preparation of material for analysis, and the most suitable parameters for analysis.

Germ and endosperm were known to have different mass fractions and compositions (Watson and Ramstad, 1987), which were quantified in order to determine the best means of separation and analysis (section 2.1). This section details the major physical and chemical characteristics of non-transgenic seed components, which were fundamental to the design of the degermer, the separation process and the analysis of the quality of separation.

The average mass, density and content of two high-quality (section 1.2.8) non-modified seed hybrids are illustrated in Table 3.1 and Table 3.2. The mass and oil content of the low quality non-transgenic parent seed (section 2.1.1) are detailed in Table 3.3. The data obtained was the most accurate that could be achieved within the limitations of manually separating the seed components, and thus were used as the 'gold standards' (section 2.1.3) by which the success of mechanical degerming was compared.

Differences were observed between all seed types in each of the characteristics analysed, including transgenic seed (section 6.4). The large difference in protein content between the two high quality hybrids was presumed to be a result of the different genetic background and purposes for use. Small differences were observed between the densities of the two components, and it was difficult to predict if the small differences in the sizes of high-quality non-transgenic seed would have any impact on the quality of degerming. Of all the differences observed, the following were most likely to impact upon the degerming quality and its assessment:

- The difference between seed component oil content was greater in hybrid 8342GLS/IT seed than hybrid 8366 seed
- Hybrid 8342GLS/IT seed, and the germ, were larger than hybrid 8366 seed and germ.
- Compared to high quality hybrids, inbred B73 seeds were smaller, and there was a substantially smaller difference between the oil content of the components.

Table 3.1 Hybrid 8366 seed and component mass and density, and oil and protein content

NON-MODIFIED SEED	HYBDRID 8366			
	Mass (g/seed)	Density (kg/m^3)	Oil (g/g)	Protein (mg/g)
Whole Seed	0.2382	1350	0.0372	1.49
Germ	0.0287	1250	0.1964	30.42
Endosperm	0.2074	1360	0.0054	0.70

Table 3.2 Hybrid 8342GLS/IT seed and component mass and density, and oil and protein content

NON-MODIFIED SEED	HYBDRID 8342GLS/IT			
	Mass (per seed)	Density (kg/m^3)	Oil (g/g)	Protein (mg/g)
Whole Seed	0.2453	1290	0.0315	22.42
Germ	0.0301	1180	0.2271	89.98
Endosperm	0.2206	1290	0.0023	7.84

Table 3.3 Inbred B73 seed and component mass and oil content

NON-MODIFIED SEED	PARENT B73 SEED	
	Mass (per seed)	Oil (g/g)
Whole Seed	0.2014	0.0269
Germ	0.0244	0.1749
Endosperm	0.1732	0.0160

The mass distributions of non-transgenic seeds are illustrated in Figure 3.1, Figure 3.2 and Figure 3.3. The high-quality non-modified seed mass fell within a narrower range than that of parent seed and transgenic seed (section 6.4), having been previously sized Medium Flat Medium (MFM). However, of this sized seed, there were approximately twice as many seeds over 50% larger than the seeds in the smallest category of size. Therefore, the range of seed sizes increased further still when seeds were not sized prior to processing e.g. parent seed B73, and so the design of the small-scale degermer was such that it could successfully operate on this wide range of seed sizes.

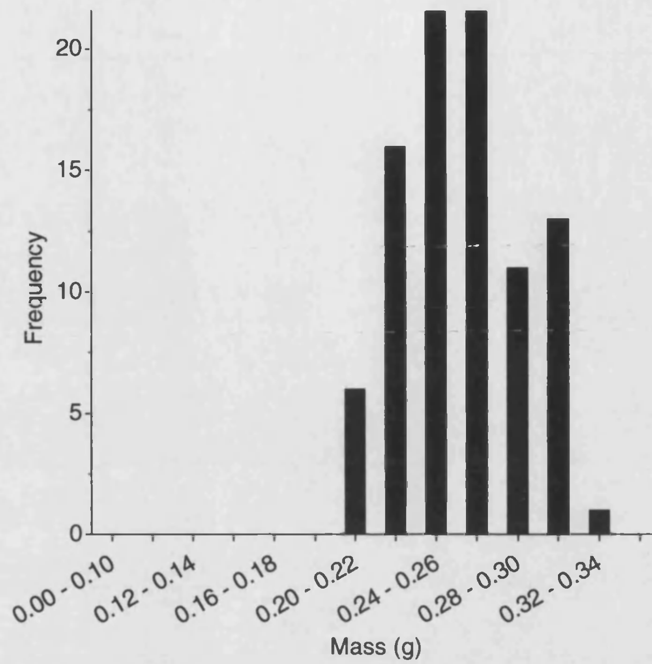


Figure 3.1 Hybrid 8366 seed mass distribution

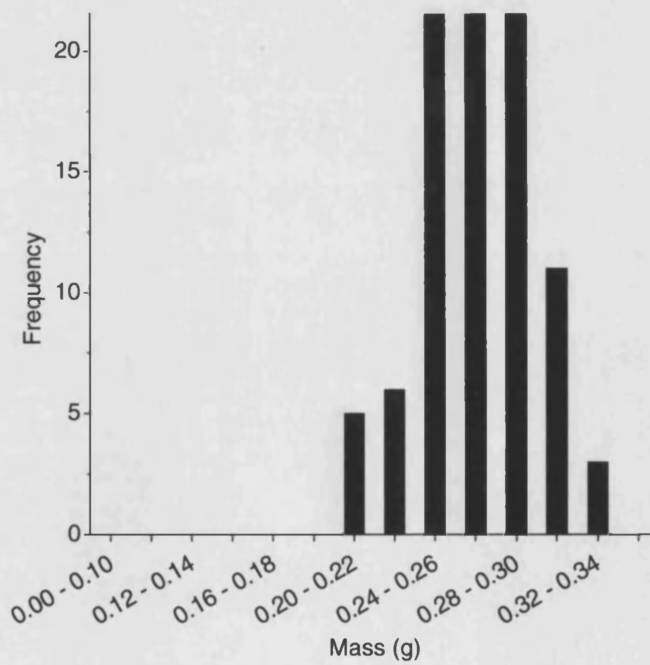


Figure 3.2 Hybrid 8342GLS/IT mass distribution

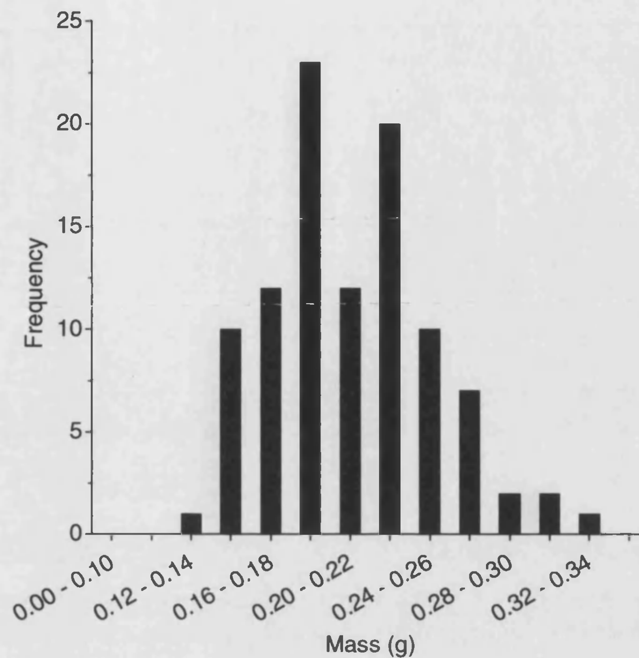


Figure 3.3 Inbred B73 seed mass distribution

The small difference in density between the two major components of the high-quality seed (<9%) was expected to present difficulties in their separation based on this physical property, by the application of the industrial methods. In addition, the density difference between germ and endosperm seed fragments produced during mechanical degerming was expected to be even smaller than the 'gold standard' values obtained by manual fractionation (above). Consequently, the possibility of separation based on density differences was tested using the method of flotation (section 4.3.3) in order to establish whether operations which separate germ and endosperm based on density differences could be applied to this system.

3.2.3 Prototype degermer design

The first prototype degermer consisted of a cylindrical copper rod mounted vertically on a motor shaft, and encased within a plastic cylinder (Figure 3.4). This configuration created an annular channel, or degerming chamber, through which it was intended for the seeds to pass. The flow of seeds was obstructed by studs of

uniform size and hemispherical shape, positioned, but not fixed, into both the rotating rod and stationary casing. The studs in fact consisted of spherical brass balls, 8mm in diameter. The studs were fitted into complimentary-shaped concave cuttings on the rotating shaft, and were held in place by the close proximity of the opposing surface i.e. the chamber wall. Studs closely fitted into holes in the chamber wall were secured in position by contact with the rotor on one side, and by contact with a secondary wall on the outside of the degermer.

Two designs of stud arrangements were tested on the surface of the rotating cylinder. The first consisted of three horizontal rows of studs, as in Figure 3.4(a), and the second consisted of one row of helically arranged studs along the length of the cylinder, illustrated in Figure 3.4(b). In both cases, three, equally distributed, horizontal rows of studs were positioned within the walls of the plastic chamber, such that one half of each stud was positioned inside the degerming chamber. Although contact between the studs in the chamber wall and the helical rotor design was not consistent due to the shape of the helical rotor, the rotor-stator clearance was too small for the studs held in the chamber wall to fall out of position.

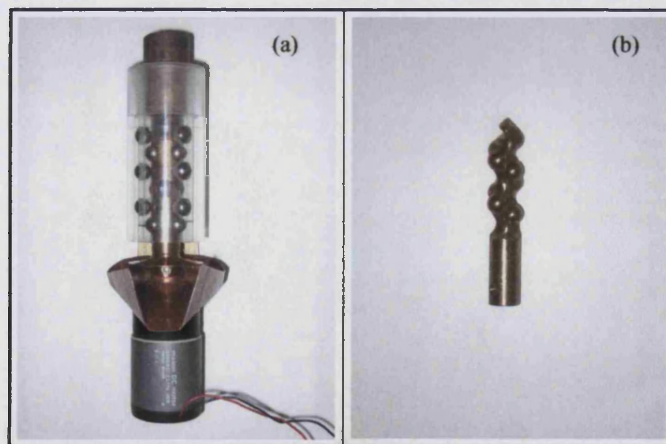


Figure 3.4 (a) First prototype degermer with horizontal rings of studs on the rotor, and (b) the second rotor design consisting of one helically arranged row of studs

The use of studs originated in the study of the Beall degermer design and operation. Therefore, in order to produce both mechanisms of degerming, as observed in the Beall degermer (section 1.3.5.a), semi-spherically shaped studs were selected to mimic the high-impact degermer interaction, and also to provide opportunity for seed-seed interaction by minimising the volume within the attrition region of the device.

A major problem with the design of the first prototype degermer (Figure 3.4(a)), with the horizontal rings of studs on the rotor, was that most seeds were too large (approximately 9mm×8mm×3mm) to enter the grinding region, and that the clearance could not be changed to facilitate entry and seed grinding. Those few seeds which were able to enter the attrition region frequently were not ground, but rested on the first ring of studs on the rotor. It was for this reason that the helical arrangement of rotor studs was tested, so that, using similar mechanised as the auger section of the Beall degermer, the corn seeds would be forced into the attrition region of the small-scale degermer. However, like with the first rotor design, if the seeds did not rest on the ring of stationary studs, they traversed the attrition region via the concave channel along the rotor, through which the gap between the rotor and stator was greatest.

The results using this prototype degermer were valuable for the identification of two important mechanical aspects of the degerming operation. The first was the need to allow seeds to enter the attrition region, by the designing of a larger cross-sectional area at the inlet through which the seeds could pass in order to enter the attrition region. The second was to then reduce this cross sectional area in order to for the rotor to do work on the seed, and to prevent the seed from passing directly through the degermer.

Possible solutions to the problem of seeds not entering the degerming region were the use of several stationary casings of different internal diameters, to suit the seed or particle size being processed. In this case, the studs would have had to be fixed into position, since opposing surfaces would no longer have kept them in place. Alternatively, a conical rotor could have been fitted into similar cylindrical casing, such that the gap between the rotor and stator would have gradually reduced from the large cross-sectional large area at the inlet along to the greatly reduced cross-sectional

area at the exit. Of the two, the conical rotor was preferable due to its capability of processing a large range of sizes of seeds in one single pass. The component parts of the device could also be fixed into position for the processing of each variety of seed, without the need for changing the casing to suit whole seed or seed fraction sizes. However, neither of these options were developed because the one design feature common to all of the degermers, that being the high speed of the rotating component, and thus the high impact between seeds or between the degermer and the seeds, was difficult to achieve using a rotor with a small diameter between the tips of the studs. The solution to increasing tip speed was to increase the diameter of rotating components which contacted seeds, by using a rotating disk design degermer.

3.2.4 Rotating-disk design

The design of a rotating disk degermer was originally conceived before investigating the different types of degermer used in industry, during which it was discovered that the disk application was already in use, in the form of the Entoleter and disk mill. The first design of a degerming device built in this style is illustrated in Figure 3.5(a):

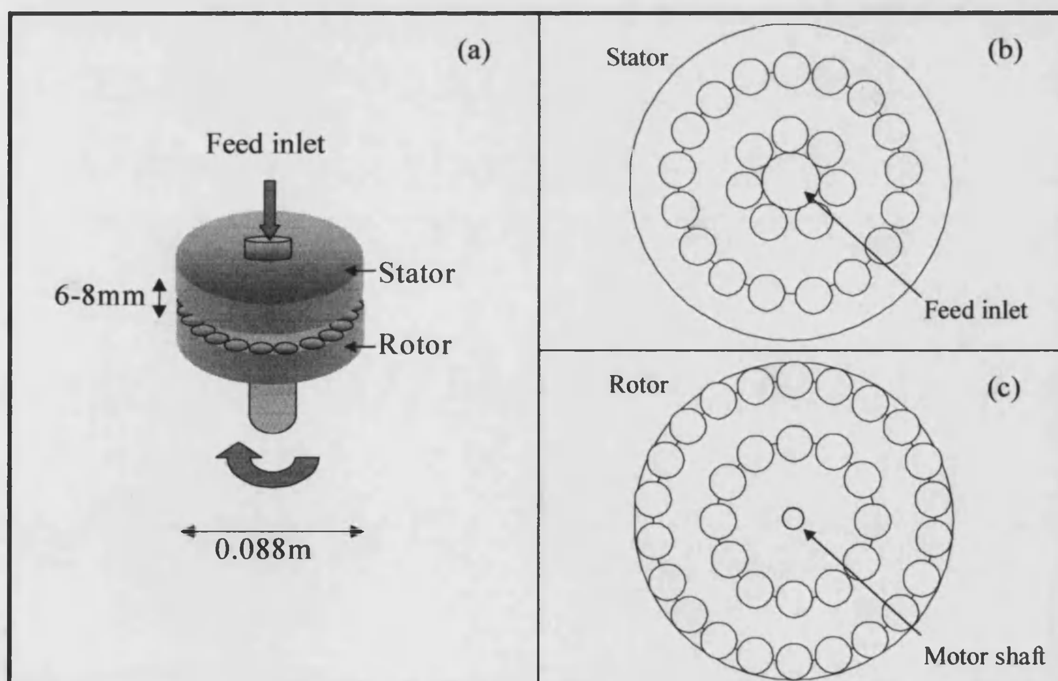


Figure 3.5 Sketch of the conceptualised small-scale disk degermer (a) working arrangements, and (b) stator and (c) rotor stud arrangements

The rotating disk degermer design was based on the findings of the prototype degermer and elements of the Beall degermer design. Not only did this solve the problem of increasing the stud tip speed, but also enabled a facile variation in disk clearance, and thus the degerming of a wide range of seed sizes by modification of the degermer volume and grinding intensity.

3.2.4.a Degermer design summary

The small-scale degermer consisted of two horizontally-mounted disks (Figure 3.6(a)), each with two rings of hemispherical studs on the facing surfaces (Figure 3.6(b) and Figure 3.6(c)). The lower disk was mounted on a fan motor drive, capable of rotating the disk at speeds up to 6000rpm, providing a stud tip-speed of $24.5m.s^{-1}$, surpassing the speeds achieved in the Beall degermer (Table 3.4). Due to the small quantity of material available (<15g per batch processed), it was not intended to replicate the head of pressure and back pressure in the small-scale device. The supposed mechanism of degerming, mostly by seed-seed interactions in the Beall degermer was substituted with a degerming mechanism based mostly on stud-seed interaction. The intensity of stud-seed interaction was varied by changing the gap between the disks in the small-scale degermer, and the speed of rotation of the lower disk.

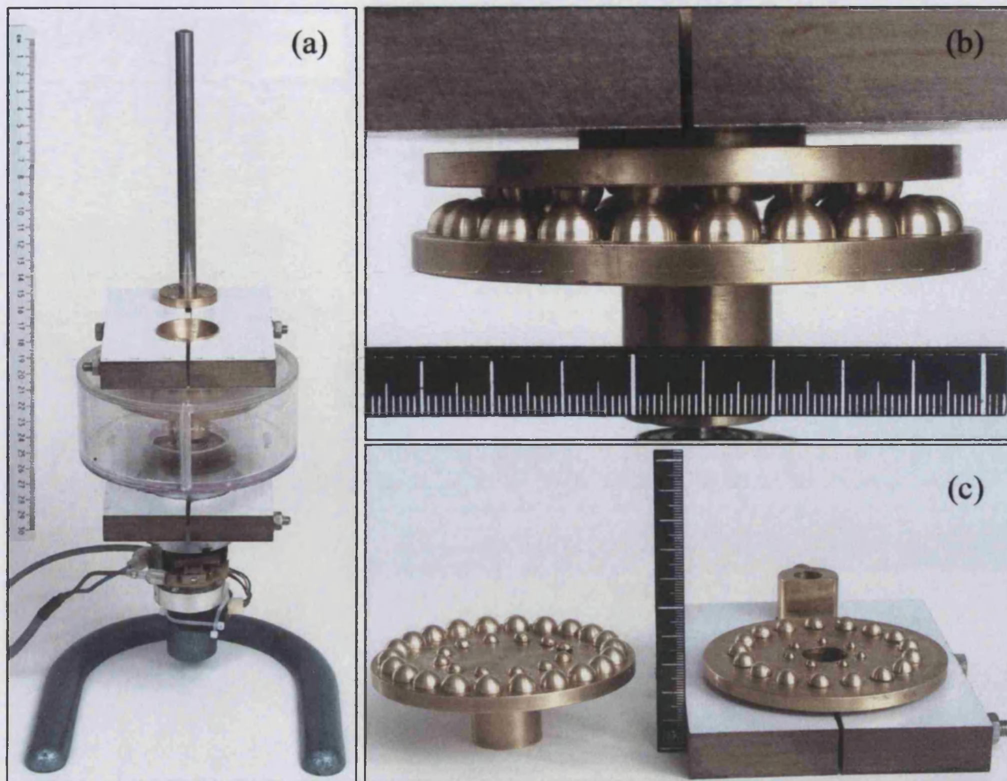


Figure 3.6 Small-scale degermer (a) contained unit operation, (b) intermeshed rings of studs, (c) increasing size of studs with distance from feed inlet

3.2.4.b Degermer operation

Individual corn seeds entered the small-scale device under gravity, via a centrally located inlet in the upper disk. The upper disk was held stationary, whilst the lower disk speed ranged from 0rpm up to 6000rpm, measured to the accuracy of the nearest 10rpm using a reflective optical sensor. The variable gap between the two disks was controlled by raising and lowering the upper disk to the required height above the lower disk. Three pairs of brass spacer bars, cut to the required heights (7mm, 8mm and 9mm), were inserted between the disks. The upper disk was lowered onto these spacer bars, and the screw clamp then tightened to hold it in place. Variations in disk clearance and the internal stud geometry, both of which affected the grinder volume, resulted in considerable variations in product size distribution and the quality of whole germ separation (Chapter 4). Seeds passed through the attrition region towards

the periphery of the device under centrifugal forces, where the products were collected in a perspex chamber.

3.2.4.c Disk speed

The tip speeds of studs varied across the length of the Beall degermer with the increasing diameter, and with different power inputs to drive the cone at different speeds. Higher rotor speeds were used in order to increase an hourly throughput, from approximately 1015kg - 2030kg operating at 700rpm, to 3175kg per hour operating at 850rpm. Other alterations to the degermer were also required to achieve these higher flowrates, such as the use of screens covering ¾ rather than ½ of the degermer cage. The higher tip speeds would have increased the magnitude of the impact between the rotor and the seeds. This impact was a contributory factor to the degree of success of degerming, and so was also an important factor to replicate in the small-scale degermer. The maximum tip speed, and hence the highest level of impact, was the most important tip speed to be determined. At 900rpm, which was the maximum sped of rotation, the highest tip speed of the Beall rotor was $18.4 m.s^{-1}$ (Table 3.4). This was the target speed for the outer ring of studs on the small-scale degermer, and was achieved by operation at 4500rpm (Table 3.5).

The speeds of stud rotation in the Beall degermer were calculated using Equation 3.1, in which r = radius (m), and N = revolutions per second (i.e. rpm/60). This equation was then rearranged for the calculation of the speeds of rotation of the small-scale degermer required to achieve the same stud tip speeds as the Beall degermer (Equation 3.2).

$$\text{Speed } (m.s^{-1}) = 2 \times \pi \times r \times N$$

Equation 3.1 Beall rotor speed

$$\text{Speed (rpm)} = (N \times 60) / (2 \times \pi \times r)$$

Equation 3.2 Small-scale rotor speed

In Table 3.4 below, the small end and large end descriptions of the Beall rotor refer to the end of the rotor at the feed inlet and at the tails, respectively (see Figure 1.3). The inner and outer radii of the small-scale degermer (Table 3.5) refer to the inner and outer rings of studs on the rotor (Figure 3.5c).

Table 3.4 Large-scale Beall degermer tip speeds ($m.s^{-1}$) at standard operating rpm

BEALL ROTOR RADIUS	ROTOR STUD TIP SPEEDS ($M.S^{-1}$) OPERATING AT:		
	700rpm	*800rpm*	*900rpm*
Small end (0.09m)	6.6	7.5	8.5
Large end (0.195m)	14.3	16.3	18.4

Table 3.5 Small-scale degermer rotor disk speeds (rpm) to match Beall rotor speeds (rpm)

SMALL-SCALE ROTOR RADIUS	ROTOR SPEEDS (RPM) TO MATCH BEALL TIP SPEEDS:		
	700rpm	*800rpm*	*900rpm*
Inner (0.022m)	2864	3273	3682
Outer (0.039m)	3500	4000	4500

The rotating disk degermer was initially tested for 'proof of concept' degerming, using the simplest methods of analysis, including the counting of unprocessed seeds and free germ. It was thought that the higher tip speeds obtainable using this disk design would crack open the seeds and release germ more successfully than operation at lower speeds, or by using the rod-shaped degermer. The use of studs in the first prototype was carried forward into this design. Initially, it was intended to use studs of universal size (5mm) which would have allowed for a minimum clearance between the disks of 6mm. However, the difficulties encountered in achieving 100% grinding with this initial stud arrangement and design resulted in their modification (section 3.2.4.e).

The seed fragments produced inside the degermer were collected in a perspex chamber surrounding the two disks (Figure 3.6(a)). This chamber consisted of two

halves of a contained cylinder, attached by a hinge on the curved, vertical surface, enabling the two halves to wrap around the degermer and be fastened together at the other side. Once fastened shut, the only two holes were at the top, above the stationary disk and which encircled the feed inlet, and at the bottom, beneath the rotating disk and surrounding the motor shaft. It was held in position by closely fitting between the upper disk and the formica support, which attached to the clamp stand and around the feed inlet, thus holding the disk in position.

3.2.4.d Stud design

Experiments using the helical-stud, cylindrical prototype degermer showed that a gap of sufficient size was essential to allow seeds to enter the grinding region. However, too great a clearance would have resulted in insufficient stud-seed interaction, and degerming. Just as it was not possible to intermesh the studs in the cylindrical prototype degermer with a helical arrangement of studs, stud intermeshing would also not have been possible with a spiral arrangement of studs on the opposing faces of the disks. Consequently, rings of studs were introduced to both the rotor and stator (Figure 3.6(b) and Figure 3.6(c)), with their positions of central radii arranged to allow the studs on each disk to intermesh.

The annular arrangement and intermeshing of studs maximised the stud-seed interaction by reducing the volume within the attrition region, and by increasing the degree of obstruction to seeds passing through the device. Due to the higher speeds and forces created inside this device, compared to the first prototype degermer, these studs were secured in position into both disks using Loctite®, a semi-permanent adhesive (Henkel Loctite Adhesives Limited, Welwyn City, Hertfordshire, UK). A 2mm cylinder was worked out of the same brass material used in the manufacture of each hemispherical stud. These cylinders were closely fitted into holes of the same dimensions drilled into each of the disks, and secured using a permanent Loctite adhesive, once the stud design had been finalised (section 4.3.3).

3.2.4.e Disk design modifications

It was found that even with the option of intermeshing the rings of studs on the two disks, processing of seed was prevented by the inability of seed to enter the attrition region. This was due to one of two reasons. Firstly, the studs in this first ring were too close to the inlet, thus preventing seeds from accelerating to a sufficient speed to be forced through the narrow gap that remained between these studs and the disks, in order to be cracked upon interaction with the studs. Alternatively, the seeds were too large and simply blocked the entry into the degerming region. It was often the case that degerming was prevented by a combination of these factors. Increasing the clearance between the disks to a gap which enabled seed to enter the attrition region also resulted in a gap which was sufficiently large for a small proportion of seeds to be discharged mostly unprocessed. With the intention of processing small numbers of seed, none were to be discharged whole. To overcome this problem, the design of the studs on the two disks was modified to facilitate the entry of seeds into the attrition region of the device.

The stud arrangement, a factor called termed geometry since it refers to the internal geometry of the degermer, was tested over three levels, in order to investigate the extent of the improvements in degerming by use of a greater range of stud sizes. The lower level of this factor consisted of all studs of equal sizes (Figure 3.5). The upper level consisted of a profiled design of the studs such that on each disk, the stud size increased with distance from the feed inlet (Figure 3.6(c), Figure 3.7 and Figure 3.8). The mid-point between these two levels consisted of the outer two rings of equal sized studs (5mm), and the inner two rings decreasing in size with proximity to the feed inlet (ring 1 stud size = 2mm, ring 2 stud size = 4mm). This profiled-design internal geometry was shown to improve the quality of degerming (section 4.3.3.c).

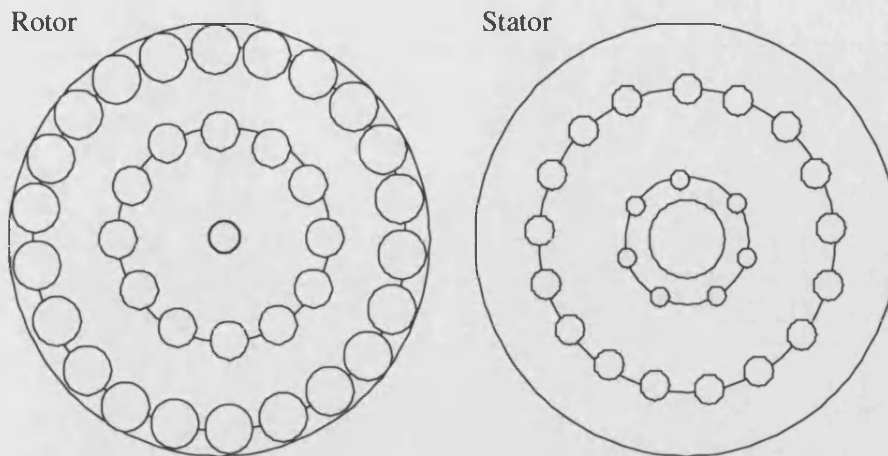


Figure 3.7 Sketch of the profiled design of the degermer rings of studs

Figure 3.8, drawn to scale, illustrates the obstruction presented to the seeds by the studs upon passage through the gap between the disks, when clearance was fixed at 9mm. Continuation of process investigations using other arrangements of studs was unnecessary since this arrangement had been shown to be capable of grinding all seeds and releasing whole germ.

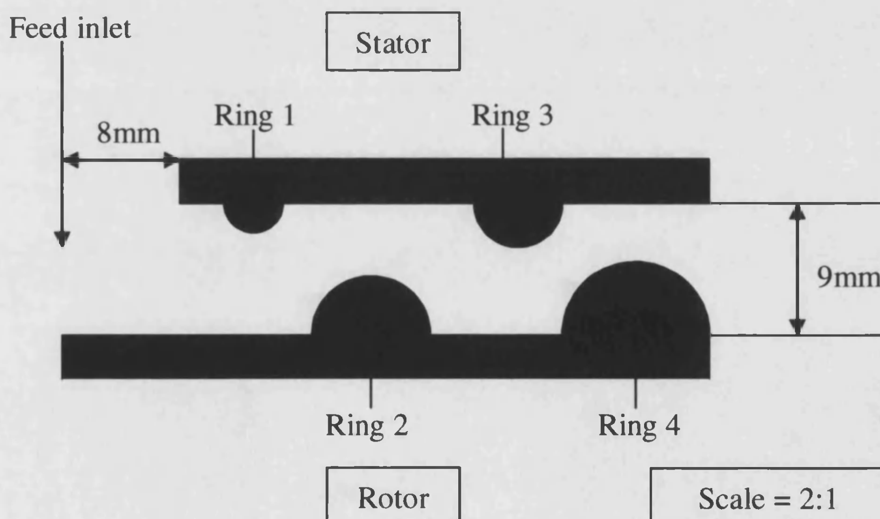


Figure 3.8 Finalised design of stud internal geometry, illustrated with a disk clearance of 9mm

3.3 Degerming Analytical Parameters

3.3.1 Oil assay

The method of oil extraction (section 2.1.7.b) was investigated using the method of factorial experiment design (section 2.3), in order to determine the required mixing time, and the necessary solids (ground corn) to liquid (hexane) ratio (Appendix B). A compromise was sought between two conflicting interests. Whereas a larger liquid to solid extraction ratio increased the quantity of oil extracted from each sample, it diluted the oil concentration, detrimentally to the assay reliability when using the larger hexane volumes. The converse situation generated values of sample oil content which were lower than the true value, but did not sacrifice assay reliability by diluting the oil too greatly. Therefore, an extraction ratio of 1:5 was selected on the basis of reasonably high levels of extraction whilst still providing an oil concentration in solution above the minimum.

3.3.2 Oil content

Germ occupied approximately 12% by mass of the seed, and contained approximately 85% of the seed oil. The 'gold standards' for degerming were determined by analysing the quantity of oil that could be extracted from manually separated germ and non-germ fractions. These are illustrated in Table 3.1, Table 3.2 and Table 3.3. An example of the magnitude of the difference in oil content between the components is given for hybrid 8366 seed in Figure 3.9, below. Germ was richer in oil than endosperm, which facilitated the analysis of the quality of separation based on the difference in oil content between the two major, separated components. The high oil content of germ also made it tougher and more malleable than the endosperm. This facilitated the separation of germ by the application of small-scale designs of conventional separation methods i.e. roller-milling and sieving (section 3.4).

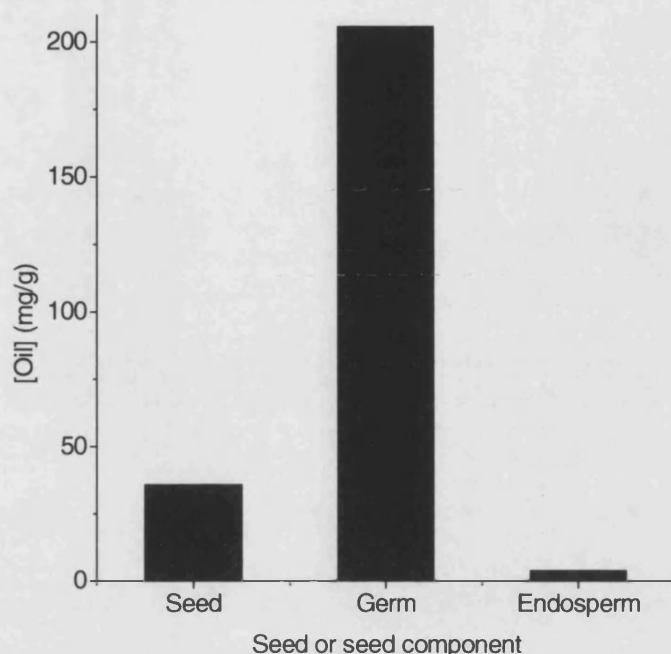


Figure 3.9 Hybrid 8366 seed and component oil content

Oil content of the product and waste streams was expressed as a percentage by mass of the respective streams, as the total oil mass contained in these streams, and as a percentage of the total oil measured in each operation of the degerming process (Appendix A(ii)).

Seed hybrid 8366 was richer in oil content than hybrid 8342GLS/IT. However, the difference in oil content between the germ and non-germ fraction was greater in 8342GLS/IT than in hybrid 8366. A greater difference in component oil content would facilitate the identification of operating conditions resulting in good germ separation in the degerming process, but might also inaccurately indicate a better quality of degerming than that achieved using varieties of seeds with a smaller difference in component oil content. Therefore, it is possible that these differences in seed properties between different seed types might have affected the reliability of this means of analysing degerming quality, for the comparison of degerming quality

between different seed types. However, sample oil content remained the best means of analysing the quality of germ separation from endosperm, since a large difference in component oil content was observed for all types of seed processed.

3.3.3 Protein content

Corn germ, the embryo of the seed, contained significantly more protein than the non-germ components. Antibody production was targeted to the endosperm of transgenic seed, and the germ was shown to contain very little antibody compared to the endosperm (section 6.4). Additionally, the endosperm of transgenic seed was shown to contain very little protein other than the product (Epicyte Pharmaceutical). Consequently, separation of the germ was valuable in the removal of endogenous seed proteins from the process stream, and analysis of this removal was potentially another useful parameter in the evaluation of the degermer separation capabilities.

The 'gold standard' of germ separation in terms of protein content was determined using a sample of the same manually fractionated material that was used in the analysis of oil content (Table 3.1 and Table 3.2). The proportional difference in protein content of hybrid 8366 seed components, illustrated in Figure 3.10, was not as great as the difference oil content. Protein content of the components of one variety of transgenic seed were also measured, in order to determine if the selected line used in transformation, and modification of the seed, resulted in substantially different protein contents of whole seeds and seed components. The difference in protein content of the components of transgenic seed (HOY3 seed protein, germ: 90.23mg/g, endosperm: 9.96mg/g) was also less than the difference observed in the oil content (HOY3 seed oil, germ: 224mg/g, endosperm: 8mg/g). Furthermore, the difference in antibody content between the germ and endosperm was shown to be greater than the difference in oil content (see below). Therefore, with two better analytical parameters available, protein content was not used as an analytical parameter in subsequent experimentation.

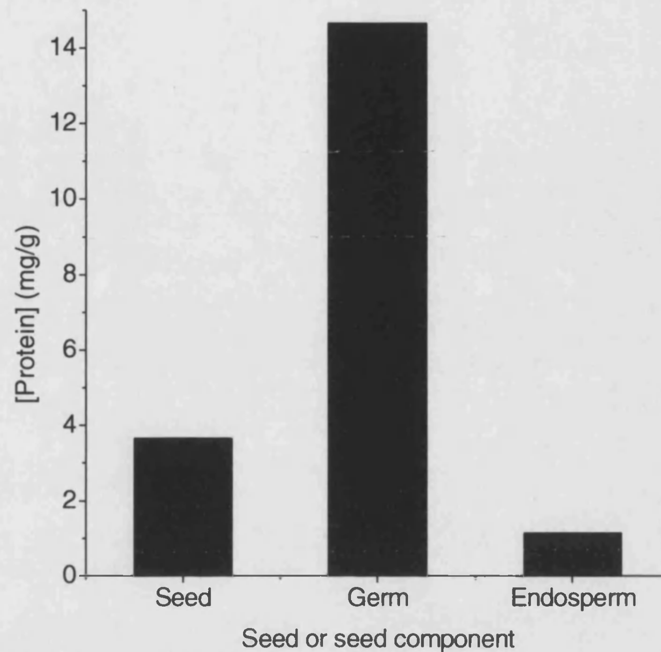


Figure 3.10 Hybrid 8366 seed and component protein content

3.4 Separation Process Design

Seed processed through the small-scale degermer alone was not separated into two suitable waste and product streams, one consisting of germ and the other endosperm, either by particle sizing (Figure 3.11 and Figure 3.12) or by the method of flotation. Therefore, a germ-endosperm separation process was designed composed of roller-milling and sieving (Figure 3.15) (section 2.2.2).

3.4.1 Sieving

Either sieving or roller-milling alone could not separate germ and endosperm. The two operations were very closely linked, and the value added by the roller-mill to the separation process was established in the comparison of the oil content of the sieve fractions generated before and after roller-milling.

Sieves of the same apertures as those used in the first sieving operation, post-degerming in industrial processing, were used in the separation of the products of the small-scale degermer. 100 corn seeds were processed through the small-scale degermer and then sieved. The results illustrated in Figure 3.11 show that particle sizing did not separate the seed components, measured in terms of mass and oil contents. The germ and endosperm fragments of this same sample were manually separated from other seed fractions and sieved separately (Figure 3.12). The mass distributions of these two fractions were very similar to the mass and oil distributions in Figure 3.11, demonstrating that additional process operations were required for germ-endosperm separation.

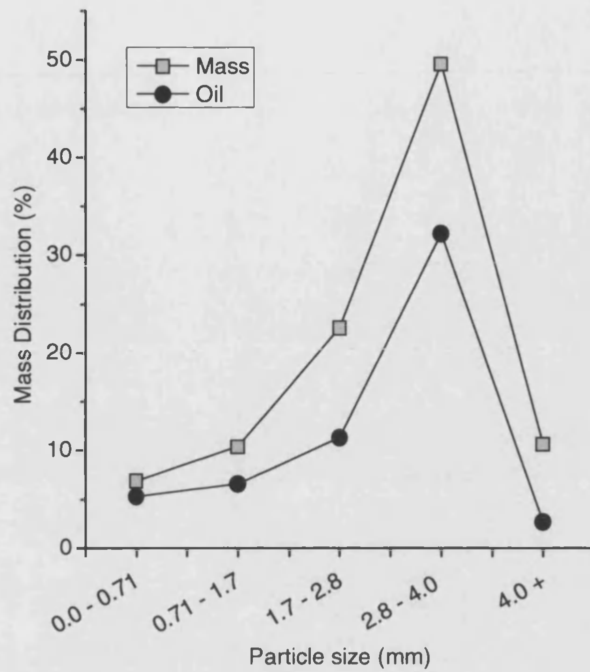


Figure 3.11 Mass distribution of degermed corn seeds

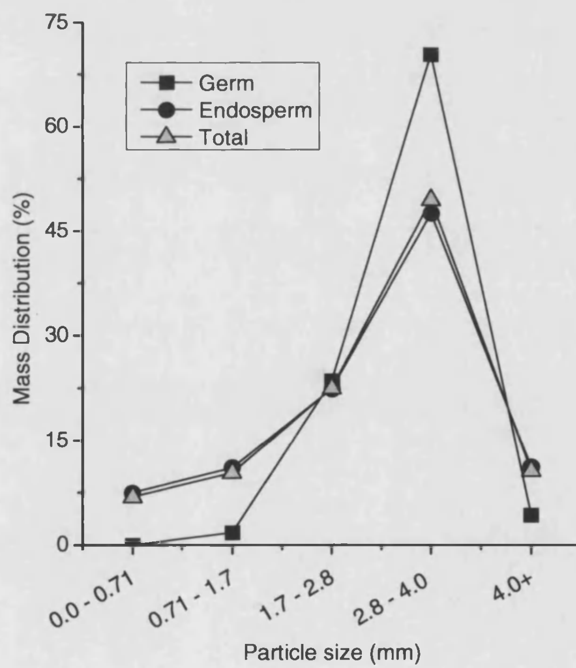


Figure 3.12 Mass distribution of degermed and manually separated corn seed components

The time required to achieve constant mass on each of the sieves was determined in order to standardise the operation before further experimentation, using 100g of seed fed through the degermer and roller-mill. The bulk of seed fraction movement between the sieves occurred with the first 10 minutes, as illustrated in Figure 3.13. Thereafter, changes in the mass of products on each sieve were much smaller and approximately constant by 25 minutes of sieving. Therefore, the time for sieving 100g of seed was set to 20 minutes, to ensure constant and reliable masses produced. Upon reduction of the feed mass to approximately 12g (50 seeds), sieving time was reduced to 10 minutes.

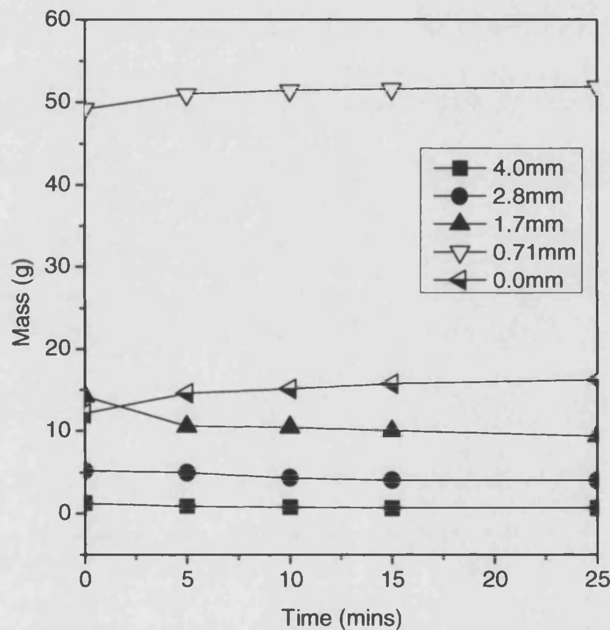


Figure 3.13 Sieving time required to achieve constant mass

3.4.2 Roller-milling

Two other options, besides the method of flotation, existed for the germ-endosperm separation, as practised in the large-scale mills. These were roller-milling and sieving, or the use of gravity separators and hydrocyclones. The latter two processes were based on the density and air-resistance differences between the two fractions. Since manipulation of density differences proved unsuccessful during flotation

(section 4.3.3), density based methods of germ-endosperm separation were not investigated any further. The more successful application of density-based operations in the dry-milling industry was a result of the vastly increased number of operations and recycle loops (e.g. Figure 1.2), which break down larger seed fractions into the constituent seed components in stages, enabling their purification in subsequent separations. Therefore, for the purpose of designing a short and effective process, roller-milling followed by sieving were selected as the unit operations for the separation of germ and endosperm.

The large-scale roller-milling separation processes are used for the scouring of degermer products, created by the speed differential between the rollers, for the release of hull and attached germ from endosperm to produce valuable, large, pure endosperm fractions. Mostly it is not intended to grind endosperm into flour. Flour is instead a by-product of processing. In this study, scouring for germ release and production of large grits was not the objective of small-scale roller-milling. Instead, small-scale roller-milling was used to grind endosperm into flour and squash the germ into larger platelets for separation via sieving.

The same sieves as used in the particle sizing of degermer products were used in the particle sizing of seeds processed through the small-scale degermer and roller-mill. Early experimentation using the manual roller-mill indicated that the oil content of corn fragments, post-degerming and rolling, increased with particle size (Figure 3.14). The processing conditions used to generate these results were selected based on earlier work which demonstrated favourable operating conditions, using the method of flotation for germ-endosperm separation. The larger particles consisted mostly of whole germ and germ fragments, as illustrated by the photograph in Figure 3.16, and constituted only a small fraction of the total seed mass. Smaller seed particles, collected on the lower sieves, contained significantly less oil than the particles collected on the upper sieves. Separation of those fractions rich in oil and low in mass would effectively be separating a large proportion of seed germ from the process output. Therefore, these results illustrated that the small-scale degerming process was capable of separating germ and endosperm. The sieves most suitable for allocation to the waste and product streams changed during the characterisation and optimisation of

the process, as the quality of degermer output improved by the application of optimised processing conditions. This is discussed in more detail in section 5.4.

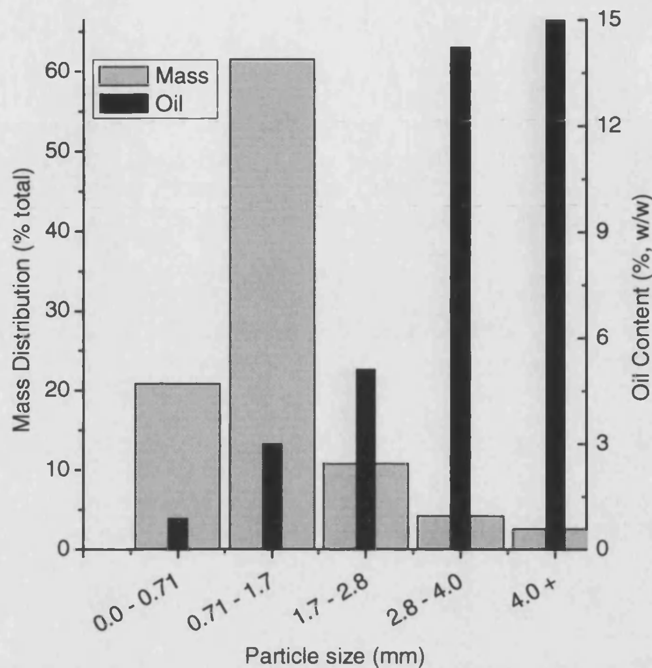


Figure 3.14 Proof of principal roller-milling for germ-endosperm separation

The oil content of the germ-rich fraction was compared to the oil content of manually separated corn germ and floated degermed samples. The germ-rich fraction of the mechanically degermed seeds consisted of the products collected on the upper two sieves. The average oil content of these fractions was 15.4% (w/w), which was significantly higher than the endosperm rich stream (3.0%), and although this was lower than the manually degermed seed germ oil content (19.6%), it compared favourably to the average oil content of germ fractions produced using the method of flotation (11.8%). The next stages of process development were to mechanise the time intensive and physically demanding roller-milling operation, and to determine the extent to which germ isolation could be achieved using this method.

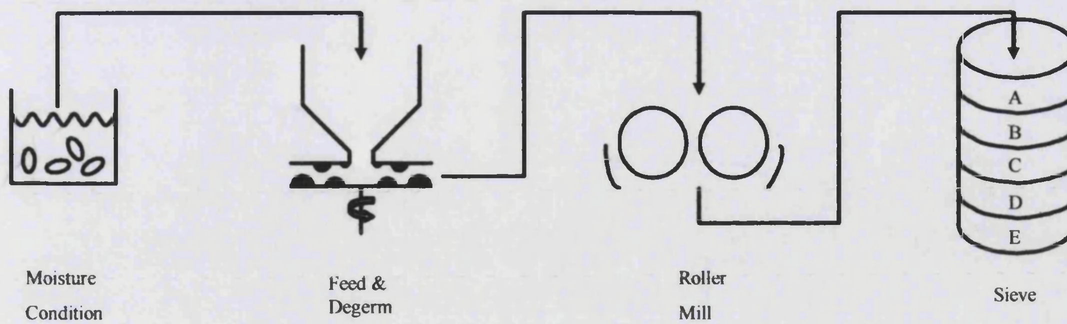


Figure 3.15 Small-scale corn degerming process flow diagram

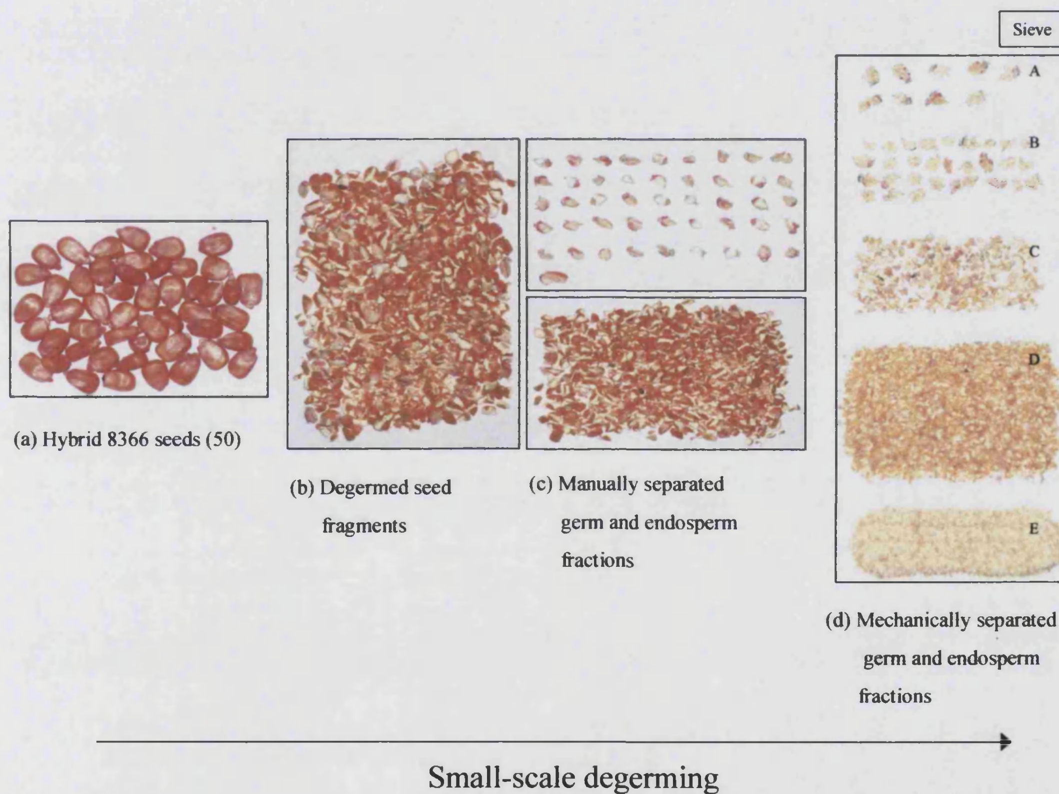


Figure 3.16 Photographs of (a) 50 hybrid 8366 seeds, (b) processed through the small-scale degermer, (c) then of the manually fractionated germ, and (d) after processing through the roller-mill and set of sieves

3.4.2.a Manual roller-milling

The number of recycles required to enable processing through the minimum gap between the rollers of the manual roller varied according to the particle size of the degermer output, which in turn, varied according to the degermer operating conditions. Certain seed particles, notably the yellow (vitreous) endosperm, were very hard and were frequently discharged out of the degermer as relatively large particles. These could not physically be crushed using this type of mill unless the gap between the rollers was gradually reduced (section 2.2.2.c). However, germ fragments were easily crushed and observed to remain mostly intact, whereas the endosperm fragments were eventually ground to flour. Sometimes as many as six recycles were required in order to enable the processing of all of the material through the smallest gap between the rollers. A factor which contributed to the need for large numbers of recycles was that the cylindrical rollers were small and their surfaces were smooth, thus there was no grip between the seed particles and rolls. In addition, because the roller-milling device was small, only small quantities of material could be ground at any one time, and thus only small fractions of seed fragments of each sample were fed through the roller-mill at each gap setting. Finally, the considerable force required for grinding caused irreparable damage to the device. These observations became the focus in the design specification of a mechanised small-scale roller-miller.

3.4.2.b Mechanical roller-milling

The impracticalities of manual roller-milling fuelled the search for a commercially available, motor driven roller-mill. Small roller-mills, mostly used in preparation for home baking, were available, but not the specifications required, which were:

- Large rollers (i.e. >25mm) to prevent seed particles collecting between the rollers
- Rollers with textured surfaces for gripping feed particles
- Variable gap between rollers (minimum approximately 20μm)
- Sufficiently powerful motor for grinding the hard seed particles

Design

The small-scale roller-mill purchased from Crankandstein (section 2.2.2.c) was modified in order to suit the requirements of milling corn fragments. The final design consisted of two 0.15m diameter rollers, of 0.045m diameter (Figure 3.17). The surfaces of both rollers were rough in texture. One roller was connected to a variable speed power drill (1050W) using a universal joint coupling, whilst the other was eccentrically mounted, providing for a gap range between 0.3mm and 3mm. Two annular channels were cut into the ends of the motor-driven roller, into which 4mm cross-section O-rings were fitted (Dichtomatik Limited, Derby, UK). These O-rings were sufficiently wide for contacting the other, eccentrically mounted roll, whilst providing for a roller gap which was wide enough to allow for a reduction in the size of particles and the release of seed components, and still not completely grinding these samples into flour. The eccentrically mounted roll was designed with small teeth at one edge, which improved contact with one of the O-rings. The two rollers therefore rotated at equal speeds, and over a range of gaps (0.3mm up to 1.2mm). The smallest gap, which was larger than that used in the prototype roller-mill by 0.1mm, was limited not by the O-rings, but by the position of the eccentric mountings.

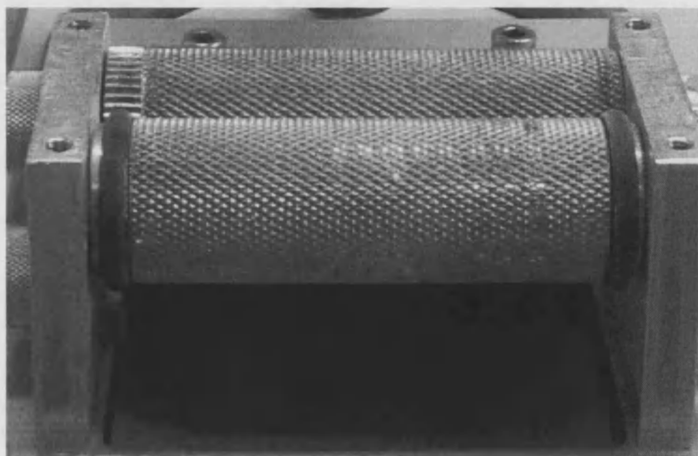


Figure 3.17 Mechanised roller-mill

For safety reasons, the small-scale roller-mill and motor shaft were both housed in perspex chambers, and these were secured onto the same formica surface to which both the roller-mill and degermer were secured. Four legs at the corners of this rectangular surface raised the equipment off the bench, thus providing space beneath the roller-mill to collect the seed particles. Feed entered the roller-mill via a slot cut into the top of the roller-mill housing across the length of the rollers, and exited beneath the mill via an area, cut out of the formica, approximating the length and breadth of the internal dimensions of the mill.

Experimentation

It was not possible to assume that this mechanised mill would perform to exactly the same standard as its manual predecessor. This was because variation in one or more of the operating characteristics (e.g. roller speed, clearance or surface texture) between the two mills might have resulted in differences in the product quality. Consequently, it was necessary to establish the operating conditions, required to achieve a high-quality of germ-endosperm separation, by experimentation.

It was important to minimise the differences in operating characteristics in the transfer of processing information from the manual model to the mechanised design. For example, the physical difficulty in the operation of the manual device resulted in recycling the seed fragments at least four times in order to process all of the material through the smallest gap available. Therefore, when operating the mechanised mill, it was important to assess the impact of recycle and varying the gap between the rollers.

Using the motorised roller-mill, there were no limitations on the minimum number of recycles, since seed fragments of any size and component were easily ground as a result of the vastly increased power supply to the mill. Therefore, grinding seed fragments through the crucial minimum gap between the rollers upon the first pass became possible. However, multiple passes with different gaps between the rollers remained a valuable aspect of processing, in order assess the extent to which attached germ was released during post-degerming processing. No more than three recycles were sufficient to indicate the importance of this recycle, which also served to reduce

the risk of germ breakage by excessive processing. The speed of roller rotation was not considered to be at all important in these experiments, since the important work done on the seed was in the crushing of the sample, and not shearing, as in the industrial mills.

Operation

The gap sizes between the rollers were established by determining the minimum gap which could be fixed between the rollers (0.3mm), the maximum that could be set whilst the O-rings on the motor-driven roll remained in contact with the variable position roll (1.2mm), and then selecting the median (0.75mm). With one pass, the degermed seed was passed through the smallest gap. Two passes and the seed fractions were first passed through the median gap, then the smallest. For three passes, seed was passed through the largest, the median and then the smallest gap.

Investigation into the effect of roller-milling, as a single factor in the germ-endosperm separation process, consisted of setting a high, a low and a mid-point value for the gap and recycle factors. These two factors were combined, so that each sample would pass through the minimum gap possible. The combination of these factors, illustrated below, also enabled a rapid determination of optimum post-degerming operating conditions for germ-endosperm separation.

Table 3.6 Combination of roller-mill gap and recycle

FACTOR LEVEL	RECYCLE	GAP (MM)		
		Pass 1	Pass 2	Pass 3
High	3	1.2	0.75	0.3
Mid-point	2	0.75	0.3	
Low	1	0.3		

3.4.3 Feeder

A prototype feeder, consisting of a conical cardboard funnel attached with tape to a pipe of known internal diameter, and a motor driven agitator held vertically from

above, was tested for its capabilities of providing a constant seed feed rate (section 2.2.2.a). Samples of the results of testing various combinations of agitator design and speed, and hopper outlet size, are given in Appendix F.

Several problems were encountered with the automated feeder. Seed flow rate was irregular and was difficult to control due to the irregularities in the sizes and shapes of the seeds. Manual modification of the agitator to suit the design was not perfect, and frequently resulted in a slightly different agitator to the design specifications, leading to incorrect positioning in relation to the hopper and pipe surfaces. Even the slightest gap between the agitator and the outlet pipe was observed to trap seeds, which would either block flow into the degermer, or jam the agitator into position. On several occasions, seed flow was stopped by just one large seed blocking the aperture at the exit of the hopper, or by one or two seeds blocking flow inside the piping at the tip of the agitator, or simply because the seeds were arching around the inlet and not being subjected to any agitation. On other occasions, using any combination of the variables tested, a torrent of seeds would pour into the device. Only a small number of conditions (emboldened in the tables in Appendix F) could achieve the target seed flow rate of 60-80 seeds per minute, which approximated 0.86kg/hour to 1.15kg/hour. However, as indicated by the error, these conditions were very unreliable. The prospect of implementing an automated seed feeder was therefore abandoned because it was not possible to control the flow rate. Feed was often either blocked, or surged, which could potentially have caused damage to the degermer. Manual feeding of the seed into the process was continued for subsequent experiments.

A positive result of the research into the feeder design was that a vessel was designed and built to facilitate the feeding of seeds into the degermer (Figure 3.18), and which also prevented the loss of seed fragments from the small-scale degermer via ejection through the feed inlet. This hopper was designed such that it supported a motor from above, if required at a later stage for the possible agitation of seeds using agitators manufactured with greater precision. This hopper and the complete small-scale degerming process are illustrated in Figure 3.19. Should it prove unfeasible to mechanically feed seeds at a consistent rate using this method, the same mechanism

as used by combines in the planting of maize could be used to achieve a very accurate flow-rate.

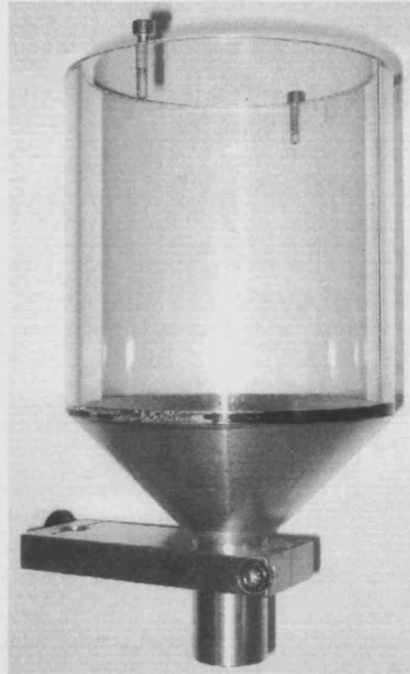


Figure 3.18 Seed hopper

3.4.4 Process enclosure

Previously, products from the degermer were contained in the surrounding chamber, and manually fed into the roller-mill once degerming was completed. In order to allow degermed seed to pass directly into the roller-mill, the chamber was modified by opening into a pipe, which in turn was connect to a delivery chamber that opened up across the length of the roller-mill feed inlet. The complete, enclosed system is illustrated in Figure 3.19.

The pipe from the degerming chamber was positioned tangentially in order to capitalise on the vortex of air and seed particles created by the high speeds of degermer operation. This vortex was significantly less powerful at the lower speeds of disk rotation, and thus a perspex disk with fitted vanes was attached to the bottom of the rotating disk to increase the turbulence within the chamber. Additionally, its

positioning near to the bottom of the degermer chamber served to reduce losses from the degermer chamber, around the motor shaft.

Figure 3.19 The contained, small-scale degerming process

3.5 Summary

The finalised small-scale degermer consisted of a rotary disk design which could operate at high speeds and a range of clearances between the two disks, enabling the testing of a large variation in the intensity of attrition. This was an important requirement for successfully degerming seeds of a predicted large range of sizes. The separation process consisted of a roller-mill followed by a sieving operation. The

major principles of operation of the manual roller-mill i.e. equal roller speeds, multiple recycles and processing though the minimum gap, were transferred to the mechanised roller-mill, in addition to the several aspects of processing which were identified as areas for improvement i.e. larger rollers with a textured surface. The process was enclosed, therefore improving safety and reducing losses, by the addition of a hopper above the degermer, and housing to capture and transfer seed fragments from the degermer and into the roller-mill.

Chapter 4 - Characterisation of the Degerming Process

4.1 Introduction

Seed moisture content was an important parameter in the degerming of corn seed. As such, its quantification and control and were both essential to experimentation. Two methods of raising seed moisture content were tested (section 2.1.8). The first, involving the soaking of seeds in water, was used in the characterisation of the degerming process. The second method involved the addition of a calculated volume of water to raise seed moisture content to a specific level, and was applied during process optimisation using the mechanised roller-mill (section 5.4). Degermer operating parameters were varied according to the limits of the design, and the predetermined levels for investigation (section 2.3).

Analysis of the product quality of the small-scale rotating disk design degermer initially involved the counting of whole seeds and whole germ in the output. Two different methods of separating germ and endosperm were tested, both of which produced two streams distinguishable by their content. One method was flotation, which separated the seed fragments immediately after processing using the small-scale degermer. Analysis of the two streams produced using this method of separation generated results from which the design of the small-scale degermer was finalised. The other method was roller-milling followed by sieving, which became an integral part of the degerming process. Analysis of degerming, using both methods of germ-endosperm separation, involved the determination of the mass, oil mass, oil concentration, and the percentage of total oil contained in the product and waste streams.

4.2 Seed Moisture content

4.2.1 Sample preparation

To enable manual fraction of seed for analysis, seed moisture content was raised to soften the seed. It was important to check that this moisture conditioning did not result in substantially different moisture contents after drying, lest the higher moisture contents diluted the oil or antibody measured during analysis. The rate of drying, and the final moisture content of seeds which had been moisture conditioned for different periods of time were investigated (Appendix A(ii)). The results showed very little difference in the pattern of drying between the seed batches which had been moisture conditioned for different lengths of time, and that sample mass during and after drying was dependent on the initial mass, and not the time the seed was soaked in water.

4.2.2 Drying requirements

Seed dry mass was determined experimentally for confirmation of seed moisture at delivery (12%), and to establish the required drying times to achieve the seed dry mass. The results (Appendix A) showed that the mass of seeds could be reduced by 12% by heating at 100°C for 6 days. The trend of falling seed mass was set to continue beyond 144 hours. In comparison to the batch of seeds dried at 80°C, the predicted loss of seed moisture, additional to that accounted for by the 12% initial seed moisture content, was believed to have been more of a result of the excessive drying temperatures and burning of the seed, rather than loss of additional seed moisture. Based on these results and the standard application of large-scale drying procedures used to dry this seed to 12% moisture content (section 2.1.8), the delivery seed moisture content was accepted as 12%. Since it was impractical and unnecessary to experimentally determine seed dry mass of every sample, Equation 2.1 and Equation 2.2 were applied to moisture content determination of all samples of seed, and were used in combination with Equation 2.3 for the calculation of water volumes for the controlled elevation of seed moisture content.

A standard drying practice was established to eliminate excess moisture from seed fractions. These fractions, which were produced either manually or mechanically, were dried typically after moisture conditioning, and prior to mass and density, and oil and protein content analysis. A balance was required between the desired rapid moisture loss, elimination of the bulk of the free moisture, and avoiding heat damage to the seed. Since the exposed surface area of seed fractions was much greater than for whole seeds, both temperature and drying time were reduced. The drying temperature for the processing of hybrid 8366 seed during process design and development was set to 70°C for a period of 72 hours,.

For the processing of transgenic seed, the drying temperature was further reduced to 37°C to avoid damaging the antibody. In addition, the drying period was also reduced from 72 hours to 24 hours, in order to rapidly analyse large numbers of degermed samples. All seed types which were compared, were processed and prepared for analysis using identical conditions (i.e. 24 hours at 37°C). Mass and fraction content data obtained prior to this point were not included in the comparison between varieties of seeds, since it was possible that different fraction moisture contents affected this data.

4.2.3 Raising seed moisture content

The effect on seed moisture content of soaking batches of hybrid 8366 seed in water, and in triplicate, is illustrated in Figure 4.1. The moisture contents achieved by soaking seeds in water for the predetermined times according to the experimental design requirements (section 2.3.2.d), were determined used these results. After 30 minutes of soaking in an excess of water, moisture content increased to 17.3%. Moisture content increased most rapidly during the first 10 minutes of soaking, by almost 4%. Beyond this point, moisture content increased at a constant rate of approximately 0.08% per minute. Using this method of raising seed moisture content, soaking seeds in water in order to achieve the low, middle and high factor levels of this factor for 2 minutes, 18 minutes and 34 minutes, raised the seed moisture content to 13.0%, 16.4% and 17.6%, respectively. Based on the experimental accuracy, these values were approximated to the nearest whole percent. However, since seed

moisture content did not increase linearly with the soaking time, the results of the factorial design experiments are presented in terms of the holding time in water.

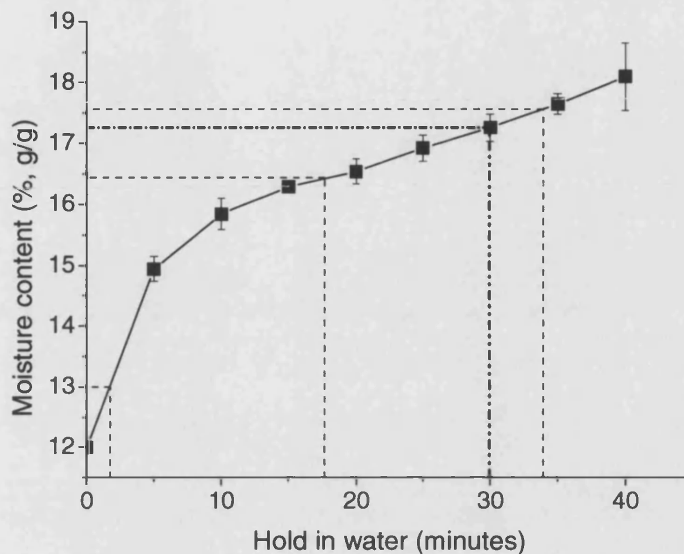


Figure 4.1 Hybrid 8366 seed moisture conditioning (error of triplicate samples)

This data shows that it was not possible to rapidly achieve higher seed moisture contents (ca. 20%) using this method of soaking in an excess of water, such as that achieved by Mehra *et al.* (Mehra and Eckhoff, 1997). By adding a calculated volume of water to seeds and lengthening the moisture conditioning time, it was possible to achieve higher and more accurate seed moisture contents, though this was at the expense of the benefits of short-duration moisture conditioning (section 1.3.4). The overall impact of the elevated moisture content was assessed using the standardised methods of analysis, and was shown to improve the quality of degerming (section 5.4). The effects of these two different methods of moisture conditioning on the quality of degerming, however, were not directly comparable, since they resulted in different final seed moisture contents.

4.3 Small-Scale Degerming

The method of factorial experimental design was selected as a means of acquiring process information from experiments involving large numbers of process variables (section 2.3). These variables were incorporated into the experiments testing the design of the small-scale degermer and the germ-endosperm separation process, its characterisation and its optimisation.

A 20-run, ½-fraction factorial experiment with 4 centre points was designed to test the effect of 5 degermer variables on a range of responses, detailed in Table 4.1, when processing batches of 30 seeds.

Table 4.1 Factors investigated using different response parameters and the method of flotation for germ-endosperm separation

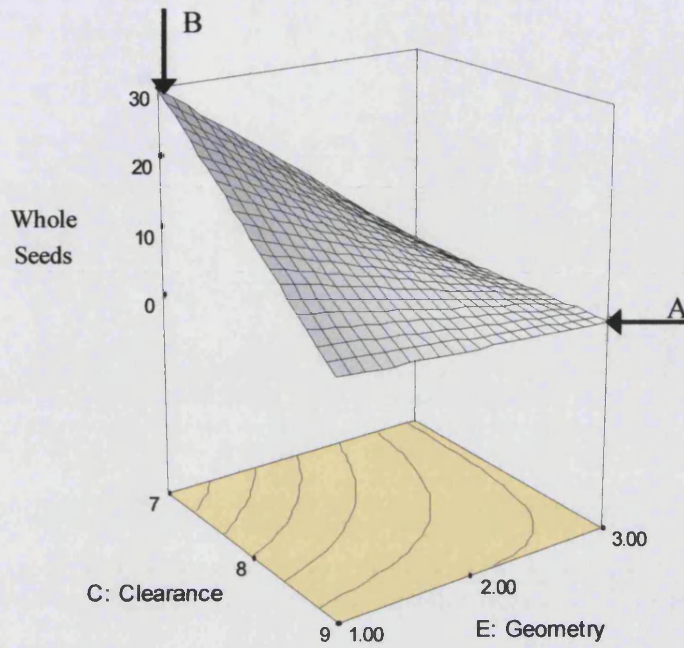
FACTORS	RESPONSES	FIGURES
Disk speed	Whole seed output	Figure 4.2
Disk clearance	Whole germ output	Figure 4.3
Degermer recycle	Germ mass (%, g/g)	Figure 4.4
Stud geometry	Germ oil (%, g/g)	Figure 4.5
Moisture content	Germ oil (g)	Figure 4.6

4.3.1 Whole seed output

The response surface design (Figure 4.2) was created in the Design Expert 5® software. It graphically represents the response of whole seeds in the degermer output as a function of degermer disk clearance and the geometry of studs in the degermer. Disk speed, recycle through the device and holding time all had very little impact on this response parameter over the range of the factor levels selected. These parameters were therefore fixed (degermer recycle: 1, degermer disk speed: 20.42ms^{-1} (5000rpm),

moisture content: 18%) at conditions which facilitated work on the degerming operation and, in the case of moisture content, more closely approximated the condition of seeds used in large scale degerming.

Figure 4.2 illustrates the range of conditions, located to the right of the contour on the base of the diagram, over which the small-scale degermer might operate without producing any whole seeds in the output. Degermer disk clearance, the internal stud geometry and the interaction between these latter two factors were the major factors which affected the quality of degerming in terms of the degermer output of whole seeds. Grinding was most successful by operation of the degermer at the highest levels of both of the factors illustrated on the X and Y axis. The closer operation tended toward the point of low levels of clearance and geometry arrangement (B), the likelihood that the level of grinding would diminish, and whole seeds would appear in the output, increased sharply. This was not entirely due to seeds passing through the degermer unbroken. It was often the result of seeds not entering the attrition region due to the obstruction caused by the inner ring of large studs, and the lack of momentum and centrifugal forces which carried seeds into and through the attrition region. This was the first indication of the limitations imposed by the use of studs of a uniform size.

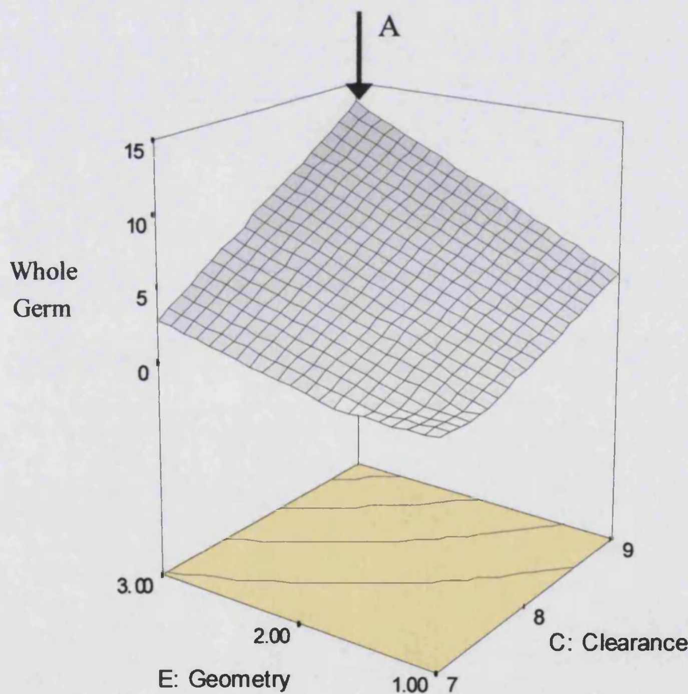


Constant Operating Conditions		
Speed = 20.42 m.s^{-1}	Holding time = 34 minutes	Degermer recycle = 1

Figure 4.2 Effect of clearance and stud geometry on the release of whole kernels from the degermer

4.3.2 Germ output

The response surface in Figure 4.3 shows a single point of operation (A), with respect to the two major variables of clearance and geometry, at which whole germ release was maximised (14 germ out of a potential 30). This point was situated at the highest levels of both clearance and internal geometry. The steepness of the response surface indicated sensitivity of the response to small changes in either of the two factors. Although it is often required that processes are operated at conditions which yield an output of a consistent quality, no such condition was available, and this was represented by the relatively steep gradient of the slope at most points along the response surface. Therefore, it was more desirable to operate under conditions which maximised the separation of germ. This point of maximum germ release also occurred within the range of conditions which resulted in 100% grinding of seeds (Figure 4.2).



Constant Operating Conditions		
Speed = 20.42 m.s^{-1}	Holding time = 34 minutes	Degermer recycle = 1

Figure 4.3 Effect of clearance and stud geometry on the release whole germ from the degermer

4.3.3 Germ separation by flotation

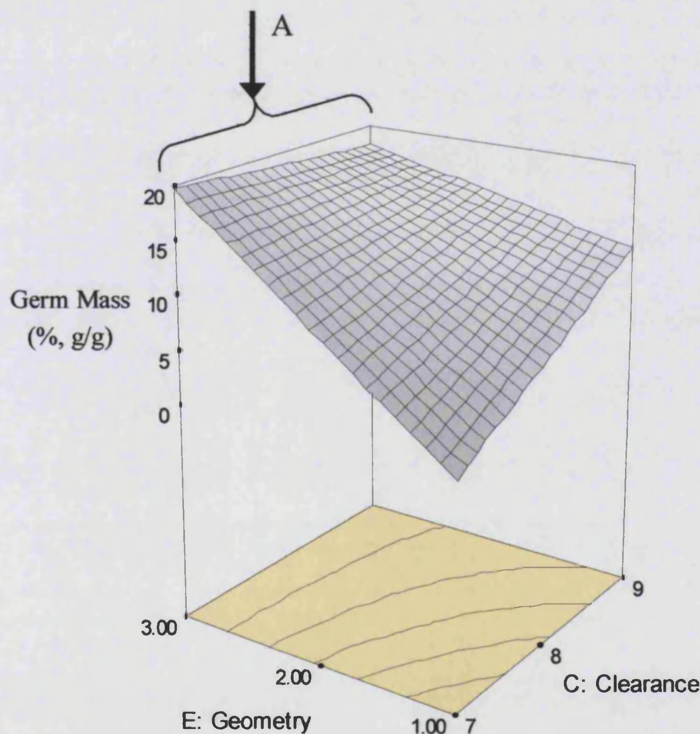
Germ was separated using the method of flotation as described in section 2.3.3.c. This simple method of separation, supported by visual observation and oil content analysis of the germ fraction, provided information which enabled the identification of design features which could be permanently incorporated into the design of the small-scale degerming device. However, this method was also limited in its application due to its poor efficacy, detailed below, and particularly in its poor suitability to the processing of transgenic seed (section 4.3.3.c)

It was observed that flotation did not perfectly separate germ and endosperm. Some whole germ and germ fragments were observed to sink into the endosperm fraction, whilst some non-germ fragments of corn seed floated with fragments of germ. The major cause of the failure of this of germ-endosperm separation was the incomplete separation of germ from the seed during degerming. The heavier endosperm fragments which were attached to the lighter germ fragments reduced the difference in density between the two. Other factors observed to be affecting separation by flotation was the surface tension of water and the attachment of air bubbles to the seed fragments. Both of these issues were mostly overcome by vigorously shaking the mixture, but this was not observed to improve the subsequent separation quality of germ and endosperm.

The small-scale degerming device did not provide the same amount of scouring of germ as achieved in the large-scale Beall degermer. Consequently, the germ fragments would not have been as pure as those produced in the large scale, in which flotation separation techniques were more successfully applied. It was not even possible to successfully detach all endosperm from germ in the manual degerming of seed. Whether or not it was a result of incomplete separation during manual or mechanical degerming, the method of flotation did not meet the required high standards for the small-scale degerming process, and so alternative methods were sought (section 4.4).

4.3.3.a Germ stream mass

With degermer operation at any level of clearance and the high level of stud geometry, indicated by the range of conditions represented by (A) in Figure 4.4, there was only a small difference in the mass of waste stream (i.e. germ mass) produced, expressed as a percentage of the feed mass. This demonstrated that the impact of geometry on this response parameter was much greater than the impact of clearance. However, this method alone provided no information about the quality of the waste stream. In order to quantify the success of degerming and the quality of the waste stream produced using this method, the oil content of the floated fractions were analysed.

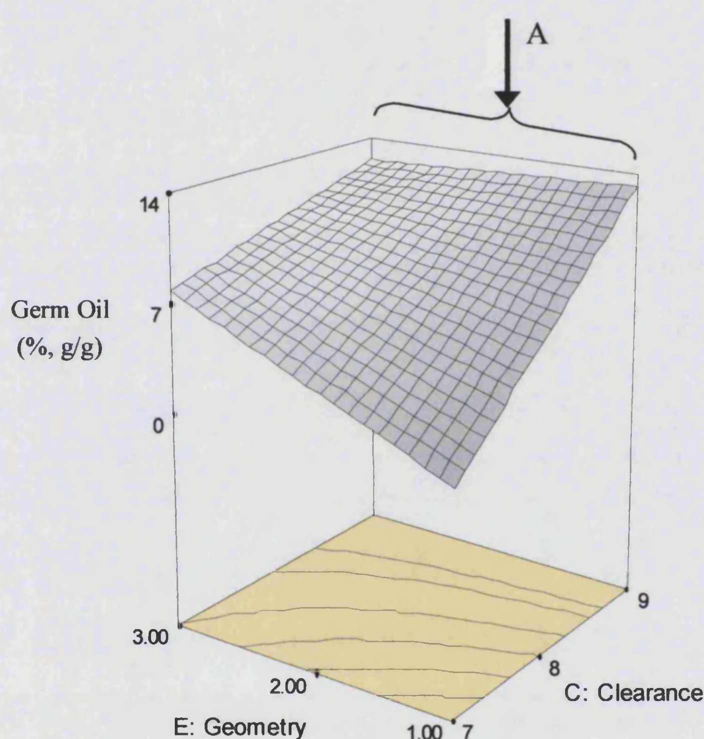


Constant Operating Conditions		
Speed = 20.42 m.s^{-1}	Holding time = 34 minutes	Degermer recycle = 1

Figure 4.4 Germ-rich waste stream mass, separated by flotation, and expressed as a percentage of the feed mass

4.3.3.b Oil analysis

The results of germ separation via flotation, and analysis of separation using the oil concentration and oil content of the waste stream, are illustrated in Figure 4.5 and Figure 4.6, respectively Both of these results again indicated toward favourable operating conditions at high levels of both clearance and stud geometry. Figure 4.5 shows the effect of stud geometry and disk clearance on the germ oil content, expressed as a percentage of the mass of germ fraction separated.



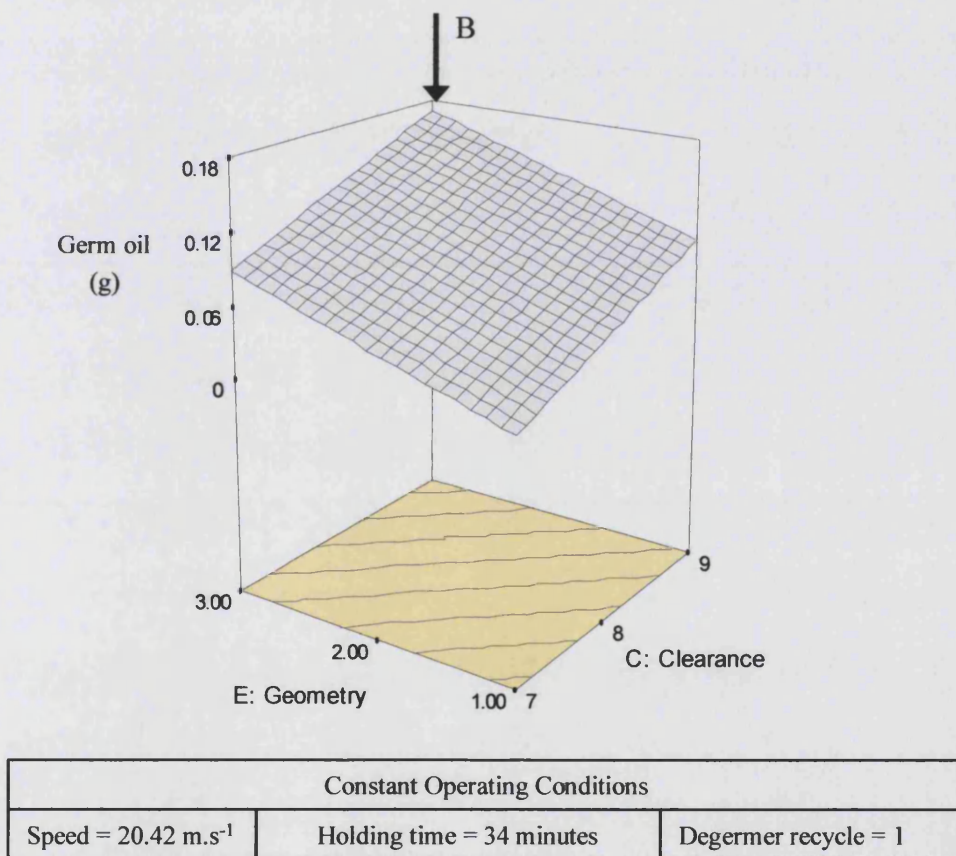
Constant Operating Conditions		
Speed = 20.42 m.s^{-1}	Holding time = 34 minutes	Degermer recycle = 1

Figure 4.5 Oil concentration of the floated waste stream

Oil concentration fell substantially with a reduction in the disk clearance at the lower levels of stud geometry. However, at the highest level of clearance, the arrangement of studs had little impact on the oil content of the floated germ, indicated by the set of conditions represented by (A). This was because higher levels of clearance increased the volume of the degermer, which greatly increased void and reduced the frequency

of impact between the seeds and the degermer, and which also greatly reduced the effect of stud design. Therefore, Figure 4.5 illustrates the extent to which using studs of the same size was detrimental to the range of conditions which could be used for the production of a high oil content waste fraction.

Figure 4.6 illustrates that the fall in oil concentration, illustrated previously, toward operation of the degermer at lower levels of clearance and stud geometry, was matched by a decrease in oil mass. Most importantly, the oil content increased as the level of the stud geometry increased over the full range of clearance, and reached a maximum at the high levels of both factors, represented by point (B).



Constant Operating Conditions		
Speed = 20.42 m.s^{-1}	Holding time = 34 minutes	Degermer recycle = 1

Figure 4.6 Mass of oil in the floated waste stream

Contrary to indications in Figure 4.5, stud geometry had a large impact on the quality of germ-endosperm separation. Although waste stream oil concentration remained approximately equal, the quantity of oil collected in the waste stream increased, and therefore more germ particles were separated, when the degermer was operated using the higher levels of stud geometry.

The results thus far acquired and analysed were sufficient to confidently finalise the design of the degermer. The studs were permanently fitted into the degermer disks according to the specifications of the higher level of internal geometry tested i.e. a profiled design with increasing stud size on each ring with distance from the feed inlet (Figure 3.7 and Figure 3.8), and as shown in the photograph in Figure 3.6(c).

4.3.3.c Suitability of flotation to transgenic seed component separation

It was likely that immersing transgenic seed fragments in a sodium nitrate solution would have resulted in the product leaching into the solution (Kusnadi, 1998b). This would only have been acceptable if this same solution was used in the extraction of product from ground non-germ seed fractions. However, although the product contained in the germ would beneficially be released into solution, it would have been accompanied by germ proteins and oil, which would have obviated the single purpose of the degerming process. It was therefore decided to avoid separation processes based on density differences.

4.4 Germ-Endosperm Separation Process

The small-scale roller-mill and sieving operation (section 2.2.2) produced five differently sized fractions of ground seed. The quality, in terms of mass and oil content, of each of these fractions was important in the analysis of the quality of degerming that was achieved. This information was used in the selection of sieves which were allocated to the waste and product streams (section 3.4.2 and section 5.4). In this section, however, degerming analysis consisted of analysing the quality of these fractions after having been combined into product and waste streams.

Roller-milling crushed endosperm fragments into smaller particles and flattened germ into larger particles for capture on the upper sieves (Figure 3.16). Analysis of these fractions, illustrated in Figure 3.14, demonstrated that small-scale degerming, followed by the roller-milling and sieving of seed fragments, could successfully separate seed into the required product and waste streams. The requirements for the two output streams are summarised below:

1. Waste stream (sieves A - C)
   Low mass and very high oil content, consisting primarily of whole germ and germ fragments, and constituting a large percentage of the total feed oil.

2. Product stream (sieves D & E)
   High mass and very low oil content, consisting mostly of endosperm and non-germ fragments, and constituting a large percentage of the total feed mass.

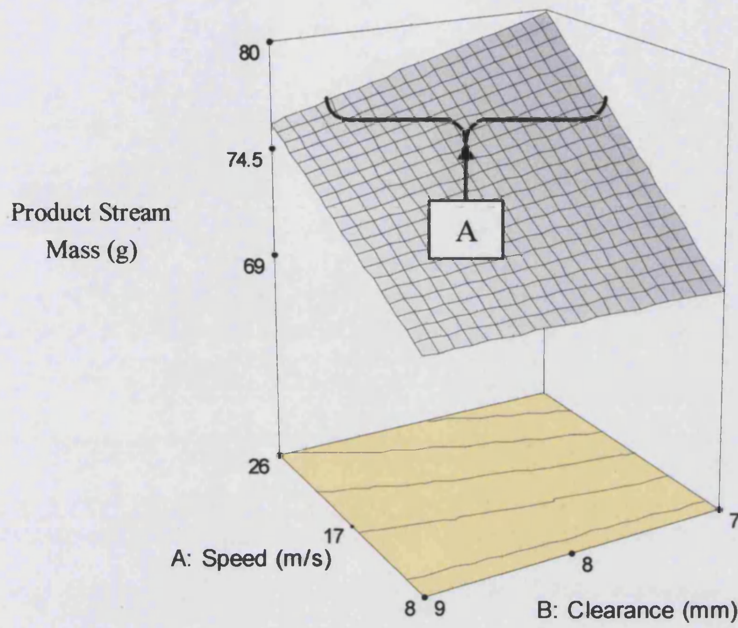
Prior to process optimisation, those sieves allocated to waste and product streams were selected on the basis of the results illustrated in Figure 3.14. The waste stream consisted of the upper three sieves (sieves A-C), and the product stream consisted of the bottom two sieves (sieves D-E). A full factorial design experiment was carried out, consisting of 20 runs and processing 100g of seed per run. The factors investigated, and the responses by which varying these factors were measured, are detailed in Table 4.2 below:

Table 4.2 Factors investigated using different response parameters and the method of roller-milling and sieving for germ-endosperm separation

FACTORS	RESPONSES	FIGURES
Disk speed Disk clearance Degermer recycle Seed moisture content	Product stream mass (g)	Figure 4.7
	Waste stream mass (g)	Figure 4.8
	Product stream oil concentration (%, g/g)	Figure 4.9
	Waste stream oil concentration (%, g/g)	Figure 4.10
	Waste stream oil recovery (%)	Figure 4.11
	Product stream oil mass (g)	Figure 4.12

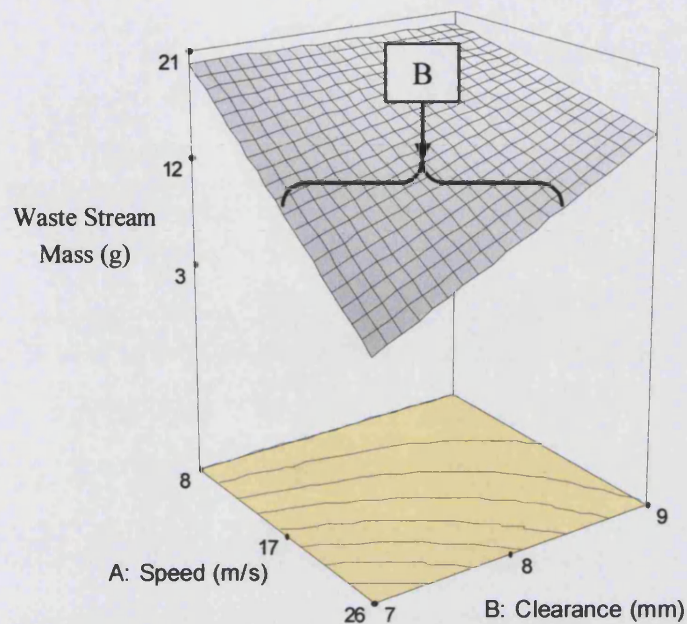
4.4.1 Mass of product and waste streams

The mass response for both the product and waste streams are illustrated in Figure 4.7 and Figure 4.8, respectively. Since germ occupies approximately 12% of the seed by mass, preferred operating conditions were indicated by regions on the response surfaces which represented the production of a high product stream mass (A) and a low waste stream mass (B). These conditions occurred at low levels of seed moisture content, clearance and recycle, and higher levels of degermer disk speed. Recycle through the degermer had a very small effect (3.6% contribution to the linear statistical model) on this response parameter, and so was omitted from the equation used to generate the response surfaces.



Constant Operating Conditions	
Holding time = 2 minutes	Degermer recycle = 1

Figure 4.7 Product stream mass as a function of degermer disk speed and clearance

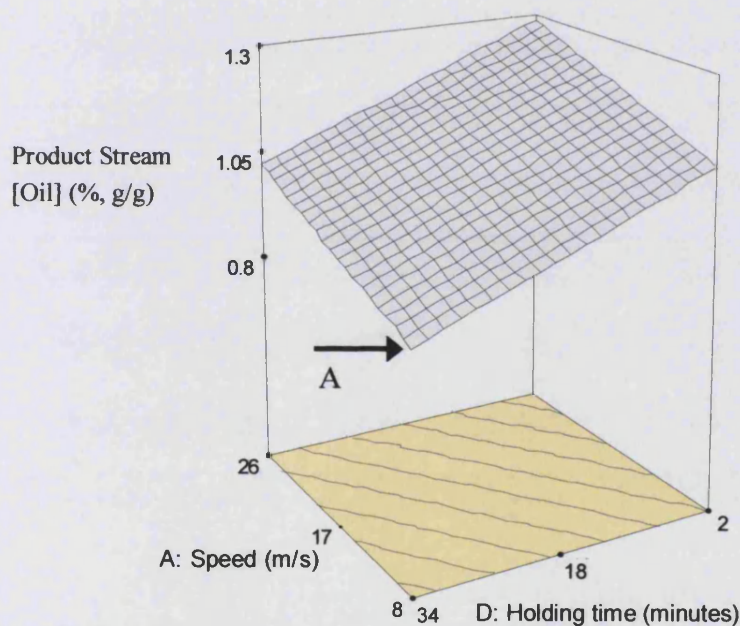


Constant Operating Conditions	
Holding time = 2 minutes	Degermer recycle = 1

Figure 4.8 Waste stream mass as a function of degermer disk speed and clearance

4.4.2 Oil concentration of the product and waste streams

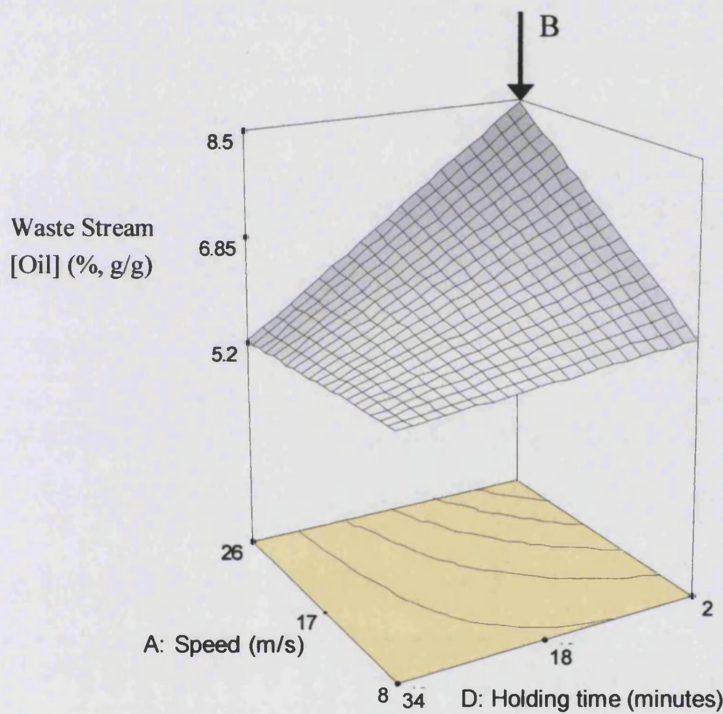
The oil concentration of the product stream, illustrated in Figure 4.9, was minimised by processing seeds of higher moisture content (18%) and disk clearance (9mm), and at lower disk speeds (A). Oil concentration of the product stream remained low in the region of operation tending toward higher moisture content and lower speeds, indicating that a high quality product stream could be obtained by operating the degermer under conditions which minimised the intensity of seed grinding.



Constant Operating Conditions	
Clearance = 9mm	Degermer recycle = 1

Figure 4.9 Product stream oil concentration

Figure 4.10 shows the effect of disk speed, moisture content, and the interaction between the two factors upon the oil concentration of the waste stream. Waste stream oil concentration was maximised by operation at the highest level of disk speed ($26 m.s^{-1}$), and the lowest level moisture content (13%), indicated by the arrow (B). At this point, the slope of the surface is steepest, indicating a region of unstable operation in terms of process reliability, and thus large variations in this response could be expected with small changes in either of the factors illustrated.

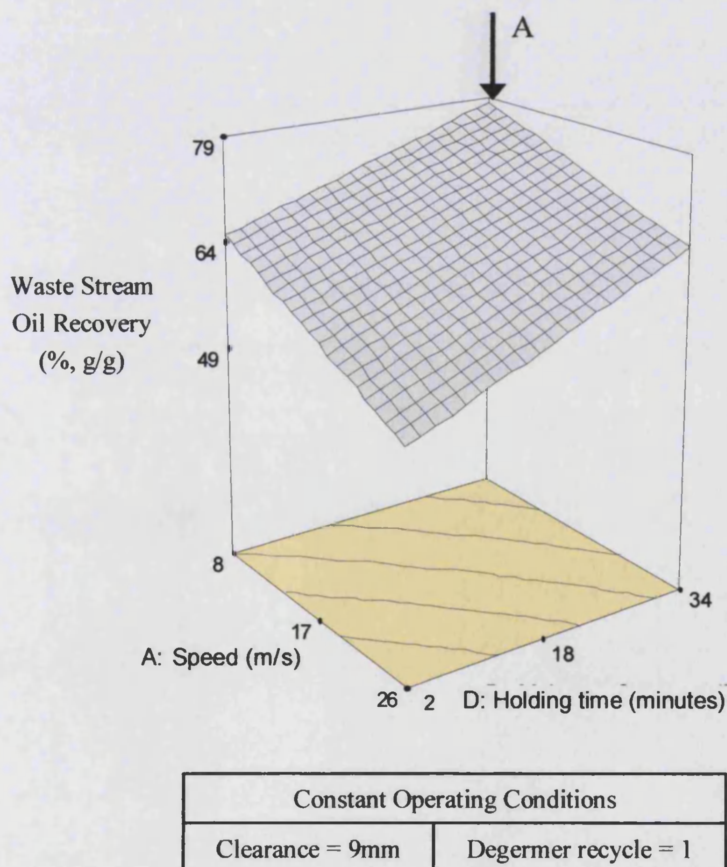


Constant Operating Conditions	
Clearance = 9mm	Degermer recycle = 1

Figure 4.10 Waste stream oil concentration

4.4.3 Oil recovery

Concentration of oil in the waste and product streams provided incomplete information regarding the separation of maximum quantities of oil. The quantity of oil in the waste and product streams was not accounted for. Although the total mass of oil was measured, a more informative method of representing the results was to illustrate the mass of oil collected, or recovered, in either the waste or product stream as a percentage of the total seed oil measured in each experiment. This method more closely illustrated the extent of oil separation, and the response was called *oil recovery*. The operating conditions required for maximising the waste stream oil recovery are represented by the set of conditions indicated by (A) in Figure 4.11. Almost 80% of seed oil was separated using the small-scale degerming process with one single pass through the degermer operating at low speeds with maximum clearance between the disks, and with seeds at the highest level of moisture content.

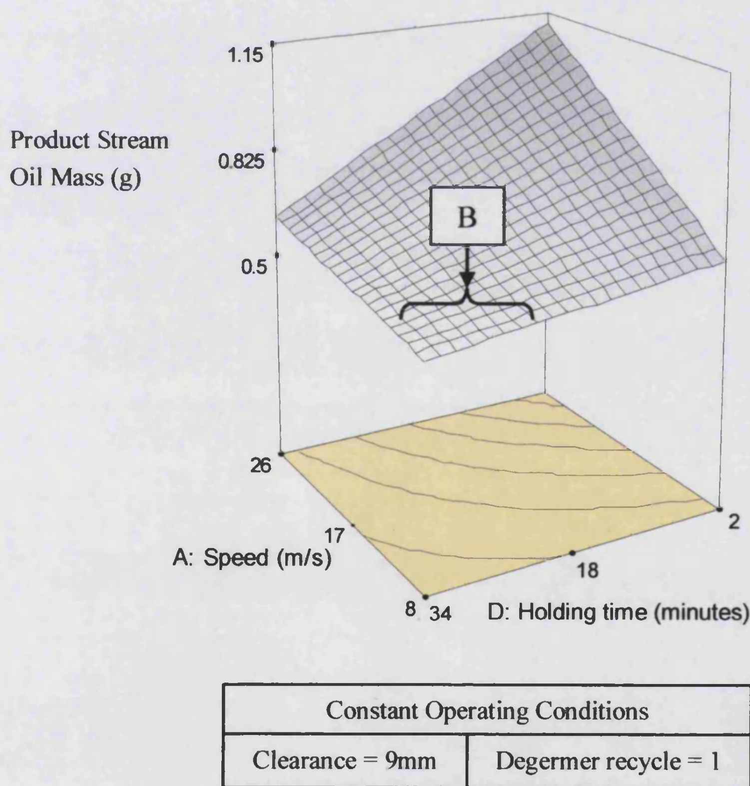


Constant Operating Conditions	
Clearance = 9mm	Degermer recycle = 1

Figure 4.11 Waste stream oil recovery as a percentage of the total oil detected

4.4.4 Product stream oil content

Expression of the waste stream oil recovery as a percentage of the total oil measured in each experiment resulted in an inverse of the response surface to that in Figure 4.11 for the product stream oil recovery i.e. operation under the same conditions yielded a product stream with minimal oil content, containing slightly more than 20% of the total seed oil. Therefore, the mass of oil, and not the mass percentage, in the product stream was selected for illustration in Figure 4.12. This response is illustrated as a function of the two factors, speed and moisture content, which most strongly influenced this response parameter. Using the data in Table 3.1, a typical batch of 100g of hybrid 8366 seeds would contain approximately 0.886g of oil.



Constant Operating Conditions	
Clearance = 9mm	Degermer recycle = 1

Figure 4.12 Product stream oil mass as a function of speed and holding time

4.5 Summary

The widest range possible of seed grinding were observed using the rotating disk design degermer, with up to 100% of seeds remaining intact after processing. Analysis of processed samples which consisted of seed fragments showed that, by observation, whole germ were released, and that, by the analysis of oil content, germ fragments were separated by flotation. Following separation by flotation, product stream oil content and oil concentration decreased as degermer operating conditions tended towards higher levels of both stud geometry and disk clearance, with seed moisture content fixed at the highest level. Consequential to these results, the highest level of stud geometry, in which stud size on each disk increased with distance from the feed inlet, became a permanent design feature of the small scale degerming device.

The results illustrated in Figure 4.7 to Figure 4.12 indicated towards a range of operating conditions which might maximise the quality of degerming. As can be seen in Table 4.3, no single set of conditions would satisfy the requirements of all of the individual responses. To overcome these discrepancies for the optimisation of the small-scale degerming process, the single response parameter (DEF) was designed which combined the mass and oil content of the product and waste streams (section 5.2).

Table 4.3 Different factor levels required to generate the required output

RESPONSE	REQUIRED LEVEL	FACTOR LEVEL			
		Speed	Clearance	Holding time	Recycle
Product mass	High	⇧	⬇	⬇	⬇
Waste mass	Low	⇧	⬇	⬇	⬇
Product oil mass	Low	⬇	⇧	⇧	⬇
Waste oil mass	High	⇧	⇧	⬇	⬇
Waste oil recovery	High	⬇	⇧	⇧	⬇
Product oil mass	Low	⬇	⇧	⇧	⬇

Chapter 5 - Optimisation of the Degerming Process

5.1 Introduction

Improvements were made to the degerming process based on the material and operating parameters which were previously observed to raise the quality of the degerming process output. These improvements consisted of increasing the seed moisture content, incorporating a mechanised roller-mill in substitution of the hand-powered device, and evaluating the output quality in terms of the degerming evaluation factor (DEF). The DEF was designed to collate the mass and oil content of the individual seed fractions produced into one single factor which facilitated the optimisation of the small-scale degerming process.

5.2 Degerming evaluation factor (DEF)

The fractions produced during small-scale degerming varied in mass and oil content as operating conditions were changed. Since both mass and oil content were the major response parameters, these changes were valuable indicators of the degerming quality achieved. Individually, the results were insufficient to identify the optimum degerming conditions. The DEF was designed to account for the mass and oil content of these individual fractions in one response parameter, and to be sensitive to small changes in the mass and oil content of the individual sieve fractions. The allocation of sieve fractions into the waste and product streams as used previously (section 4.4) was carried forward for the design and optimisation of the DEF and the degerming process.

Individual analysis of the waste and product streams consisted of taking the oil mass and seed mass of these two streams as fractions of the total oil mass and seed mass measured in both streams. Calculation of the degerming evaluation factor was based on the differences between the two fractions representing the waste (Equation 5.1) and product (Equation 5.2) streams:

$$Xa = \frac{Oil^{A-C}(g) / TotalOil^{A-E}(g)}{Mass^{A-C}(g) / Mass^{A-E}(g)}$$

Equation 5.1 Waste stream degerming fraction

$$Ya = \frac{Oil^{D-E}(g) / TotalOil^{A-E}(g)}{Mass^{D-E}(g) / TotalMass^{A-E}(g)}$$

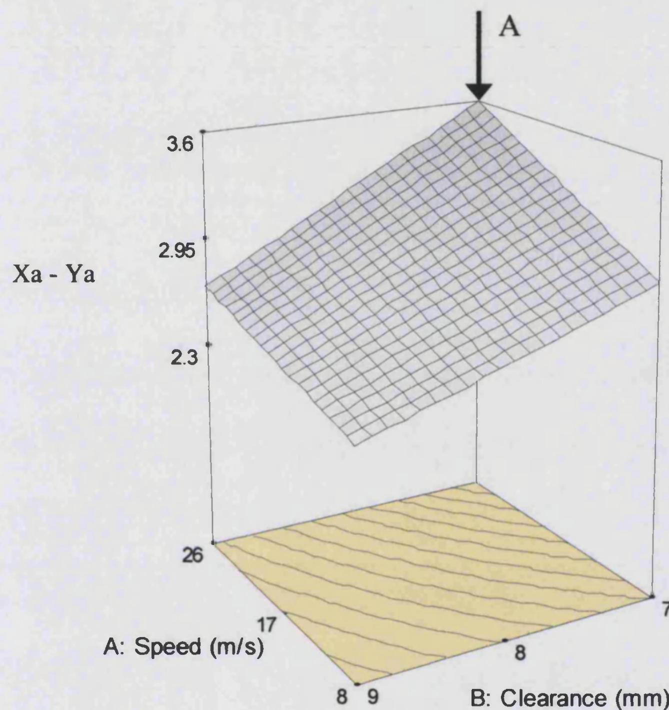
Equation 5.2 Product stream degerming fraction

The greater the difference between the dimensionless analytical parameters of the two streams, the better the degerming. Response surfaces (Figure 5.1 to Figure 5.4) were generated to illustrate the effect the factors upon several variations of the DEF response parameter detailed in Table 5.1, for the development of the DEF analytical parameter, and for the optimisation of the small-scale degerming process.

Table 5.1 Factors investigated using different DEF response parameters, and the method of roller-milling and sieving, for germ-endosperm separation

FACTORS	RESPONSE		FIGURE
	DEF	Equations	
Disk speed	Xa – Ya	Equation 5.1 &	Figure 5.1
Disk clearance	Xa / Ya	Equation 5.2	Figure 5.2
Degermer recycle	X – Y	Equation 5.3 &	Figure 5.3
Moisture content	X / Y	Equation 5.4	Figure 5.4

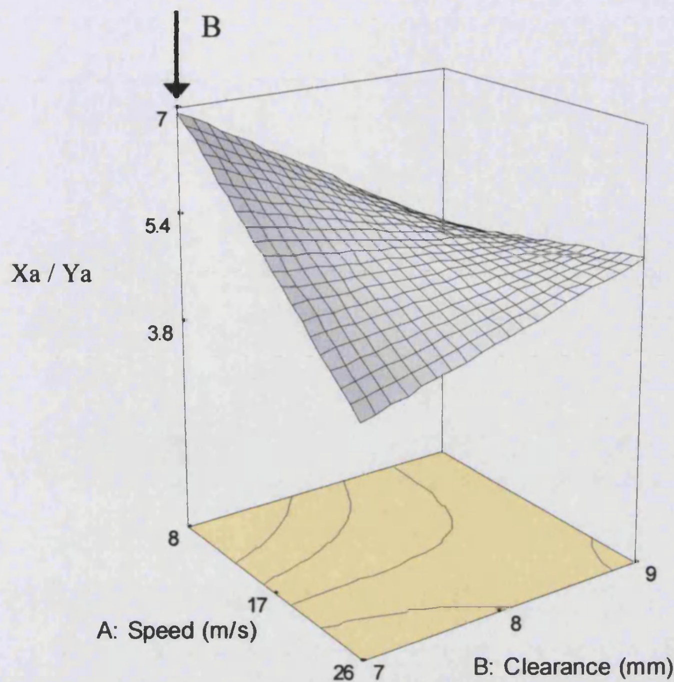
The response surface in Figure 5.1 indicated that the conditions which optimised operation (A) of the degermer also maximised the intensity of attrition and work done on the seed inside the device. This resulted in the production of very small quantities of whole germ, which were required for better separation via roller-milling and sieving, and the retention of a greater mass of seed fragments in the waste stream.



Constant Operating Conditions	
Holding time = 2 minutes	Degermer recycle = 3

Figure 5.1 Xa – Ya degerming analysis

It was necessary to modify the DEF in order to account for the notably small yet important differences between the low (i.e. <1) Ya values, and for the small mass of high oil content fractions collected on the upper sieves. Since Xa-values typically did not exceed Xa=5, and the small Ya-values were mostly less than Ya=1, the solution which best represented the lower Ya values, and thus accentuated the difference between DEF values, was to measure the degerming quality in terms of Xa/Ya (Figure 5.2)



Constant Operating Conditions	
Holding time = 34 minutes	Degermer recycle = 1

Figure 5.2 Xa/Ya degerming analysis

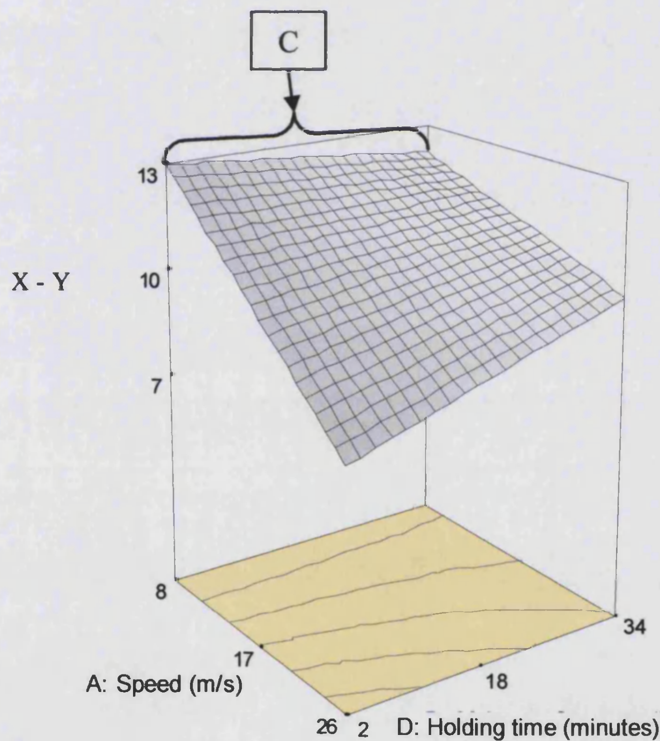
Figure 5.2 (point B) illustrates very different optimum degermer operating conditions to those in Figure 5.1. In order to provide a more accurate representation of the fractions collected on the upper sieves, the method for the calculation of the DEF was modified. This modification consisted of changing Equation 5.1 and Equation 5.2 to account for the mass and oil content of the individual sieves, within each of the streams. The two equations from which the DEF was calculated are given below (Equation 5.3 and Equation 5.4), and two response surfaces (Figure 5.3 and Figure 5.4) illustrate the results using similar variations in the DEF to those used previously i.e. X-Y and X/Y.

$$\mathrm{X} = \sum_{C}^{A} \left(\frac{Oil\,(g)i \big/ TotalOil\ (g)}{Mass\ (g)i \big/ TotalMass\ \ (g)} \right)$$

Equation 5.3 Waste stream degerming fraction

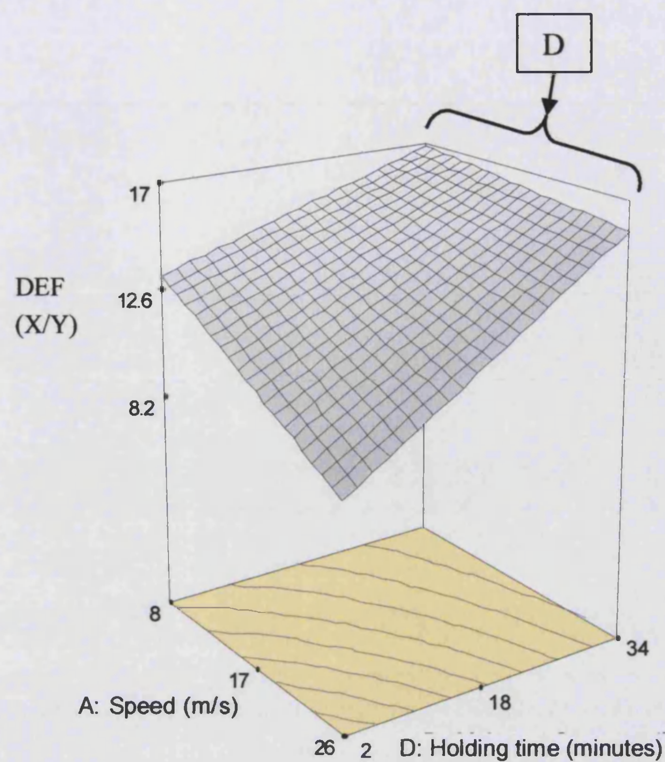
$$\mathrm{Y} = \sum_{E}^{D} \left(\frac{Oil(g)i \big/ TotalOil(g)}{Mass(g)i \big/ TotalMass(g)} \right)$$

Equation 5.4 Product stream degerming fraction



Constant Operating Conditions	
Clearance = 9mm	Degermer recycle = 1

Figure 5.3 X-Y degerming response



Constant Operating Conditions	
Clearance = 9mm	Degermer recycle = 1

Figure 5.4 DEF (X/Y) degerming response

The different operating conditions required to maximise the response using each of the four methods are illustrated in Table 5.2. The optimum operating conditions represented by (D) in Figure 5.4 (X/Y) occurred within close proximity to the maximum DEF (C) in Figure 5.3 (X-Y), indicating an improved reliability of assessment by accounting for individual sieves. Process reliability is discussed in more detail in the following section (section 5.3.3)

Table 5.2 Factor levels required to maximise the DEF

DEF RESPONSE	FACTOR LEVEL			
	Speed	Clearance	Holding time	Recycle
Xa – Ya	⇧	⬇	⬇	⇧
Xa / Ya	⬇	⬇	⇧	⬇
X – Y	⬇	⇧	⬇	⬇
X / Y	⬇	⇧	⇧	⬇

Statistical analysis of the data represented by the two different methods of analysis i.e. X/Y and Xa/Ya, showed that the reliability of the analysed results in the X/Y format (F-value = 11.9) greatly exceeded that for the Xa/Ya results (F-value = 3.3). The optimised operating conditions, determined used the (X/Y) DEF, were:

- Degermer disk speed: $20.42 m.s^{-1}$
- Degermer clearance: 9mm
- Degermer recycle: 1
- Holding time: 34 minutes

The DEF was therefore calculated using X/Y for the analysis of all subsequent experiments designed for the optimisation of the small-scale degerming process.

5.3 Mechanised Roller-Milling

5.3.1 Comparison of the mechanical and hand-powered roller-mills

100g batches of hybrid 8366 seeds were processed according to a ¼-fraction factorial design experiment, which tested the effect of mechanisation of the roller-milling operation and variations in its operating conditions (section 3.4.2.b), in addition to the degermer variables and seed moisture content range previously investigated using the manually operated roller-mill. The result was an overall decrease in the DEF

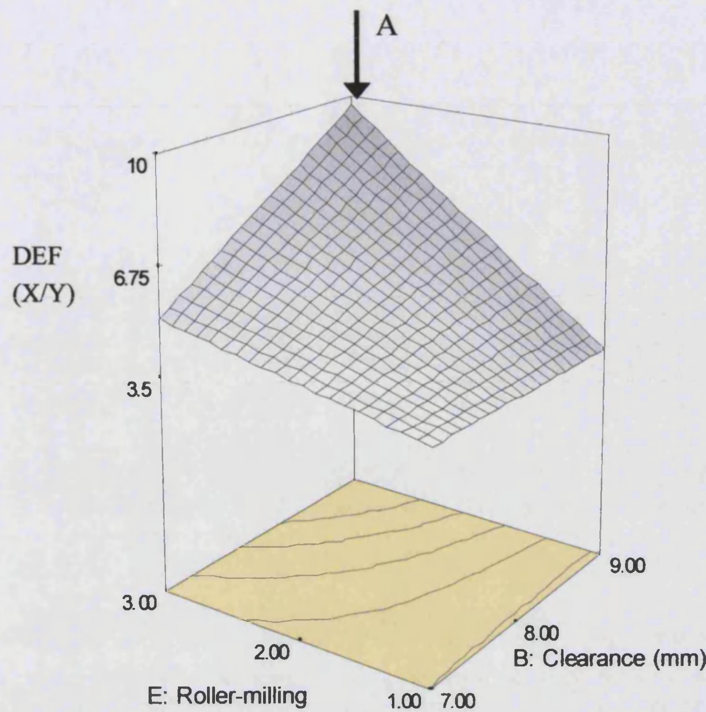
response (Figure 5.5). However, the maximum value of 9.68 (A) still indicated a high level of separation of the two seed components.

5.3.2 ANOVA for the identification of the major processing factors

Analysis of variance enabled the selection of the factors and factor interactions which had the largest contribution to the equation (Equation 5.5) used to produce the response surface in Figure 5.5. These factors were B – Clearance (15.18%), D – Moisture Content (9.42%), E – Roller-milling (49.40%), and the interaction BE – Clearance-Roller-milling (17.89%). No transformation of the response was required to maximise the model fit value (F-value) at 14.17, which indicated a good fit of the model equation to the experimental data. Under these conditions, there was a negligible probability (0.62%) that experimental noise contributed to the model. The effect of processing of hybrid 8366 seed using the small-scale degerming process, in terms of the DEF (X/Y) response parameter, was therefore summarised by Equation 5.5, below, expressed in terms of the coded factors (i.e. +1 or -1 for the high and low levels of the factors, respectively).

$$DEF = 4.62 + 1.08B + 0.85D + 1.95E + 1.18BE$$

Equation 5.5 Calculation of the DEF in terms of the major processing factors



Constant Operating Conditions		
Speed =20.42 m.s^{-1}	Degermer recycle = 1	Holding time = 34 minutes

Figure 5.5 DEF using the mechanised roller-mill for germ-endosperm separation

5.3.3 Data and process reliability

With robust experimental design (G-efficiency 83.3%) and statistically sound data (F-value = 14.17), it was not necessary to expand this fractional factorial design experiment. The extent of curvature of the response surface in Figure 5.5 shows a moderate level of interaction between clearance and roller-milling. At high levels of both factors, at which the DEF was maximised, slight changes in the level of either one had a large impact on the DEF. The steepness of the response surface at the point which maximised the DEF indicated of a region of processing instability with regards to the quality of the product represented by the degerming evaluation factor. The instability at these conditions was reflected in the large 95% confidence interval of DEF ($7.40<DEF<11.01$).

5.3.4 Evaluation of the DEF

The sensitivity of DEF contributed significantly to the large confidence interval and the apparent process instability. It was designed for the purpose of accentuating small differences in the mass and oil content of the products of degerming. Variations in product quality were not unexpected, since seeds were not identical in size, shape and content, and the exact mechanism of degerming within the process would vary with each seed. Therefore, although the DEF provided a useful means to characterise and optimise the degerming process, there were limitations in its use as a response parameter, by the inaccurate representation of certain degerming conditions. On occasion, when germ separation was observed to be relatively good, but too small a sample mass was collected on the upper sieve for individual analysis, such that it had to be combined with the lower sieve, the DEF value plummeted. This was observed in the triplicate degerming of seeds at an elevated moisture content (section 5.4), in which the average DEF of duplicate results (DEF = 20.3) greatly exceed the result of the triplicate batch processed (10.2). This incorrect indication of poor degerming exemplified the sensitivity of the parameter, and thus its limitations for comparing the quality of degerming.

5.3.5 Confirmation of the optimum operating conditions

Optimum operating conditions of the degerming process, represented by the maximum DEF (A), occurred under the following conditions:

- Disk speed = $20.42 m.s^{-1}$
- Clearance = 9mm
- Degermer recycle =1
- Moisture content = 18%
- Roller-milling = 3 (i.e. 3 passes, each with different gaps: pass 1 gap = 1.2mm, pass 2 gap = 0.75mm, pass 3 gap = 0.3mm)

Confirmation of the degerming capabilities of the small-scale device operating at the optimum conditions involved processing one batch of seed, in triplicate, and

determining the DEF for each of the runs. Two of three of the DEF results (10.90, 7.58, 13.23) fell within the 95% confidence interval for the model equation. The one which did not fall within this limit fell favourably above the highest level, thus demonstrating the large variation expected from this response parameter, and the performance of the small-scale degerming process, on batches of the same type of seed.

5.4 Modification of DEF with elevated seed moisture content

The DEF increased with seed moisture content over the full range of moisture content tested. In addition, the feed to industrial degermers is often higher in moisture content than the range of moisture contents investigated thus far. It was therefore decided test the impact of increasing seed moisture content beyond 18% on the quality of degerming that could be achieved using the small-scale degerming process. The result of degerming duplicated batches of 50 seeds of hybrid 8366 at 21% moisture content approximately doubled the average DEF from 10.57 to 20.29. It was likely that this vast increase in the DEF was a result of both the increase in seed moisture content, and the change in the method of moisture conditioning. This improvement created the possibility of reducing the waste stream from 3 sieves (80% seed oil in 35% seed mass) to the upper most 2 sieves (70% seed oil in 15% mass), which served to greatly reduce the loss of endosperm to the waste stream, whilst still separating a large proportion of the seed oil (Figure 5.6).

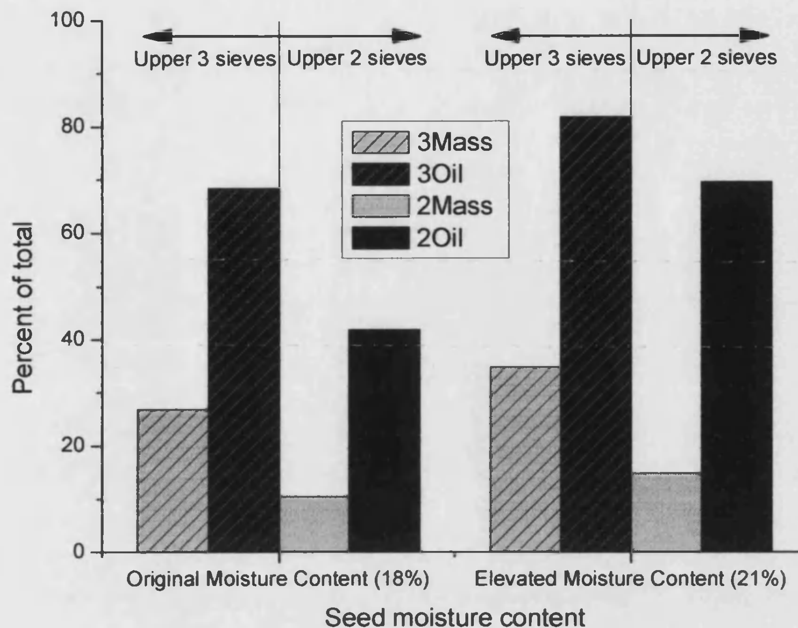
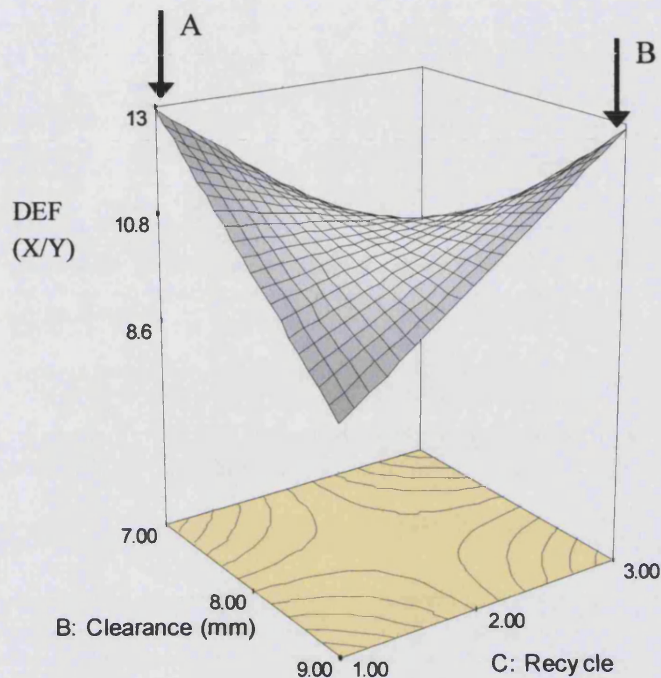


Figure 5.6 Reduction in the number of sieves allocated to the waste stream

The effect of elevated seed moisture content on the quality of degerming was investigated over the same range of each of the other four factors previously tested, using the same ¼-fraction experiment design (section 5.3.1), to test whether the influence of any other factor changed at the higher levels of seed moisture. Batches of 50 seeds -of hybrid 8366 were processed at high and low levels of moisture content of 15% and 25%, respectively, and the results are illustrated in the response surface below (Figure 5.7).

The response surface in Figure 5.7 was significantly different to Figure 5.5, indicating that changes were required to the operating conditions in order to achieve maximum germ separation. These changes would involve operation at the conditions represented by points (A) or (B) marked on the response surface. However, none were made due to the decrease in the reliability of the equation generated to fit the experimental data (F-value = 6.43, noise contribution = 2.6%). It was possible that this decrease in reliability was caused by the reduction in sample size (section 6.2.1 and section 6.2.2) when compared to the size of samples (100g) used previously. However, the relatively small increase in the magnitude of the DEF, from 9.68 to

12.93, by operation of the degerming process at different levels of either clearance or recycle to those previously established (section 5.3.5), were not substantial enough to warrant changes in the processing conditions.



Constant Operating Conditions		
Speed =20.42 m.s^{-1}	Moisture content = 21%	Roller milling = 3

Figure 5.7 Change in the DEF response when processing seeds of higher moisture contents

The operating conditions established using the lower range of moisture contents were carried forward, but consequential to the reduction in mass and increase in oil content of the upper sieves, such that only the upper two constituted the waste stream, the method of moisture conditioning was changed in order to achieve a feed seed moisture content of 21%.

5.5 Summary

The small-scale degerming process was evaluated in terms of the oil contents and mass fractions of both the product and waste streams using a single response parameter, the Degerming Evaluation Factor (DEF). The DEF was optimised to suit the requirements of processing, by equally weighting the importance of each seed fraction produced. Application of the DEF enabled the identification of one single set of operating conditions at which degerming was optimised, the allocation of just two sized seed fractions into the waste stream, and a reduction in the sample sizes required for processing to a suitable scale for transgenic seed processing (section 6.2.1). The optimum operating conditions of the degerming process were as follows:

- Disk speed = 20.42m.s^{-1}
- Clearance = 9mm
- Degermer recycle =1
- Moisture content = 21% (achieved using long duration moisture conditioning)
- Roller-milling = 3 (i.e. 3 passes, each with different gaps: pass 1 gap = 1.2mm, pass 2 gap = 0.75mm, pass 3 gap = 0.3mm)

As a result of the combination of the sensitivity of this response parameter, with the inherent variability in process output between replicates, and perhaps more so when using small batches of seed, the DEF was unsuitable for the comparison of degerming quality between different seed types. Therefore, the DEF response was substituted for the mass and oil content of the product and waste streams. This method of analysis was the most effective of the methods available for the evaluation of the degerming quality of the different seed types processed at the optimum operating conditions (Chapter 6).

Chapter 6 - Application of the Small-Scale Degerming Process to Different Types of Seeds

6.1 Introduction

Observed differences in the quality of large-scale fractionation between different varieties of non-transgenic corn seeds are commonplace (Brekke, 1970; Peplinski et al., 1989). Consequently, differences were also expected between transgenic and wild-type seeds, as discovered by Mott *et al.* in the processing of transgenic rapeseed for the production of polyhydroxybutyrate (Mott et al., 2000). These differences were analysed in terms of the mass and oil content of the individual fractions produced on each sieve, and in the combination of these fractions for the production of product and waste streams.

Before transgenic seed was processed, it was necessary to reduce the standard sample sizes to suit seed availability whilst preserving the process and assay reliability. This was based mostly on reducing the mass of sample required for accurate oil analysis. Other factors were investigated which might have had a significant impact upon consistency of processing and the quality of degerming, particularly with the use of smaller sample sizes. These were seed and component size and content, and seed shape.

Triplicate batches of 50 seeds of five different types, two of which were transgenic, were processed through the optimised small-scale degerming process (section 5.5). The products of the degerming process are graphically illustrated in terms of the mass, oil content and antibody (transgenic seed only) content of fractions separated according to particle size, and expressed as percentages of their respective totals measured in the process output. In this chapter, comparisons of degerming quality are made between the different qualities of non-transgenic seeds, between these different seeds and transgenic seeds, and between two types of transgenic seeds which originated from two different transformation events.

6.2 Process sample size scale-down

6.2.1 Feed mass reduction

Sample sizes were reduced until the processing of these samples resulted in the collection of an insufficient mass for analysis on the upper-most sieve. The results (Table 6.1) show that there was no trend in the change of the DEF as the sample sizes were reduced. Although a barely adequate size of sample was retained on sieve A when using 10g of seed as feed, a sample mass of approximately 12.5g was selected for subsequent processing to account for seed size variations between seed varieties. Approximately 50 seeds constituted the 12.5g feed mass of hybrid 8366 seed, and so this was established as the standard sample size for all seed varieties subsequently processed.

Table 6.1 Change in the DEF with a reduction in processing sample size (duplicated results)

SAMPLE SIZE (g)	DEF	Range (DEF)
100	9.2	7.6 to 10.9
50	15.1	12.6 to 17.8
25	16.3	15.3 to 17.4
12.5	10.3	9.8 to 10.8
10	11.5	11.5 to 11.6

6.2.2 Analytical sample mass reduction

The mass of seed fragments required for analysis was reduced to 0.4g, by changing the method of sample grinding, by reducing the quantity of ground sample required for oil content analysis, and by demonstrating that the duplication of oil content results did not significantly change the overall assessment of degerming quality.

6.2.2.a Improved method of sample grinding

The coffee grinder was substituted with the Geno/Grinder, and the efficacies of the two are detailed in Figure 6.1. The quality of grinding achieved using the Geno/Grinder exceeded that of the coffee grinder on three accounts:

- A larger proportion of the sample was reduced to the required particle sizes i.e. <0.71mm (97%) than the coffee grinder (53%).
- A greater quantity of oil was extracted from samples of the same origin
- A reduction in the variability between triplicate measurements of oil content.

It was necessary to maximise the sieving throughput for extraction because the smallest mass fractions collected in the output from the degermer contained the largest quantities of oil, and consequently were the most difficult to separate via sieving due to particle agglomeration on the sieve. The most likely causes of the higher oil extraction and the reduction in variability between oil content measurements were the smaller sizes of particles which were produced using the Geno/Grinder, and the increase in the consistency of particle sizes produced. It was essential to produce smaller particles not only for the oil assay efficacy, but also to enable a near instantaneous washing of the recombinant protein from broken cells of the transgenic seed, and to minimise the effect of slow intraparticle diffusion (Bai and Nikolov, 2001). However, the particle sizes of the samples produced were not measured. Also, by substitution of the coffee grinder with the Geno/Grinder, it was not possible to compare oil content or oil concentration data between results generated using the two methods of grinding. It was for this reason that this oil content data was expressed as a percentage of the total oil measured.

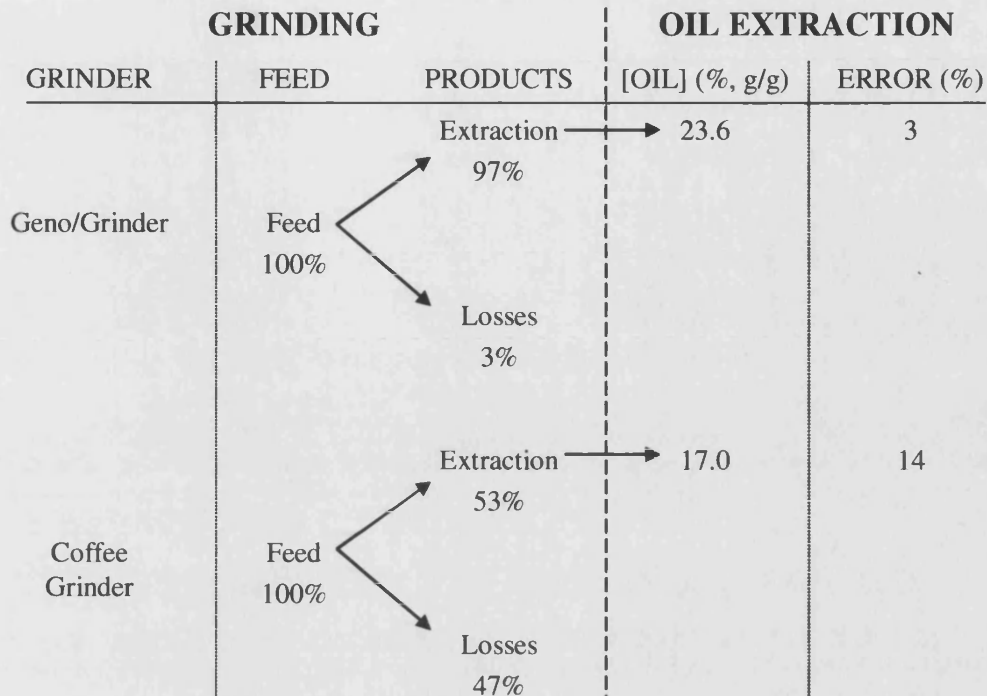


Figure 6.1 Schematic comparing grinding and oil extraction efficiency

6.2.2.b Reduced sample mass for oil content determination

Oil extraction sample mass was reduced to 0.15g by the analysis of oil content, and reliability in its measurement, with reducing the mass of sample from 0.2g to 0.05g by 0.05g (±0.005g) decrements. Firstly, reliability of extraction using 0.2g samples was determined for comparison with the reliability using smaller masses. The results, in Table 6.2, show the range of oil concentrations between sieves, and the extent of experimental error between triplicates.

Table 6.2 Oil measurement reliability (triplicate 0.2g samples)

SIEVE	[OIL] (%, G/G)	ERROR (%)
4.0	13.7	1.6
2.8	18.1	4.2
1.7	8.9	16.9
0.71	5.3	3.6
0.0	5.6	1.6

Table 6.3 shows the large decrease in assay reliability with decreasing sample sizes, and the decrease in the accuracy of oil content measurement represented by the very small differences in the oil content between the different sieve fractions. Smaller sample masses greatly reduced the value and reliability of the assay. Consequently, a compromise was reached between the two extremes of extraction mass and ratios tested, and one which tended more closely toward the conditions which yielded a greater range in the oil content detected across the five sieves of the degerming process output. This compromise consisted of the use of 0.15g samples. The oil concentrations and the error between triplicate runs are illustrated in Table 6.4.

Table 6.3 Oil measurement reliability (triplicate 0.05g samples)

SIEVE	[OIL] (%, G/G)	ERROR (%)
4.0	27.8	7.7
2.8	20.2	28.7
1.7	20.0	9.7
0.71	20.2	12.2
0.0	20.2	8.7

Table 6.4 Oil measurement reliability (triplicate 0.15g samples)

SIEVE	[OIL] (%, G/G)	ERROR (%)
4.0	15.4	4.2
2.8	17.0	13.7
1.7	9.2	8.8
0.71	7.1	2.6
0.0	7.9	1.3

6.2.2.c Evaluation of single sample oil extraction reliability

The DEF was used as a means to compare the accuracy of degerming quality analysis, with taking either single or duplicate oil measurements from each fraction produced during degerming. Triplicate 100g batches of seed were processed at the optimum operating conditions. The average DEF calculated using the first replicate of oil contents of the different fractions produced was 10.6 (±27%). The second replicate of oil assay, using the same product fractions, yielded a DEF of 11.1% (±34%). With such a small difference between the two DEF values, and large error in the DEF between the replicate batches processed, duplicating the oil content assay samples had no significant impact on the DEF, and consequently no more than single sample oil content analysis was required.

6.3 Effect of different seed sizes on the response parameters

6.3.1 Sizes and contents of seed components

The size and oil content of components of seeds of the same hybrid (hybrid 8366Bt), but of different sizes, were analysed in order to establish whether these seed properties might also have affected the DEF. The seeds making up the batch of seeds of different sizes, which were used for comparisons with seeds of a single size, were size-classified to the same degree of accuracy as MFM seeds used for process design. The batch of mixed seeds consisted of 10 SP (Small Platelets), 10 MFM, 10 MRM (Medium Round Medium) and 10 LP (Large Platelets) seeds. The mass and oil content of whole seeds and the two major components are reported in Table 6.5.

There was a larger difference between the two samples in germ mass and germ oil mass than in endosperm mass and endosperm oil mass. In terms of degerming assessment, equal successes of germ separation using the small-scale degerming process, in terms of the frequency of released germ per seed, would not be fairly represented by the DEF. In this case, the batch of mixed sized seeds would yield a greater proportion of oil in the waste stream, and so the difference in DEF between

processing the two different batches of seed would partly represent the difference in the quality of seed, or suitability of this seed, to the small-scale degerming process. The extent of the potential difference in the DEF resulting from different seed properties was not investigated.

Table 6.5 Hybrid 8366Bt seed and seed component mass and oil content comparisons between 40 MFM-sized seeds and 40 seeds of a controlled mixture of sizes

	MFM Seeds (g)	Mixed seeds (g)	Size Factor Mixed seeds / MFM seeds
Dry mass	7.608	9.8463	1.29
Germ mass	0.9374	1.3224	1.41
Germ oil	0.2345	0.3359	1.43
Endosperm mass	6.5822	8.4580	1.28
Endosperm oil	0.0408	0.0452	1.11
Total oil	0.2753	0.3811	1.38

It may prove beneficial to introduce a factor to account for the differences in seed component masses, proportions and contents. However, in the absence of sufficient transgenic material to determine such factors for transgenic seed processing, the most suitable criteria for the assessment and comparisons of degerming quality of different varieties of seeds were the direct use of the mass and oil contents of the product and waste streams.

6.3.2 Degerming batches of seeds of different sizes

6.3.2.a Degerming quality relative to seed size

The effect of seed size on degerming quality was investigated by milling five batches of 50 seeds, four of which consisted of the same hybrid of seed (hybrid 8366Bt), but of different sizes. The fifth batch of seed consisted of approximately equal quantities of these different sizes of seed. Twelve seeds of each size (section 6.3.1) were used apart from 14 MFM seeds. The results in Table 6.6 show that the quality of degerming was lowest for the size of seed (MFM) used in process design. This result was surprisingly low, based on the confidence interval previously determined (section 5.3.3). Seeds of all other sizes degermed to a higher quality, and there was no

consistent change in degerming quality with respect to the change in the size of seeds processed.

Table 6.6 DEF variation with processing seeds of different sizes (duplicated experiments)

SEED SIZE	AVERAGE DEF	RANGE (DEF)
SP	10.9	8.3 to 13.5
MFM	5.3	5.8 to 5.7
MRM	10.4	8.8 to 12.1
LP	9.2	8.7 to 9.7
Mixed Sizes	7.8	7.5 to 8.2

6.3.2.b Effect of seed size on DEF variability

The variation between results of the duplicated experiments indicated that the quality of degerming was more consistent when processing larger seeds than smaller seeds. This was thought to be a result of the difference in the intensity of grinding within the degermer. With a fixed, large gap between the degermer disks (9mm), smaller seeds were less likely to be subjected to similar intensities of abrasion than the larger seeds.

6.3.2.c Degerming quality relative to seed shape

The degerming quality using the sample of seeds of a mixture of sizes was lower than that for other sizes of seed, excluding MFM. This supported the low DEF data obtained from processing MFM seeds alone. Therefore, it is possible that seed shape bore greater significance on the degerming quality than seed size. The germs in flat seeds were typically sunken into the endosperm on the flat sides of the seeds, and thus were not as immediately accessible for removal via impact in the degermer as seeds of other shapes. The germs in these rounder seeds were sometimes observed to protrude from the seed surface, or were positioned near to an edge of the seed, and thus were more accessible for removal upon impact with the studs in the degermer.

6.4 Transgenic seed properties

There was a much greater range in the mass of transgenic seeds than for the high quality seed hybrids (section 2.1.2.b), and this range is illustrated in Figure 6.2 and Figure 6.3. The extent of variation in seed size between batches selected for processing was minimised by selecting seeds as close to the average size as possible, within the limits of availability of these seeds. The average seed and seed component mass, oil content and antibody content of these 'sized' seeds are detailed in Table 6.7. Although the endosperm in transgenic HVF1 seed contained no detectable oil, it is unlikely that the endosperm component contained absolutely no oil. This result was an indication of the limits of reliability of this assay at very low oil concentrations.

Table 6.7 Transgenic seed mass and content of oil and antibody

MODIFIED SEED	HVY2			HVF1		
	Mass (g)	Oil (g/g)	Antibody (ng/g)	Mass (g)	Oil (g/g)	Antibody (ng/g)
Whole seed	0.2125	0.0387	1169	0.2500	0.0378	998
Germ	0.0256	0.2232	32	0.0289	0.2062	15
Endosperm	0.1815	0.0040	1042	0.2106	0.0000	803

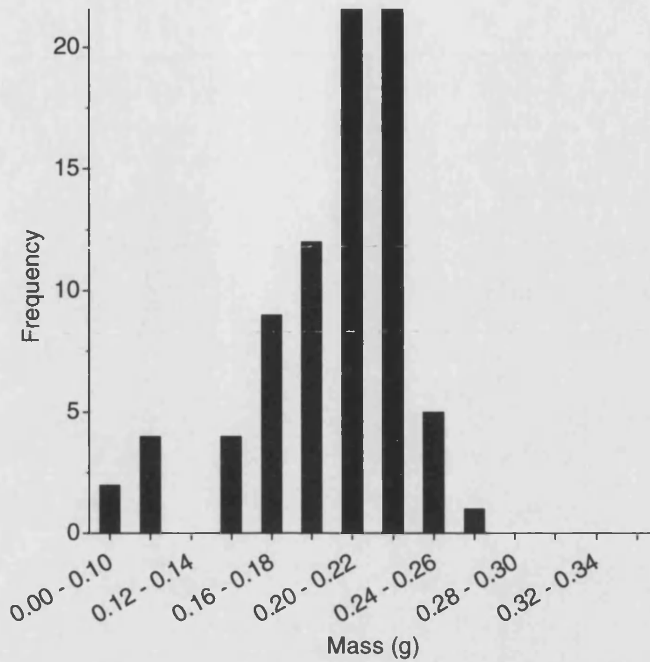


Figure 6.2 Transgenic HVY2 seed mass distribution

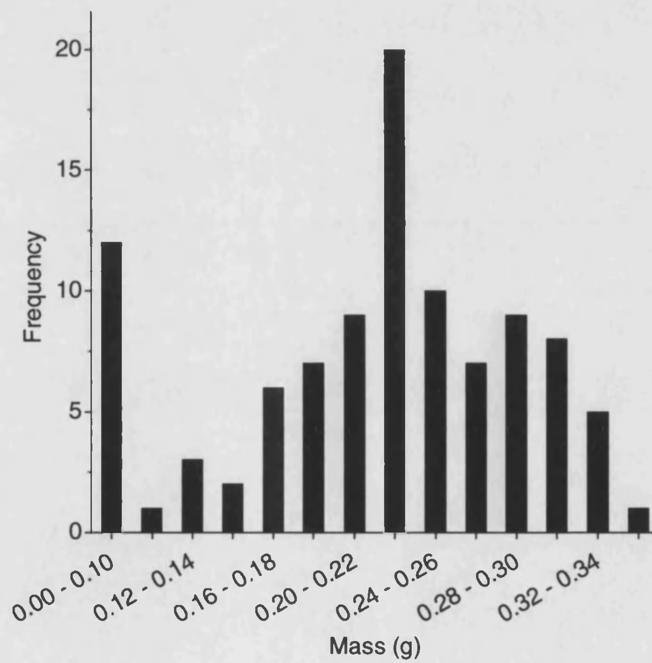


Figure 6.3 Transgenic HVF1 seed mass distribution

Large variations in antibody expression levels were detected between transgenic seed varieties of different transformation events, between siblings taken from the same transformation event (section 2.1.2.d) (Figure 6.4), and between one type of transgenic seeds taken from the same transformation event (data provided by Epicyte Pharmaceutical, Inc.) (Figure 6.5). By keeping the seeds from different transformation events separate (e.g. HVY and HVF), it was possible to investigate if there were any differences in the quality of degerming between these seeds. With the additional analytical parameter of antibody content, it was possible to assess degerming quality in terms of the parameter on which the feasibility of the process would ultimately be determined.

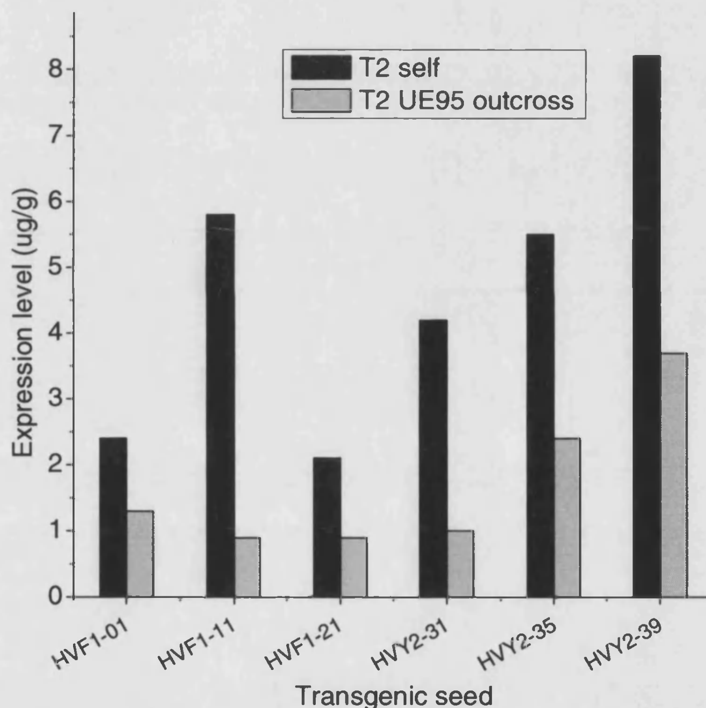


Figure 6.4 Bulk testing of (T2) seed expression levels

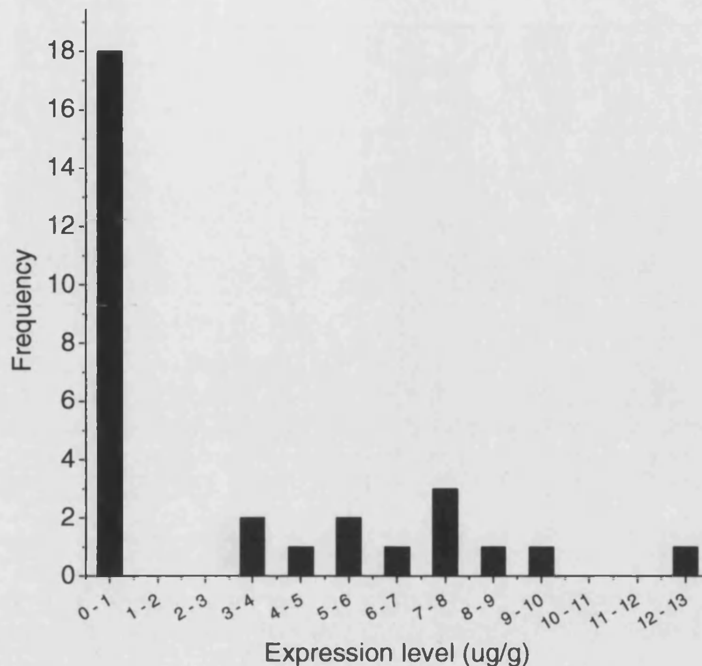


Figure 6.5 Transgenic HOY3: single seed expression levels

6.5 Processing different types of seed

6.5.1 Two high quality, non-transgenic seed hybrids

The quality of degerming of both high quality hybrids 8366 and 8342GLS/IT was expected to surpass that of other varieties of seeds, since the small-scale degerming process design and optimisation was based on hybrid 8366, and only a small difference was observed between the two seed hybrids in terms of the measured physical properties (section 3.2.2). The results of processing using the small-scale degermer are illustrated in Figure 6.6 and Figure 6.7. The values obtained for the degerming of all non-transgenic seeds are detailed in Table 6.8, below:

Table 6.8 Non-transgenic seed mass balances across the degerming process (triplicate experiments)

Seed Type		Feed	Product stream	Waste stream
8366	Mass (g) (Error)	12.76	11.00 (±0.3%)	1.20 (±1.1%)
	Oil (mg) (Error)	432	118 (±24.0%)	245 (±5.0%)
8342 GLS/IT	Mass (g) (Error)	13.16	10.01 (±1.1%)	1.99 (±18.0%)
	Oil (mg) (Error)	386	120 (±25.4%)	284 (±32.7%)
B73	Mass (g) (Error)	10.54	8.15 (±3.1%)	1.85 (±4.0%)
	Oil (mg) (Error)	271	61 (±42.7%)	149 (±9.1%)

There was little difference in the overall quality of degerming between the two high quality hybrids. The high variation between replicates was a result of the variation in processing quality between replicates. Hybrid 8342GLS/IT contained a higher proportion of seed oil in the waste stream than 8366 seed, but the mass of 8366 seed product and waste fractions more closely approximated the required proportions of separation. The major differences between the two were the lower mass of hybrid 8342GLS/IT product stream, and the significantly higher oil content of the fractions collected on sieve 4.0 (Figure 6.7). However, when these individual fractions were combined into waste and product streams, the difference in oil content was not significant, but the different proportions of mass (Table 6.9) indicated toward a better degerming of hybrid 8366 seeds.

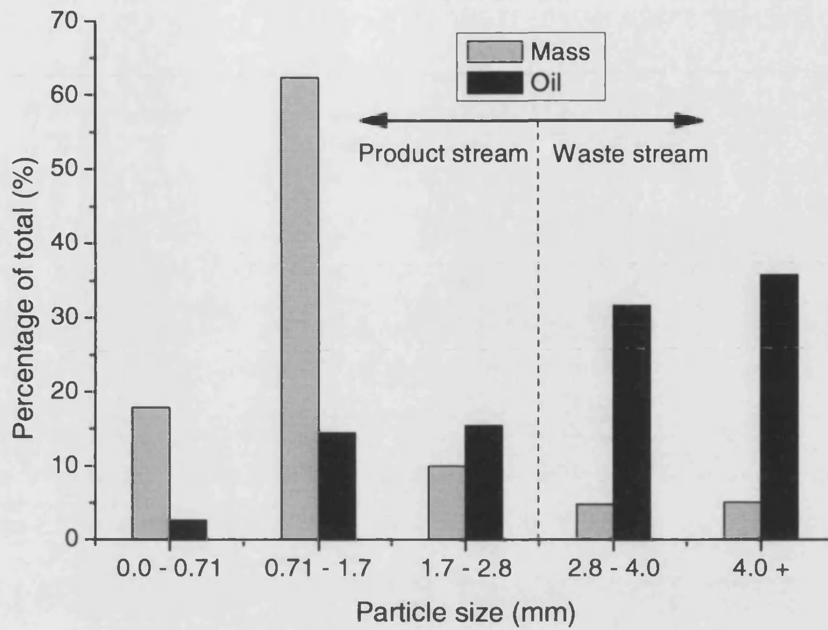


Figure 6.6 Small-scale degerming products of hybrid 8366 seed

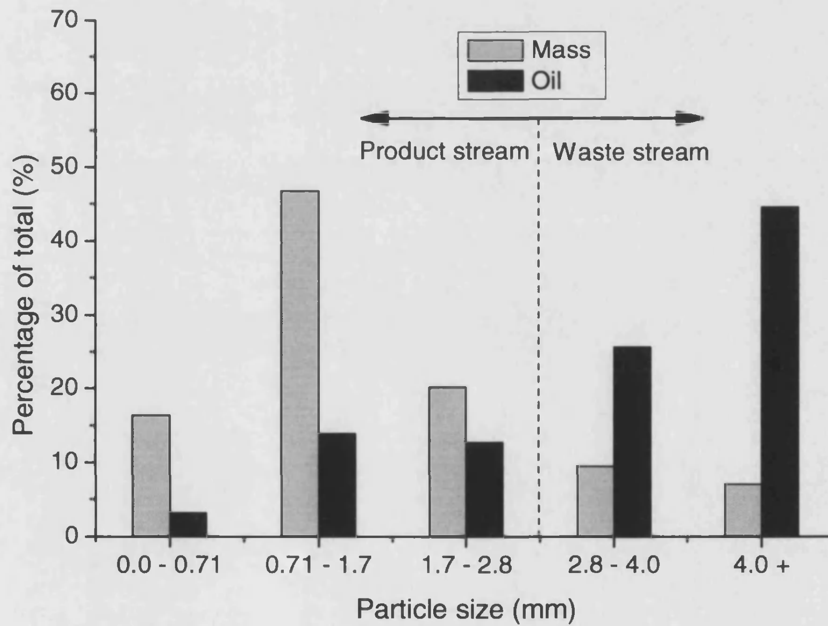


Figure 6.7 Small-scale degerming products of hybrid 8342GLS/IT seed

6.5.2 One high quality hybrid and one low quality non-transgenic seed

The quality of small-scale degerming was compared between non-transgenic hybrid 8366 seeds and the low quality parent B73 seeds. Due to the large differences in seed properties, notably the size and oil content of B73 seeds (Table 3.3) in comparison to the high quality hybrids (Table 3.1), degerming results were compared in terms of the proportions of mass and oil separated into product and waste streams (Table 6.9).

Table 6.9 Degerming process product streams of all seed types processed

Seed Type	Product Stream		
	Mass (%)	Oil (%)	Antibody (%)
Non-transgenic seed			
8366	90.2	32.5	
8342GLS/IT	83.4	29.7	
B73	81.5	29.3	
Transgenic seed			
HVY2	84.2	46.8	91.1
HVF1	82.3	41.7	89.6

Table 6.9 shows that there was no significant difference in the product stream oil content between these two types of seed. However, due to the larger mass of the waste stream produced, the overall degerming quality was better using hybrid 8366 seed than the parent seeds.

The distributions of mass and oil of processed B73 seed across the output sieves, illustrated in Figure 6.8, were very similar to those in Figure 6.7 representing the degerming of hybrid 8342 GLS/IT. A higher quality of degerming was expected by the degerming of hybrid 8342GLS/IT than parent B73 seeds, due to differences between their genetic backgrounds (section 2.1.1). This, however, was not the case. Although it was possible that the lower quality of degerming of hybrid 8342GLS/IT was a result of the crossing of the inbred UD70 with BD68, or the sizing of the seeds prior to processing (section 6.3.2.c), this cannot be ascertained using this data.

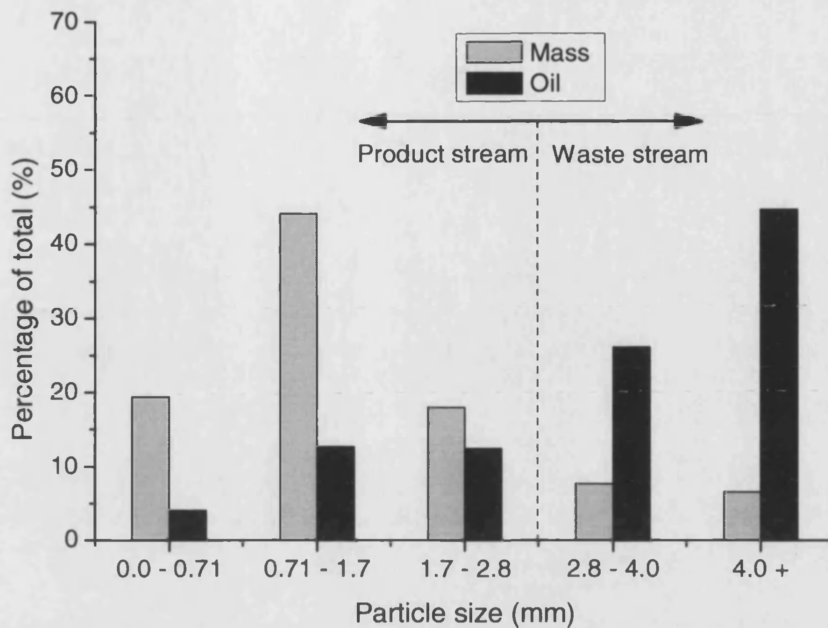


Figure 6.8 Small-scale degerming products of B73 seed

6.5.3 Transgenic and high-quality non-transgenic seed

The small-scale degerming process was shown to be capable of degerming transgenic seed, as illustrated by the separation of a large proportion of seed oil in a small proportion of seed mass (Figure 6.9). In comparison with hybrid 8366, a lower quality of degerming was achieved using transgenic seed on two accounts; in the separation of a smaller proportion of seed oil in the waste stream and the entrainment of a greater quantity of endosperm therein, given by the retention of a greater mass than if only germ had been separated (Table 6.9). The mass balances for the degerming of transgenic seed are reported in Table 6.10, and discussed in section 6.5.5.

The major difference in the pattern of oil distribution across the sieves occurred in the oil content of HVY2 particles in the size range of 2.8mm – 4.0mm (see Figure 6.9), resulting in a less clear distinction between those sieves designated for the product and waste streams. There was no distinct decrease in fraction oil content between sieves 2.8mm and 1.7mm, which indicated that a substantial quantity of oil, which ought to have captured on sieve 2.8, had passed through onto sieve 1.7. However, due

to the large mass of seed and larger proportion of antibody on sieve 1.7mm, this fraction belonged in the product stream, despite the relatively large oil content.

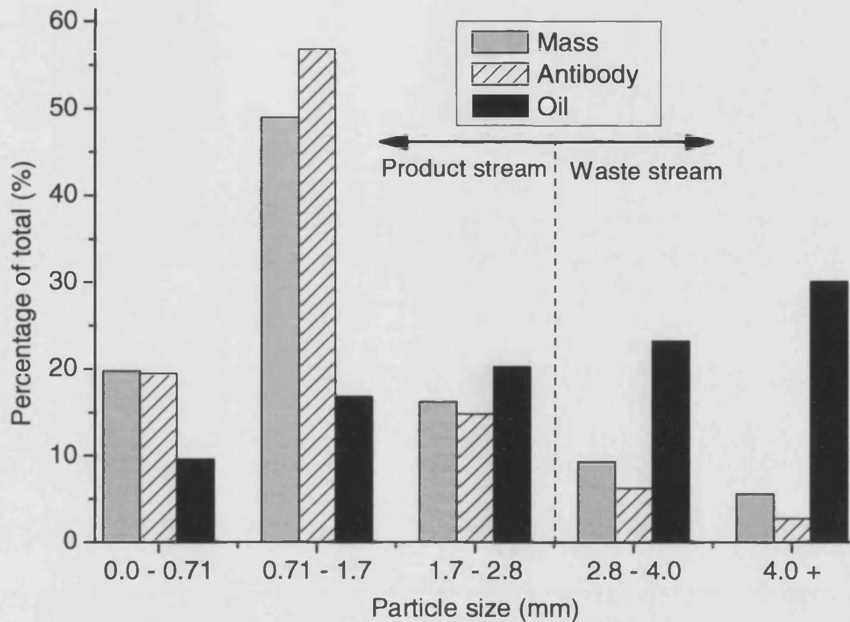


Figure 6.9 Small-scale degerming products of transgenic HVY2 seed

6.5.4 Transgenic and low-quality non-transgenic seed

By physical appearance, B73 seeds were similar to the transgenic seed. By comparing the figures illustrating the mass and oil distributions for these seeds (Figure 6.8 and Figure 6.9), it is clear that both seed types were successfully degermed, given by the low mass of oil-rich waste stream. However, there was a greater difference between the oil contents of the product and waste streams of degermed B73 seed than transgenic HVY2 seed (Table 6.9). Therefore, despite the introgression with UE95, the degerming quality of transgenic seeds was lower than that of B73 seeds.

6.5.5 Two transgenic seed varieties: HVY2 and HVF1

The two product streams of degermed transgenic HVY2 and HVF1 were similar in the proportions of mass, oil and antibody (Table 6.9). By graphically comparing the degerming quality of HVY2 (Figure 6.9) and HVF1 seeds (Figure 6.10), there was a greater distinction in the mass and oil content in the degerming of HVF1 seeds, between those seed fractions designated for product and waste streams, than for HVY2 seeds. The magnitude of this difference was significant, as shown by the oil contents of the waste and product streams in Table 6.10. One possible cause of this difference was the difference in characteristics between these transgenic seed varieties (Table 6.7). Although richer in oil concentration, the germ of HVY2 seed contained slightly less total oil than HVF1 seed. Also, because HVF1 seed and seed germ were larger, the same extent of degerming would have released larger germ and germ fragments of a sufficient size for retention on the upper sieves.

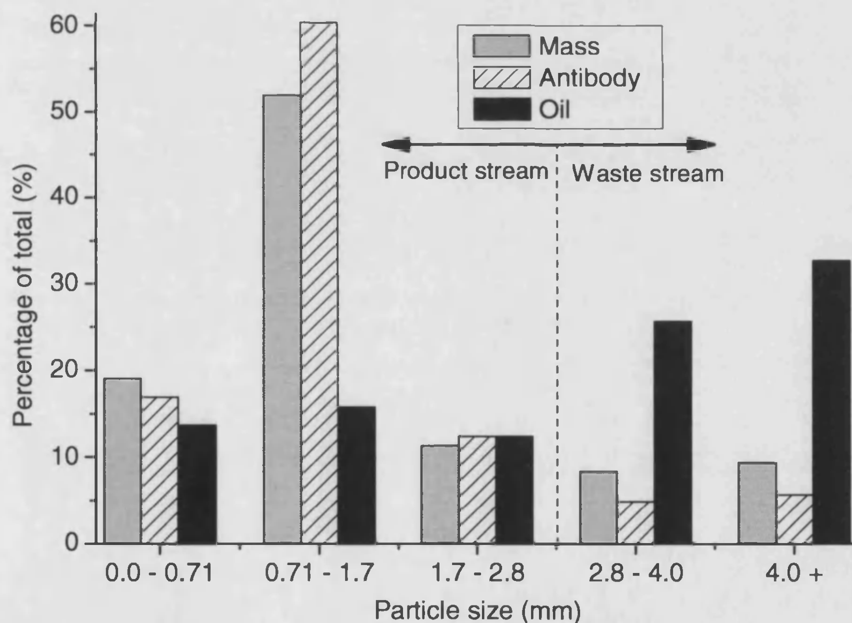


Figure 6.10 Small-scale degerming products of transgenic HVF1 seed

Table 6.10 Transgenic seed mass balances across the degerming process (triplicate experiments)

Transgenic Seed		Feed	Product	Waste
HV2	Mass (g) (Error)	10.71	8.73 (±6.6%)	1.53 (±6.7%)
	Oil (mg) (Error)	411	166 (±24.6%)	189 (±3.5%)
	Antibody (μg)	58.47	72.73 (±13.5%)	7.12 (±2.3%)
HVF1	Mass (g) (Error)	12.75	9.75 (±0.3%)	2.10 (±2.6%)
	Oil (mg) (Error)	481	203 (±1.2%)	283 (±1.3%)
	Antibody (μg)	54.23	70.41 (±8.5%)	8.22 (±30.9%)

There was very little difference in the distribution of antibody with particle size between the two varieties of transgenic seeds processed. Approximately 90% of seed antibody collected in the product stream of both varieties processed. Whereas manually fractionated endosperm contained 97.0% of seed antibody, HVY2 product stream contained 91.1% of the total detected, and HVF1 product stream contained 89.6%. The balance between the loss of approximately 10% of total antibody and the separation of 53.2 and 58.3% of seed oil from HVY2 and HVF1 seed, respectively, would be the important factors to consider in the assessment of the value of this process in the preparation of material for extraction and purification.

Whereas losses in mass and oil were observed across the degerming process, the quantity of antibody increased. It was not possible that a greater quantity of antibody would leave a process than had entered it. The most likely cause of this imbalance was that the additional processing through the small-scale degerming process improved the extraction yield. The extra processing might have resulted in a smaller average particle size than those produced by the application of the standard analytical procedures, thus improving the washing of the antibody from the broken cells and reducing the effect of slow intraparticle diffusion (section 6.2.2.a). It was also possible that roller-milling degermed corn fragments increased the extraction yield by producing an effect similar to the flaking rolls as used by Bai *et al.* in the processing of transgenic canola, in which it was discovered that a greater extraction yield of rGUS was always achieved from ground flakes compared to ground flour of similar particle size (Bai et al., 2002).

6.6 Results extrapolation exercise for high quality transgenic seed degerming

Continuation of the introgression programme was expected to lead to improvements in plant physiology, and the production of high quality transgenic seeds (section 2.1.2). It follows that the quality of transgenic seed degerming using the small–scale process was expected to improve, and to approximate the quality achieved by processing hybrid 8366 seed.

If it were possible to predict the quality of the waste and product streams based on the performance of degerming other types of seed, then it would be possible to reasonably accurately predict the degerming quality of high quality transgenic seed based on the data produced from processing hybrid 8366 seed. Although the viability of this prediction was debatable, since it has not previously been possible to predict the degerming quality of any variety of seed based on the degerming quality of another variety, it was a useful exercise to demonstrate the extent of product and oil separation from transgenic seed that might be possible using the small-scale degerming process (calculations are provided in Appendix G). Using the known masses of germ and endosperm in each stream, and the experimentally determined seed and processing data of hybrids 8366 and HVY2 seeds, processing high-quality transgenic seed through the small-scale degerming process would yield a product stream containing approximately 97% of seed antibody, 32.5% of seed oil, in 90% of seed mass.

6.7 Summary

The highest quality of degerming was achieved by processing the seed which was regarded to be of the highest quality (hybrid 8366). The difference in degerming quality was not the result of one single factor, such as a low oil content of the product stream, rather it was the combination of the factors specific to the design of the

Degerming Evaluation Factor (section 5.2). Both the mass and oil content values of the fractions produced in the degerming of high quality seed differed to those obtained in the degerming of transgenic seed, but neither significantly enough individually to conclude, with confidence, that a higher quality of degerming was achieved when processing the high quality hybrids. However, it was demonstrated that, by combining the individual fractions into product and waste streams, the high quality hybrid was degermed to a higher quality than the low quality non-transgenic seeds and the transgenic seeds. It was also demonstrated, by tracking the antibody across the process, that a large proportion of the antibody collected in the product stream, which also contained less than half of the seed oil. These results illustrated that the small-scale degerming process was capable of degerming a variety of types of seeds with a wide range of physical characteristics, including transgenic seeds.

Chapter 7 - Degerming Scale Comparisons

7.1 Introduction

Currently, no commercially available methods exist for the small-scale temper-degerming dry-milling of corn seed (section 1.4). Small-scale degerming processes exist (section 1.4.4), including a wet-milling process of 100g capacity, which was developed for the reduction of sample sizes and labour time requirements for the determination of wet-milling characteristics of corn samples (Eckhoff et al., 1996). The product yields were compared to those achieved using a 1kg wet-milling process, and were shown to be statistically equivalent. However, the wet-milling process is not suitable for the processing of transgenic seed (section 1.3.1). Similar comparative data between scales for the dry-milling of transgenic corn seed would be of great value (section 1.4.5), but a 1kg capacity dry-milling degerming process was not available for comparison with the small-scale degerming process in this study. Therefore, the products of the small-scale degerming process were compared to those produced by the pilot-scale degerming of the same type of seed.

One batch of 50kg of inbred B73 seed (non-transgenic) was processed through the pilot-scale Beall degermer. Samples of through-stock and tails (section1.3.5.a) were analysed separately in order to assess the extent of germ separation in the Beall degermer, and to assess the particle size range and oil content of these different output streams. The small-scale germ-endosperm separation process (i.e. roller-milling and sieving) was applied to the processing of the two output streams produced by the pilot-scale Beall degermer, in order to assess the quality of these streams and the extent of germ-endosperm separation that had been achieved using just the Beall degermer. Samples from the two output streams were then combined in their correct proportions, equivalent to the mass of 50 B73 seeds, and were moisture conditioned in order to reach the same moisture content as small-scale degermed seed, before processing through the small-scale germ-endosperm separation process (i.e. roller-milling and sieving). The quality of germ-endosperm separation of Beall degermed seed, using the small-scale separation process, was then compared to the quality of

degerming achieved using the same seed processed through the complete small-scale degerming process (section 6.5.2).

7.2 Degermer products analysis

Seed processed through the small- and pilot-scale (Beall) degermers were immediately separated according to particle size, without roller-milling, and the oil content of these sized fractions was analysed. The results of pilot-scale-degermed and small-scale degermed seed are illustrated in Figure 7.1 and Figure 7.2, respectively. The mass and oil distributions of the products of the two degermers, without further processing, differed considerably. The pilot-scale degermer produced considerably more fines in the range of 0.0μm to 710μm, and oil was distributed more widely across the sieves, than the products of the small-scale degermer. The small-scale degermer yielded a very small fraction of fines, with much of the degermer output and oil collected on the largest two sieves.

7.3 Beall through-stock and tails analysis

The pilot-scale degermer through-stock consisted of 68% of feed mass, and the oil content (4.7% w/w) was approximately twice that of the tails stream (2.4% w/w). Additional processing was required to separate the released germ from the mixtures of seed particles of various sizes. Although when processing at larger scales, the two streams are often combined prior to further processing, they were processed separately in order to more closely observe the extent of degerming which had been achieved in the degermer. The results of processing the through-stock and tails, through the roller-mill and sieving operations of the small-scale germ-endosperm separation processes yielded the results as illustrated in Figure 7.3 and Figure 7.4, respectively.

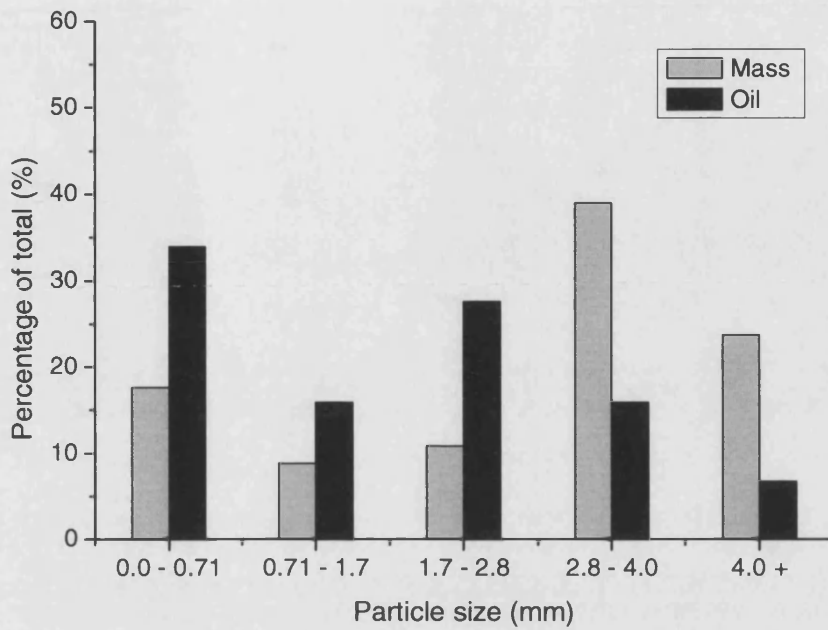


Figure 7.1 Pilot-scale degermed inbred B73 seed mass and oil distribution

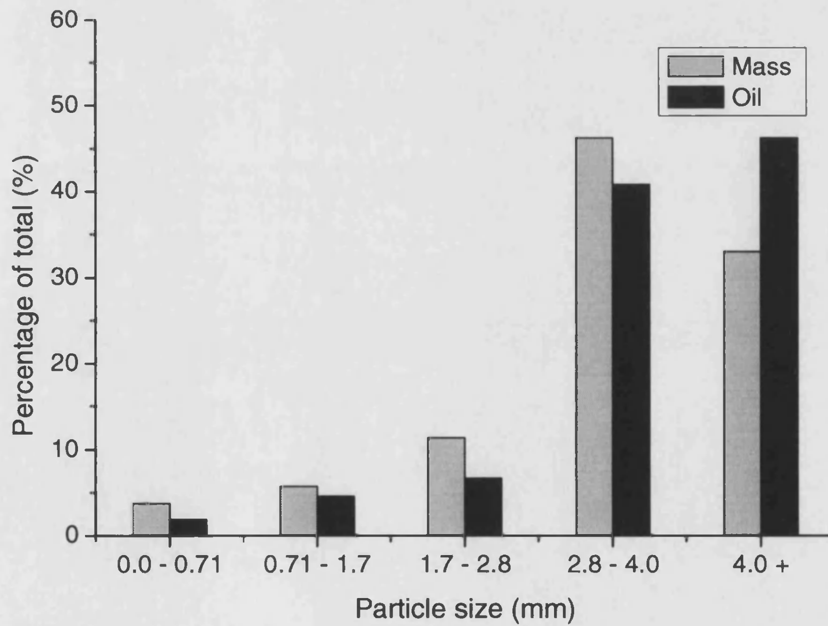


Figure 7.2 Small-scale degermed inbred B73 seed mass and oil distribution

Both figures show that a high proportion of the feed mass was reduced to the smallest range of particle sizes, 0.0mm-0.71mm (ca. 25%). The major differences in the mass distribution between the two fractions, was in the collection of a greater mass of larger fragments in the processed through-stock stream (A), and a much larger mass of fragments in the 0.71mm – 1.7mm particle size range in the tails stream (B). In the through-stock stream, the production of larger fragments after roller-milling, which were also very high in oil content, was indicative of the collection of a large proportion of germ in the fraction of large particle sizes.

There was a greater difference in the distribution of oil between the through-stock and tails than in the distribution of their mass. Much of the oil in the through-stock was contained in the small mass of large fragments, and the relatively large mass of fines. The tails stream also contained relatively large quantities of oil in the small mass of the largest category of particle size, which indicated that germ was entrained in this stream. However, the mass of the large fragments collected in the upper two sieves of the tails stream was small (0.44g) compared to that collected in the processing of through-stock (0.80g), and so the total oil content of this fraction (0.047g) was less than half of that of the through-stock (0.107g).

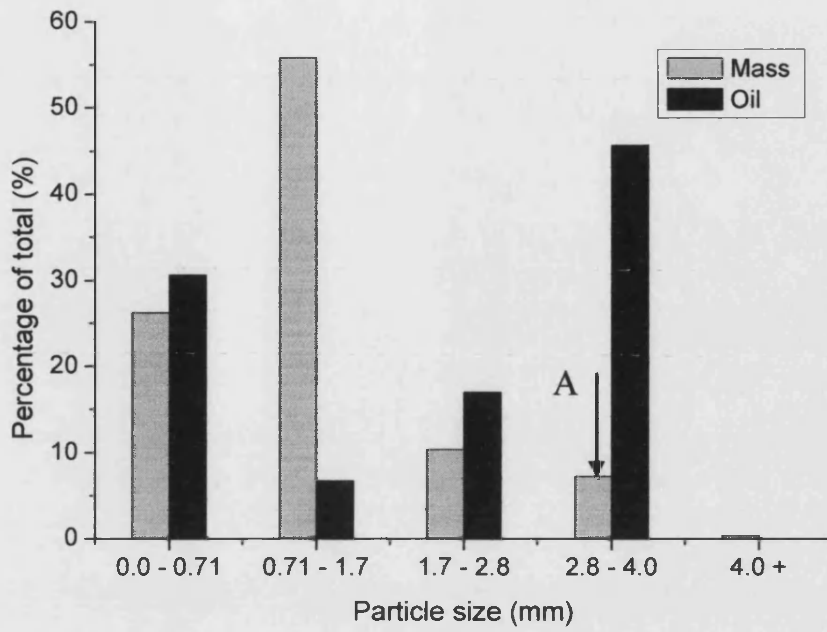


Figure 7.3 Pilot-scale degermed through-stock mass and oil distribution

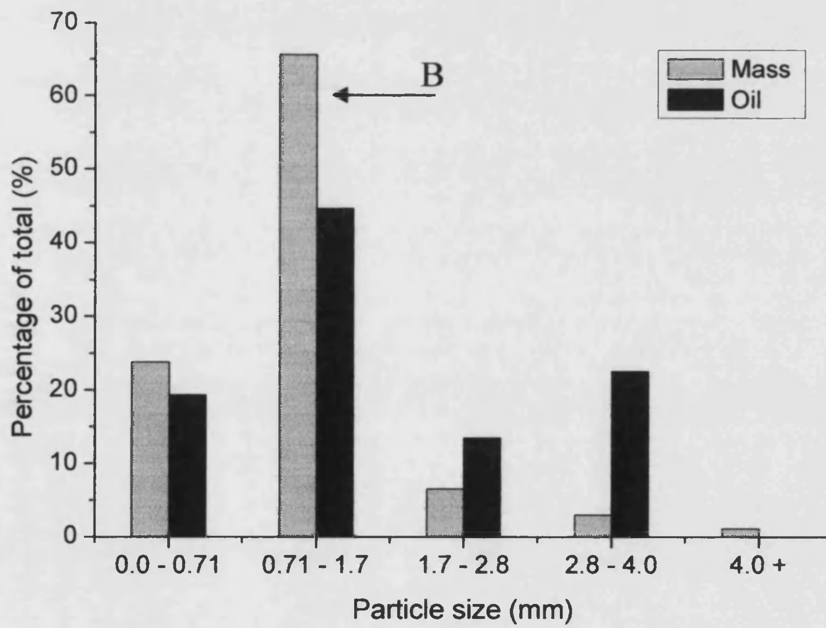


Figure 7.4 Pilot-scale degermed tails mass and oil distribution

7.4 Pilot-scale degermed seed separated via roller-milling and sieving

Seed which had been processed through the pilot-scale degermer, and the two output streams combined for processing using the small-scale germ-endosperm separation process, were not separated into distinctly different waste and product streams (Figure 7.5) with regard to their proportions of mass and oil.

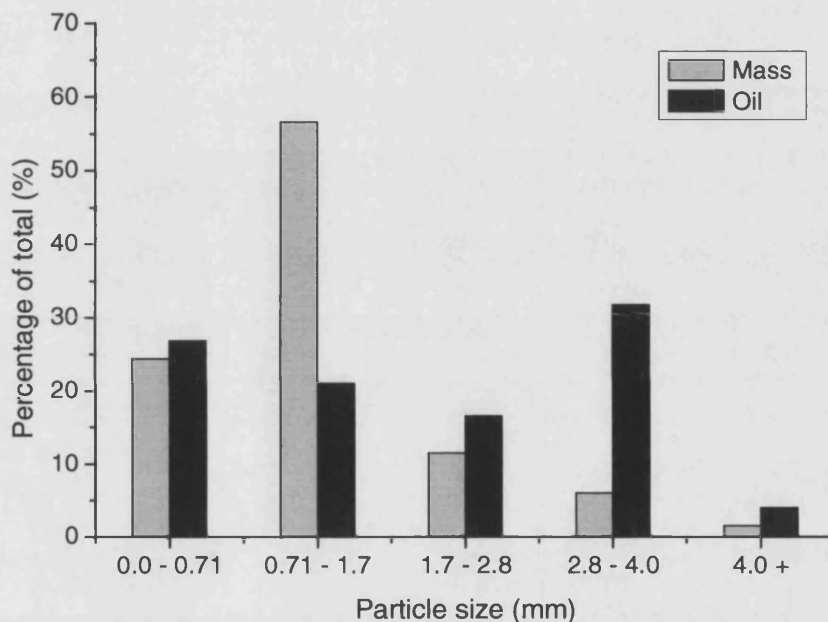


Figure 7.5 Pilot-scale degermed inbred B73 seeds mass and oil content distribution, with products separated using the small-scale germ-endosperm separation process

The product stream contained a high proportion of seed oil (64.3%) in a very high proportion of seed mass (92.5%). The proportion of oil in the product stream was much higher than that produced in the separation of small-scale degermed seed (29%) (Table 6.9). This was mostly a result of the smaller mass of fragments collected in the waste stream, and the very high oil content of the fines produced (i.e. particles <0.71mm). Although, to a small extent, the seed fragments were separated into two streams, one of which was richer in germ than the other, the overall quality of degerming using this arrangement of process operations was poor. This could be

attributed to the high levels of abrasion in the pilot-scale degermer. The high abrasion levels were required for the production of large, germ-free grits (section 1.3.2), but were not suitable to the small-scale separation process which was ill-equipped for the separation of mixtures of very small fragments of germ and endosperm.

7.5 Transgenic seed degerming at pilot-scale

The separation characteristics of pilot-scale degerming transgenic HVY2 seed was predicted based on approximations using the data obtained from processing B73 seed. Again, it was not possible to reliably predict the degerming quality based on a different type of seed (section 6.6). However, in the absence of sufficient quantities (50kg) of the transgenic seed to test directly, the exercise was valuable simply to observe the extent of transgenic seed degerming using a pilot-scale degermer that might be achieved, based on the quality of degerming of seeds which were similar in description to the transgenic variety.

In the transition from small-scale to pilot-scale degerming of B73 seed, followed by the small-scale separation process, the oil content of the product stream approximately doubled, and the product stream mass increased by approximately half the mass of the waste fraction, as shown in Table 7.1. Assuming that similar changes would occur in the mass fraction and oil content of the product and waste of HVY2 seed, if processed through the Beall degermer, the product stream would consist of approximately 92% of the output mass, and would contain approximately 73% of the oil and 96% of the antibody.

Previously, product and waste stream antibody content was predicted for the degerming of high quality transgenic seed (section 6.6). It was not possible to predict the quality of degerming high-quality transgenic seed using a pilot-scale degermer, since two important aspects of processing, the seed type and scale, were different. In order to make such a prediction, one of the high-quality non-transgenic hybrids would have to be processed through the pilot-scale degermer. Similarly to the comparisons used previously, the change in the quality of degerming of the high-quality non-transgenic seed between scales would be combined with the small-scale degerming

results of the transgenic seed, for the prediction of the quality of degerming a high-quality transgenic seed.

Table 7.1 Experimental and predictive data for mass, oil and antibody content of product streams of inbred B73 seeds and transgenic HVY2 seeds

RESPONSE	EXPERIMENTAL DATA			PREDICTIVE DATA
	Small-scale		Pilot-scale	Pilot-scale
	B73	HVY2	B73	HVY2
Mass (%)	81.5	84.2	92.5	92
Oil (%)	29.3	46.8	64.3	73

7.6 Summary

As a result of the production of a large quantity of oil rich fines, and the significantly higher oil content of other fractions in the product stream, substitution of the small-scale degermer by the pilot-scale Beall degermer resulted in a substantial decrease in the quality of germ-endosperm separation. Since the Beall degermer is used in industrial dry mills for the production of low oil content endosperm products, it must be a part of an extensive process for the successful separation of the wide range of fractions produced. The small-scale separation process, consisting of three recycles through the roller-mill and one sieving operation, was successfully applied to the processing of small-scale degermed seed fragments, but was entirely unsuitable for the separation of pilot-scale degermed seed.

Chapter 8 - Conclusions and Future Work

It was intended to devise a process which was capable of separating corn seed embryo, or germ, from whole corn seeds. The first operation in this process consisted of a small-scale degerming device. The design of this device incorporated several design features of the major large-scale degerming devices in order to subject corn seeds to the required high levels of impact. The products of the degermer consisted of a mixture of seed components of different sizes. Separation of the germ and endosperm fragments was accomplished using a roller-milling and sieving process, which was based on the separation processes used at industrial scales, and which manipulated the differences in physical properties between the seed components. This shortened separation process was demonstrated to be capable of separating corn fragments into two distinctly different streams, with regard to the mass and oil content of these streams (Figure 3.14).

Using the method of factorial experimental design, the effects of operating factors and seed moisture content were investigated. Statistical analysis of the results enabled the selection of the major factors for the production of multiple linear regression models (Equation 5.5) which represented the selected response parameters in terms of these major factors, and the production of response surfaces using these equations to illustrate the results. The finalised design of the degermer was such that it would allow for the highest frequency of germ separation, and the greatest flexibility in operating conditions to account for the widely different seed physical characteristics (Figure 3.5 to Figure 3.8).

The quality of degerming using the small-scale degerming process varied between seed types. The extent of variation in any one of the mass or oil response parameters was insufficient to confidently conclude that the degerming quality was any better or worse with one variety compared to another. The degerming evaluation factor (DEF) was designed for use as a tool for the identification of the optimum process operating conditions. This index incorporated the mass and oil requirements of the waste and product streams into one single, dimensionless value. Point calculations enabled the

identification of the set of operating conditions and the required seed moisture content which maximised the DEF value (Figure 5.5). This point represented the maximum degree of germ-endosperm separation, and the optimum operating conditions of the small-scale degerming process (section 5.5).

Batches of 50 seeds of five different types were processed through the small-scale degerming process. The greatest difference in the quality of degerming, as defined by the mass and oil contents of the product and waste streams produced, was observed between the high quality seed hybrid 8366 and both varieties of transgenic seeds. The quality of degerming of the high quality seed exceeded that of the transgenic seed, by the generation of a product stream which was both higher in mass and much lower in oil content, by approximately 10% (w/w) and 15% (w/w), respectively. The major cause of this difference was believed to have been a result of the genetic background of the seed, since the sizes of seeds were shown to have had little effect on the degerming quality over the range of sizes tested. Seeds which differed in their genetic backgrounds have been demonstrated to vary in suitability to degerming (Kirleis and Stroshine, 1990). It follows that the variability observed in this study might also have resulted from the different densities and hardness of the seeds, and possibly the different strengths of the bonds between the different seed components. However, a high level of transgenic seed degerming was achieved, as the product stream contained less than half of the major contaminant of the seed (i.e. < 47% (w/w) seed oil), in 83% (w/w) of seed mass and containing 90% (w/w) of the detectable antibody.

A comparison of scales of degerming involved the substitution of the small-scale degermer with a pilot-scale Beall degermer. Both degermers were operated at conditions which maximised the quality of the output, and the products of both were processed through the small-scale germ-endosperm separation process. The small-scale degerming process in its entirety proved to be much more effective in the separation of germ and endosperm (Figure 6.6 compared to Figure 7.5). The difference in the overall degerming quality between the two degermers was consequential to the higher level of attrition in the pilot-scale degermer, which produced more small fragments of seed than the small-scale degermer. In particular, smaller germ fragments were produced with much larger quantities of oil-rich fines.

The small-scale degerming process was not capable of separating these smaller seed fragments into distinctly different product and waste stream as effectively as the longer, large-scale separation process post-degerming.

As demonstrated by the quality of the product stream of degermed hybrid 8366 seed (Table 6.9), the small-scale degerming process was capable of separating a large proportion of germ and endosperm. The highest quality of germ-endosperm separation achieved using the small-scale degerming process generated a product stream which contained 1.1% (w/w) oil, which compared favourably to the product stream of the pilot-scale degermed seed (1.7% (w/w) oil), but was richer in oil than 80% of the endosperm fractions produced using the Beall degermer in industrial scale dry-milling process (oil <0.8%, w/w) (Brekke, 1970). The product stream of transgenic seed degerming contained 1.9% (w/w) oil, thus demonstrating that the small-scale degerming process could be applied to the processing of transgenic seed. Other applications of the small-scale degerming process include:

- The assessment of the improvements in the quality of transgenic seed, with regard to the suitability to degerming, upon introgression with high quality varieties of seeds.
- The provision of processed seed of a similar quality to that which would be produced in large-scale degermers, for the development of an extraction and purification process
- Continuous operation for the production of sufficient quantities of material for clinical trials.
- Use of the operating characteristics as the basis for scale-up to meet future capacity requirements

Future work

During the course of this research, several aspects of the small-scale degerming were identified as opportunities for further study, some of which might lead to further improvements in the quality of the product stream. These include process modifications, the incorporation of additional operations, scale-up, by linking the

small-scale process to large-scale operations, and analysis of the quality of antibody extraction and purification from degermed and non-degermed corn seeds.

Process modifications

- The use of rollers in the roller-mill with less coarse surfaces, particularly for the final passes through the roller-mill, in order to minimise damage done to the germ and germ losses into the textured surface, and to maximise the size of germ platelets.
- Introduce factors to account for the differences in degerming quality between different types of seeds, resulting from the different proportions and contents of seed components (section 3.3.2)

Additional operations

- Increase use of sieves for early separation of fines or large germ fragments before roller-milling or between the recycles through the roller-milling operation, or introduce more sieves to provide a more distinct cut off point between those fractions allocated to the waste and product streams
- Explore the use of low capacity hydrocyclones for the separation of small quantities of hull and small germ fragments from the endosperm, based on differences in densities and aerodynamic properties.

Scale-up

- Configure correlations between the small- and large-scale degerming devices, ultimately for the prediction the quality of large-scale degerming for any type of seed, based on the quality achieved on the small-scale.
- Test large-scale germ-endosperm separation process in experimentation using products generated from the small-scale degerming device.

Extraction and purification

- Evaluation of the degerming process by comparing the antibody extraction and purification yield from degermed and non-degermed transgenic seed.

Prospects for Transgenic Crops

The future of recombinant protein production in transgenic crops will be determined by the success of their integration into the environment, their containment, and the final product expression levels within the target host system. Each product and each host system must be considered on an individual basis. Extensive agricultural practices, processes and an assortment of regulations exist and can be directly applied to the production of transgenic crops. Currently, one of the major hurdles to the widespread production of pharmaceutical crops is that no production system has yet been established which satisfies all of these issues. It is possibly a matter of time before all of the requirements of production, and the potential advantages inherent to this production system, are combined and applied to set the precedent for the production of recombinant proteins in transgenic crops.

Chapter 9 - Process Economics

9.1 Introduction

The economic advantages of plant-based production of recombinant proteins have been reported in the literature (Evangelista et al., 1998; Stoger et al., 2001). Some researchers have compared transgenic plant production systems to more traditional methods of production i.e. cell culture (Hood et al., 2002), whilst others have covered the economics of extraction depending on the species of plant (Kusnadi et al., 2001), or focused on the minimum production and purification targets for economical production (Mison and Curling, 2000). In some of these studies, the costs of production have been investigated based on the application of standard protein extraction and purification techniques to recover the product from an extract taken from transgenic material. None, however, have quantified the potential costs savings that might be achieved by applying existing agricultural processes for the improvement of feed quality into the extraction and purification process.

This chapter provides an overview of the major differences in the costs of recombinant protein production between transgenic plants and fermentation production systems. The emphasis of this comparison is on the cost of manufacture of the required volumes of product, post development and prior to downstream processing, indicated by the alternative routes in the production schematic illustrated in Figure 9.1. This part of the production process was believed to incur the greatest cost savings for plant based production.

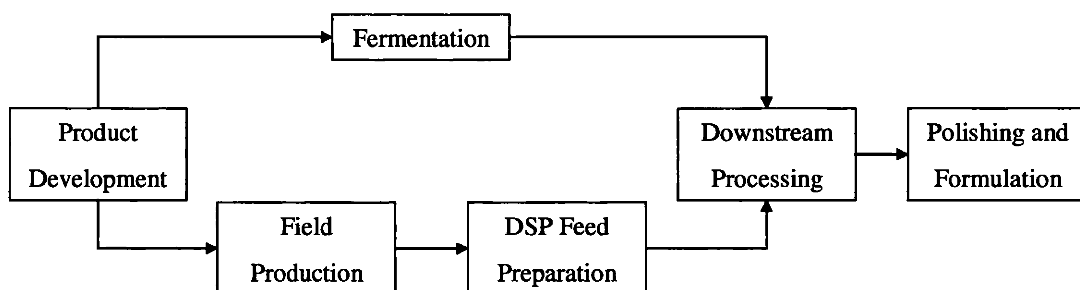


Figure 9.1 Production of recombinant proteins by fermentation or by transgenic plants

9.2 Estimated Production Costs of Transgenic Corn

Plant-based production of recombinant proteins has been estimated to cost approximately 10-50 times less than production in cell culture (Daniell et al., 2001; Kusnadi et al., 1997) (section 1.2.10). The sources of this large cost difference are in production prior to downstream processing, in which sunlight, soil and water in plant-based production systems replace expensive nutritional media, contained buildings and steel upstream processing vessels, such as fermentors. Adhering to the regulations associated with field production of transgenic pharmaceutical plants (section 10.2) significantly raises the costs of production when compared to non-transgenic plants. However, it is still widely believed that the application of an existing infrastructure, practices and processes would significantly reduce the cost of production prior to downstream processing and purification when compared to production by fermentation (section 9.2.2), even with the use of dedicated farming equipment, methods of transportation and specialist storage facilities.

9.2.1 Effect of target purity and the scale of production

The varying cost of antibody production in corn is affected by many factors, primarily product expression levels, the required level of purity, and the scale of production (Evangelista et al., 1998; Mison and Curling, 2000). The costs and scale of production are product specific. Scales range from as little as 250kg-500kg per year of highly purified, or parenteral grade, antibody, up to 1000kg-5000kg per year of intermediate grade antibodies such as anti-HSV antibodies and other prophylactic anti-infectives, such as those held in Epicytes's product portfolio (section 1.5.1). Transgenic plants are particularly suited to the production of large volumes of product, in which the favourable economies of scale apply in the use of existing, low cost, large-scale, agricultural practices and processes. For the production of smaller volumes required in higher purities, in which the benefits of agriculture contribute much less significantly to the overall process economics, plants may not prove to be the most economical system. This is particularly true in light of the generally low expression levels that have been achieved in plants (section 1.2.5), such as antibody

expression levels within the range of 0.35% to 2% of total soluble protein (Giddings, 2001), and the increasing titres, up to 2g/L (Chadd and Chamow, 2001) that have been obtained in mammalian cell fermentations.

9.2.2 Costs of agricultural production

Based on achievable expression levels of 1.5mg/g grain, the production of approximately 7850kg/hectare, and an extraction and purification yield of 40%, the total operating costs for the manufacture of recombinant proteins in corn seeds has been estimated as US$49.4/g of product with an annual output of 100kg per year (Mison and Curling, 2000). Corn production costs were estimated to be $0.7/g, which tallied closely with those presented by Crosby ($0.65/g) (Crosby, 2003), but were substantially higher than previous estimations ($0.20/kg) (Evangelista et al., 1998). Other costs included in the total cost estimate were consumables, utlities, personnel and capital. In the study by Mison and Curling, the favourable economies of scale of plant-based productions were demonstrated for production at up to 100 metric tons per year, at which operating costs were as low as $4.5/g. Whereas capital related costs and labour costs represented the largest part of the production costs for the production of 100kg per year, these costs became less significant at the larger scales. The largest fall in costs occurred over the increase in capacity over the lower end of the scale, such that an increase in production by one order of magnitude reduced the cost from $49/g to $13.8/g. These costs are substantially lower than those reported via animal cell cultures (Hood et al., 2002; Khoudi et al., 1998; Smith and Glick, 2000), and also lower than the current target cost of goods of mammalian cell production ($50/g), which is of 1-2 orders of magnitude smaller than current cost (Chadd and Chamow, 2001).

9.3 Interface Between Agricultural Processes and the cGMP Facility

9.3.1 Product extraction from unprecedented quantities of biomass

Epicyte Pharmaceutical had planned on processing 50 000 metric tonnes of corn over 300 day campaign for the annual production of 5000kg of antibody. In order to

process approximately 166 666kg of seed per day, and based on the use of a low extraction ratio of one unit mass of seed to two units of mass of water, 333 333L of high quality water would be required daily for the extraction operation alone. It was estimated that an additional 216 666L per day would be needed for processing. The daily requirement of at least 550m^3 greatly increases the costs of utilities. The cost of water, alone, has been calculated to represent up to 66% of utility costs in the production of approximately 100 metric tonnes per year of antibody (Mison and Curling, 2000). Based on the extraction ratios used for the antibody assay (section 2.1.7.d), it is likely that the required volumes as predicted above would increase in order to satisfactorily extract the product.

9.3.2 Impact of corn degerming on processing costs

Degerming transgenic seed is likely to improve process economics in several ways. In the absence of experimental data, it can only be assumed that degerming would reduce the costs of DSP by reducing the maintenance costs i.e. cleaning, and the frequency of membrane replacement. Complete separation of oil- and protein-rich germ from the processing stream would reduce the processing volumes by approximately 10%, which would reduce the pumping requirements in terms of the volume and viscosity of the process stream, reduce the sizes of pipes, vessels, and several unit operations e.g. chromatography columns, and reduce the volume of high grade water required for extraction and DSP.

9.3.2.a Increase in the crispness of operations resulting from the degerming of seed

The advantages of degerming have been summarised above, and in sections 1.2.6 and 1.4.4. Whereas the viscosity of plant-based biosolutions has been likened to that of pea soup, degermed seed would produce a less thick soup containing smaller quantities of endogenous proteins and oils, which would result in much crisper subsequent separation interfaces (Epicyte Pharmaceutical). The financial value of this added ‘crispness’ is difficult to quantify without experimental data to draw comparisons between the extraction and purification efficiencies when extracting from degermed and non-degermed seed. The one study which compared these efficiencies, using a three step chromatographic purification, did so for the production

of rGUS which had been targeted to the germ of corn seed (Kusnadi et al., 2001). This research group found that neither starch nor oil affected the purification yield and final purity, and concluded that the choice of starting material to the purification process would depend on the process economics, including seed fractionation, downstream processing and the potential revenue from co-products. These conclusions, however, are very process and product-specific. Different unit operations would be used for the purification of antibodies compared to enzymes, such as product-specific chromatography columns to manipulate the binding properties of antibodies.

9.3.2.b Demonstrating the true value of degerming by the application of realistic purification processes

The pampering conditions of the extraction and purification processes of the laboratory are often designed to demonstrate the maximum product yield achievable, and for the production of sufficiently high quantities of high purity product for characterisation studies. Outside of the laboratory, the prohibitive costs of large-scale production using the same pampering conditions, result in the need for economically, and environmentally sound, alternative methods. It would be essential to the determination of the true value of the degerming operation to compare the efficacy of the extraction and purification process, as perceived for the large scale operations, on degermed and non-degermed seed.

9.3.2.c The effect of degerming on the production of topically applied antibodies

Production of antibodies for topical application would not require the use of extensive and expensive chromatographic separations as required in order to attain the high-purities of parenteral grade products. It was believed that membranes might be used to achieve the bulk of product separation and purification (Epicyte Pharmaceutical). Under these circumstances, the presence of seed oils and endogenous proteins would have a large impact on the quality of separation, either by fouling the membrane and forming a filter cake which would obstruct the passage of antibody, or by passing through the pores and contaminating the product stream. The quantification of this

effect would have to be determined preferably by experimentation, which would require the use of a degerming device for the production of a stream containing scarce quantities of seed oils and proteins, for comparison with whole ground seeds. Alternatively, quantification of the effect of protein and oil removal might prove possible by estimation based on the processing information provided by the suppliers. Although the formation of filter cake might, to some extent, serve to add to the filtration of debris, it most often results in a sharp decrease in throughput and yield of product. Newer separation methods exist, such as vibrating membrane surfaces (Sellick, 2003; Wilson et al., 2003), effectively decouple the forces that force the feed material into a processing system, from those forces that keep membranes clean. Using this operation, it is possible that the efficiency and lifetimes of membranes might be increased during the processing of streams produced from both degermed and non-degermed seed extract.

9.3.2.d The effect of degerming on the production of parenteral grade antibodies

For the production of parenteral grade antibodies, it is possible that the necessary, and very expensive, chromatographic operations might be adversely affected by seed oils which would foul the resin. This has been demonstrated by Kusnadi *et al.*, in the observation of a reduction in flow rate through the columns in the purification of rGUS (Kusnadi et al., 1998a). Furthermore, methods to reduce the protein loads to membrane and chromatographic separation processes would greatly improve their efficiency. Other methods to achieve this reduction in protein load, in addition to degerming of seed prior to processing, include the precipitation of native proteins or the product out of solution for separation using less expensive processes. Degerming would be the preferred option of the two due to the lower equipment, processing and waste disposal costs. The subsequent reduction in process stream protein content, with concomitant increase of the antibody fraction of the total soluble protein, would improve the efficacy and lifetime of membranes and reduce the chromatography column size and resin cost (Evangelista et al., 1998).

9.3.2.e Potential revenue from waste material

A major issue with processing vast quantities of transgenic material is in the safe and efficient disposal of waste materials. These include the spent solids i.e. the stems, leaves and seedless cobs, the ground corn filter cake produced after extraction and filtration, and the large volumes of water separated from the extract solution during clarification of the extract and product purification. Some transgenic products, such as human serum albumin or haemoglobin, are so safe that the waste material could be fed to animals. Others, however, may be so hazardous that only incineration may be appropriate. Most by-products can be disposed of by fermentation, which would provide additional revenue to an otherwise costly disposal process (Crosby, 2003). Some of the regulations associated with this issue are discussed in the following chapter (section 10.4.5).

Separated germ could add to the potential sources of revenue of the various by-products of transgenic crop processing. This was based on the assumption that the manufacture of each by-product would comply with the regulations associated with the recombinant proteins produced. Corn oil could be produced by extraction from the germ in its relatively pure form (i.e. dry-milling output quality). Corn oil extraction is not possible using anything other than the separated germ. Similarly, the production of ethanol using corn starch i.e. refined solids separated from the product stream after extraction, is a more successful process in the absence of seed germ. Therefore, degerming increases the range of potential applications of waste material for revenue-generating by-products, which would serve to offset the costs of processing and reduce the disposal costs of waste products.

9.4 Downstream processing

Estimates of downstream processing costs are difficult to make, since no plant derived antibodies are yet produced on a commercial scale. However, there are examples of other commercially produced proteins, discussed in section 1.4.3 and section 1.4.4, and Evangelista *et al.* have provided an economic evaluation of one such process for the purification of rGUS (Evangelista et al., 1998). This process, however, did not

extract from degermed corn seed, rather the product was extracted from whole ground corn.

9.4.1.a Criteria for the evaluation of the benefits of degerming in DSP

The introduction of degermed seed into the purification process, as opposed to whole ground seed, might drastically alter the suitability and feasibility of certain operations. Therefore, it is important to consider which of these operations might benefit the most from a feed produced from fractionated seed, in terms of the capacity for proper operability, and the costs of operation, maintenance and replacements. It has been reported that the extraction process, represented by the flow diagram in Figure 9.2, could potentially sufficiently purify antibodies to be used directly either for non-parenteral, or topical delivery, or as a high quality feed to further purification processes. The extraction, filtration and chromatography operations are all likely to benefit from degerming, for the reasons as detailed previously (section 9.3.2).

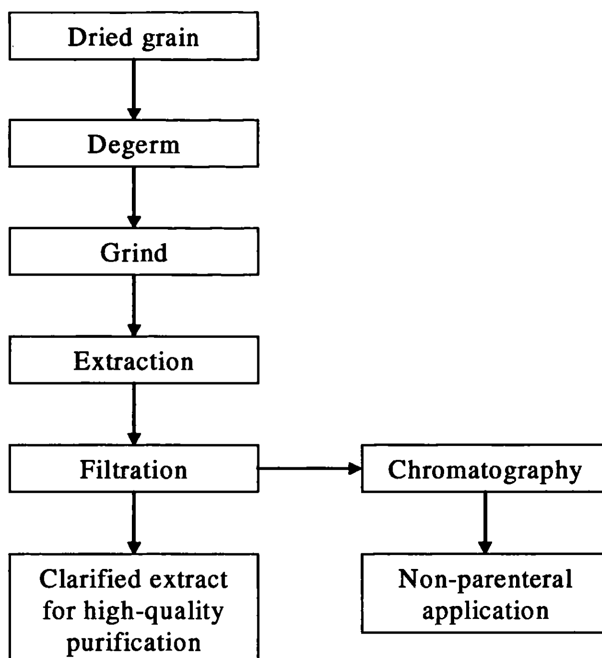


Figure 9.2 Extraction of recombinant proteins from transgenic corn

9.4.1.b Food grade GMP processes for the preparation of feed to DSP

Using food grade GMP unit operations, it is possible to generate an intermediate product of a suitable quality to be used as a feed to the purification process, and which would also be of a similar quality to that required for the purification of products from cell cultures. Apart from the degerming operation, this extraction process is based on known and readily scaleable technologies, which is greatly beneficial to the rapid implementation of suitable operations into a process for the recovery of recombinant proteins from transgenic crops.

9.4.1.c Application of standard DSP operations

Since a high quality intermediate can be produced using food grade GMP facilities, the purification process of plant derived antibodies (Figure 9.3) is expected to be very similar to that used in the purification of antibodies produced in cell cultures. Consequently, the costs of such a process can be estimated using widely available, cell culture processing data.

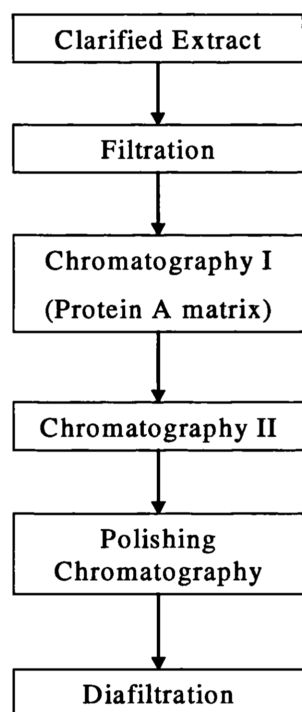


Figure 9.3 Antibody purification to parenteral quality

Omitted from the schematic in Figure 9.2 are operations which might be required for the separation of herbicide and pesticide residues (Miele, 1997), and possibly for the removal of toxins and alkaloids found in tobacco, for example (section 1.2.2.a). Also omitted from Figure 9.2 are those operations which are vitally important for the clearance of viruses and prions from the process stream (Stoger et al., 2002), which are produced using animal cell fermentations. It is likely that these operations will be the major differences between the two purification processes. Another beneficial aspect of plant-based production is that corn is consumed daily by the majority of the populace, and so any possible contaminants are generally regarded as safe (GRAS) and are well characterised from an allergenicity perspective. This greatly reduces the extent of regulatory compliance for the production of antibodies intended for use in topical applications. Although economic comparisons have not been made comparing the effect of the different operations in the process, it is widely believed that the costs of downstream processing would be approximately equal between the two methods of production. One such estimation for the operating costs for the production of 100kg/yr of a monoclonal antibody is US$81/g of product (Sinclair et al., 2002).

9.4.1.d Scale restrictions of essential DSP operations in large scale seed processing

The large capital required for the building, operation and maintenance of an extraction and purification process would constitute the greatest proportion of the final product costs (Kusnadi et al., 1997). By increasing the scale of production, it is possible to reduce the final product cost (section 9.2.2) (Mison and Curling, 2000), although it must be noted that some purification operations are restricted in scale-up, and in order to increase capacity beyond a certain threshold, multiples of these operations must be purchased. This is apparent from the reduction in product costs with increasing scale. Cost reduction of recombinant protein production was greatest during the scale up from 0.1 tons per year to 1 ton per year, from US$49.4/g to US$13.8/g, and then reduced much less significantly up to 100 ton production capacity (US$4.5/g).

9.5 Summary

The successful fusion of agriculture with the pharmaceutical industry has the potential to cost effectively produce vast quantities of recombinant proteins. Pivotal to the success of production are the product expression levels, and the capability of safely and effectively extracting the product from the large quantities of biomass produced. Attainable, and generally regarded as rule of thumb, expression levels of 1-1.5mg of target protein per gram of seed, are required to keep costs low (Mison and Curling, 2000). Below these values, as in the case of Epicyte's intended 0.5mg per gram of seed, production costs were predicted to increase sharply. The impact of degerming corn seed on process economics, for the production of a product-rich endosperm fraction, has not been studied experimentally. Economical benefits of degerming include improvements in extraction and purification efficiencies, resulting from a less viscous stream containing fewer endogenous proteins and oils, and additional revenue from by-products and a reduction in waste disposal costs.

Chapter 10 - Regulatory Considerations

10.1 Introduction

It is important that in striving to optimise the economics of plant-based antibody production, it must be coupled with responsible product stewardship. The introduction of transgenic crops of dubious environmental safety could be disastrous to the environment and to the future acceptability of this relatively new host production system. It is possible that transgenic crops might prove detrimental to the ecosystem, and to the agricultural industry by the transfer of genes into non-modified food crops, and the possible cross-species transfer of genes resulting in the unintended and undesirable production of new breeds of herbicide resistant weeds. This chapter provides an overview of the major regulatory issues involved in the large scale production of transgenic crops. In particular, the discussion is focused upon the regulatory requirements to demonstrate containability of the plant genetic material, and the fusion of good agricultural practices (GAP) with the good manufacturing practices (GMP) in order to meet the high standards required for the production of pharmaceutical proteins. The penultimate section in this chapter summarises the regulatory considerations for the development of transgenic corn expressing pharmaceutical proteins.

10.2 Production system requirements

The application of established methods of biomass production, from field preparation to crop storage, is beneficial to the production in transgenic plants since the technology has already been validated by the appropriate regulatory authorities. Additional regulations apply to the production of plants expressing proteins for human and animal pharmaceutical application, and these guidelines differ between geographical locations. Since Epicyte Pharmaceutical was planning for production within the USA, the production guidelines provided by the US Department of Agriculture (USDA) and the Food and Drugs Administration (FDA) are used in this study.

Systems for the preservation of identity of value enhanced grains such as corn have been in existence for many years. The identity preservation systems of value enhanced crops (VEC's), such as food grade corn, white, waxy and high-oil corn can vary in tolerance for contamination of non-target grain, but the prospect of out-crossing to commodity corn is of no particular concern. For the production of recombinant protein products, however, the regulatory considerations are fundamentally different, and result in much stricter containment and quality control requirements than those for VEC's (Hood et al., 2002). For this reason, new methods of containment have been developed for the handling, managing and processing these new crops, and it has been suggested that even new terminology would be needed in order to distinguish between identity preservation systems for food and feed uses, and for those used for biopharmaceutical and industrial proteins (Nikolov and Hammes, 2002). Similarly to the variation in identity preservation systems of VEC's, all plant systems used for the production of recombinant proteins would require the following:

- Isolation from other crops to prevent out-crossing
- Identity preservation from seed production to final product formulation
- Agricultural and pharmaceutical regulatory compliance
- Quality control and quality assurance programs, and standard operating procedures to ensure final product quality and regulatory compliance

10.2.1 Concerns regarding transgenic pharmaceutical crops

With increasing public awareness regarding the production of pharmaceutical proteins for animal and human use in crops which have previously used for food or feed purposes, the industry is being placed under unprecedented scrutiny. Following the inadvertent introduction of Aventis Crop Science's StarLink brand corn into the food chain, the industry and regulatory agencies are being asked to demonstrate that measures are being taken to completely prevent future incidences of this type. While the public and non-transgenic corn growers have cause for concern, so does the industry, since a single similar incident with a pharmaceutical plant would be devastating to the company involved, and would have a significant negative impact on the transgenic plant industry. While companies understand the importance of

developing procedures to ensure complete containment of the pharmaceutical product at all stages during product development through to large scale production, the need for total containment introduces a novel and complex element that has a large impact on the regulatory process.

Concerns of those resisting the approval and use of genetically modified organisms (GMOs) and products derived from them are several-fold, and cover the impact on the environmental, food and feed supplies, and corporate ethics. Different types of genetically modified crops include those designed for resistance to pests, herbicides, drought or saline conditions, for the enhancement of desirable food characteristics, and for the production of pharmaceutical proteins. Wrongly have numerous concerns which have been reported over pest resistant or superior yielding crops, for example, also have been associated to pharmaceutical crops. Each crop, and each use of the crop, must be evaluated on an individual basis.

10.2.2 Isolation

The extent of crop isolation required is dependent upon whether the crop is self-pollinating or capable of cross-pollinating adjacent crops. Whereas self-pollinating crops e.g. tobacco, are separated by a reasonable distance to ensure that there is no mixing with non-transgenic varieties, cross-pollinating crops, such as corn, must be separated by a minimum of 201 meters (660 feet) and grown under 'notification', in accordance with the USDA guidelines. This distance is considered reasonable in order to minimise the out-crossing between crops and the subsequent introduction of the transgene into commodity corn. However, for the production of pharmaceutical proteins in transgenic corn intended for either animal or human applications, this distance is increased to 1500 meters and grown under a USDA "permit" (Nikolov and Hammes, 2002), and the crops are also isolated temporally, by manipulating the differences in the timing of pollination between the neighbouring commodity crops and the transgenic lines.

The spatial separation of transgenic corn presents significant challenges since it is difficult to find land suitable for growing the crop with the required distance from

other crops. High humidity increases the lifespan of corn pollen, and low humidity reduces it. Consequently, the Corn Belt states in the USA are excluded due to the prohibitively small sizes of fields and diversity of ownership. The High Plains present a more suitable production environment, with its low humidity and thus limited pollen life, and large farms for easy coordination (Crosby, 2003).

10.2.3 Identity preservation and containment

Identity preservation procedures currently used with speciality crops are imperative for the quality control of the transgenic product and in preventing contamination of the commodity crops grown for food or feed uses with the transgene of interest. Grain production starts with soil preparation and ends with storage and processing. The highest level of quality control is required for the production of planting seed, and fortunately for the handling of transgenic seed, this practice is already common in the seed industry and can be applied directly. Although intended to accomplish two different goals, the procedures to ensure containment and product identity are linked, and would become a part of the documentation supporting product approval.

All items of farming equipment used for the planting and harvesting of transgenic crops must be cleaned out prior to, and after handling the transgenic material. For GMP-compliance, all equipment which comes into contact with the grain or the plant must be stainless steel or specialist plastic. Clean-out of the planters is simple and ensures high purity of the transgenic crop, and prevents contamination of the commodities with the recombinant crop. Since the cleaning of the harvesting equipment is much more difficult, especially with grain crops such as corn, it might prove easier to harvest small volumes by hand, and then use dedicated combines once volumes have increased.

The high levels of containment and clean out apply throughout the entire process of seed manufacture, including drying, storing and transporting to the processing facility. Validation of these steps is important to ensure that no plant material is introduced into the environment at any point. There are a number of additional systems in place which form the overall quality control and quality assurance program. Examples include the grow-out of parent lines, the essential GMP training, and documentation

of training, of all workers producing pharmaceutical crops, and the use of validated data collection and storage systems (Crosby, 2003).

Conventional methods can be used to address the quality and containment of the crop once it reaches the processing plant. However, if processing is outsourced, rather than being processed in a dedicated, and company controlled facility, then additional challenges lie in the development of processing strategies to meet product specifications and customer requirements e.g. GMPs.

Gene containment strategies which are currently being investigated, additional to spatial and temporal isolation, include incompatible genomes, control of seed dormancy or shattering, counter-selectable markers, suicide genes and maternal inheritance (Daniell et al., 2001; Stoger et al., 2002). Of particular interest is the use of inducible promoters, which may alleviate regulatory problems related to field production by separating the biomass growth and protein synthesis steps. Plants tissues harvested from the field may be induced to promote foreign protein during storage in a GMP facility. As a result of the greater control of conditions during protein synthesis, when compared to the use of constitutive promoters and outdoor plants, better product yield and less variation in product quality can be expected (Doran, 2000).

10.2.4 Regulatory compliance

All aspects of recombinant protein production in crops are covered at some point by at least one regulatory agency. In order to plant any recombinant crop, permits must be obtain from the USDA prior to issuance of feed or food tolerances. The USDA is also the controlling agency in the movement of viable seeds that contain the recombinant protein, up to the point of processing. It is the responsibility of the pharmaceutical company to have an operation a system which monitors the required permits and assures that movement of the recombinant material is accompanied by the required documentation. This system would form a part of the overall production plan.

Once seed reaches the manufacturing plant, the handling and processing of seed containing proteins for human and animal pharmaceutical application must comply with the FDA regulations. All phases of manufacturing, from tempering and degerming through to formulation of the pharmaceutical product must be conducted in strict compliance with the existing regulations and guidance used for other pharmaceutical proteins. However, no one set of regulations apply to all products and processes. The great variation in plant-based production systems, each best designed to address the different crops, products and the associated containment requirements, must be considered individually for the identification of, and compliance with the appropriate regulations.

The USDA and the FDA have provided extensive guidance on the appropriate regulatory considerations, data requirements and regulatory processes for transgenic plants and pharmaceuticals. The combination of the regulations provided by both the USDA and FDA is being examined by both the agencies themselves and companies developing new products. New, and nonetheless very important, procedures can be identified en-route to production, and companies would be prudent to practice these procedures, pre-empting their introduction into the essential requirements stipulated by the regulatory agencies at a later date. Additional sources of guidance which could help to steer a company in the right direction, include numerous 'Points to Consider' documents on the FDA website (www.fda.gov), and the outcome of government sponsored meetings and industry sponsored work groups, such as the Human Therapeutics in Transgenic Plants Industry Group Draft White Paper (Price et al., 1999). The latter two provide guidance to understanding the specific data that are likely to be required to support product safety. A sample of this process of meeting regulatory requirements is given in section 10.4.

One particularly complex, and contentious regulatory issue is that of achieving and demonstrating complete containment of human pharmaceuticals produced in plants, when a portion of the manufacturing process has been moved into an open area where variables are a constant factor. It would be illogical to regulate drugs produced in transgenic crops differently from those produced by transgenic fermentation. Also, excessive level of regulatory oversight based on perceived human or environmental

risk would be unworkable. Therefore, perhaps the most difficult and important aspect of meeting regulatory requirements, arises in the definition of the manufacturing process for pharmaceutical-expressing plants (Emlay, 2002).

In comparison with the production of a pharmaceutical product in a "closed system", a major part of the production process in transgenic plants occurs in an uncontrolled environment or "open system". In order to achieve regulatory approval, it is essential that the manufacturing process is well-defined and performs to an approved and consistent standard. Even subtle changes in the process can result in minor but undesirable changes in the finished product. Therefore, the production of pharmaceuticals in an environment where controls are very limited introduces a unique element to the manufacturing process. The regulatory agencies and the companies producing these products must tackle the scenario whereby the processes and controls represented by a cGMP (current Good Manufacturing Process) facility are now missing during much of the manufacturing process. It follows that the definition of an acceptable manufacturing process must lie somewhere between the ideal of complete control of the manufacturing process, and acceptance of the conditions inherent to plant-based production.

10.2.5 Quality control and quality assurance

A successful production system must have well designed quality control procedures that can be validated and that are relatively easy to administer and follow. All aspects the production process must be addressed. The company responsible for production must clearly communicate the requirements to the grower either contractually or in a growers guide. During processing, quick and accurate analytical tests will be required to monitor and assure the quality of the process stream i.e. product quantity, quality and purity, in to demonstrate that each of the operations in the process are operating as specified prior to approval. Standard operating procedures (SOPs) can be utilised to ensure consistency in processing and as a means to communicate the important operations to any third parties involved. Finally, in order to assure the product quality and to satisfactorily comply with the regulatory agencies, documentation

supporting the validation of these procedures must be provided, and this provision must be built into the production system.

10.3 Monitoring production

The detailed plans which must be submitted for the production of transgenic plants must be accompanied by detailed plans describing the monitoring of production. These plans should include validated experimental protocols for the monitoring of production, containment and the methods of reporting the results, in addition to the documentation demonstrating the implementation of standard agronomic practices i.e. soil fertilisation and pesticide treatment (Price et al., 1999). Procedures should be established for the monitoring of containment before and after plant production, and for the validation of those methods which are intended to detect the type of changes in the environment resulting from failure of the containment measures (Kjellsson and Strandberg, 2001).

A thorough environmental risk assessment of this open production system would be an essential regulatory requirement, particularly since the traditional processes of pharmaceutical protein production have been conducted in their entirety in cGMP facilities. This risk assessment would involve the identification of all potential hazards, and the assessment of both the risk of that hazard occurring and the magnitude of the harm it may cause (Dale and Kinderlerer, 1995).

10.4 Regulation of large-scale transgenic corn production

In order to obtain regulatory approval for the production of antibodies in corn, a company must demonstrate that all aspects of production and processing are contained and consistent. The manufacturing process can be divided into four major components which tackle the major issues involved in the transition from crop development and the stable expression of the product, through to formulation and

production of the finished product. The first component is concerned with the data and procedures to demonstrate consistency in the expression of the product. The second is the uniform application and recording of field production procedures to ensure containment and product integrity. Thirdly, the pre-processing of the harvested plant material, analogous to the introduction of raw material into the cGMP facility of cell culture productions, must be completed in a controlled environment. The final component covers the production of the finished product in a cGMP facility. Although the procedures used to obtain regulatory approval of a cGMP downstream processing facility are well established, the difficulty with this final component is in defining the stage at which cGMP procedures should be applied.

10.4.1 Consistency of product expression

The FDA requires documentation which demonstrates stable integration of the target gene into the plant genome, and consistent expression of the intended product between generations. This data includes the characterisation of the DNA construct used to generate the transgenic plants, consisting of details of the quality control of the transgene construct assembly, cloning and purification, and detailed characterisation of the transgene coding and regulatory sequences (Price et al., 1999).

It is absolutely essential that the intended product remains unchanged between generations and between production locations. In order to achieve this, it may be necessary for companies to carry out additional tests which are not yet stipulated by the regulatory agencies. Such procedures may involve, for example, the monitoring of product expression at different stages of production in order to be certain that it is present as the intended product.

In preparation for large-scale seed production, it is first of all necessary to identify and characterise a suitable host production system, then to fully describe the methods used to transform the plant for the expression of the target product, and finally to characterise the seed stock produced by these transformed plants for large-scale product manufacture. The host plant must be characterised, by the provision of details such the variety name, known phenotype and relevant genotypes, history of

use, the part of the plant to be utilised in product manufacture, and the presence and identity of known potentially harmful constituents such as toxicants and allergens. A detailed description of the method of transgene introduction must be provided, including all procedures used during the generation of plants with germline alterations, and the established or novel plant transformation techniques.

Characterisation of the transgenic seed stock involves the use of sound, and sensitivity established, methods of analysis for the identification of plant seed stock and progeny transgenic plants. Tests confirming that the seed stock is producing the desired product within acceptable limits must be described. It must also be demonstrated that the transgene in the seed stock is structurally stable, using methods such as Southern blots, in order to counter the possibility that there will be a rearrangement or deletion of all or some of the transgene copies during or subsequent to integration. Expression stability is equally important, since it can vary upon interaction of the transgene with the genetic background of the host plant. Therefore, stable expression must be demonstrated both within a generation and through several breeding generations.

The final requirement prior to large-scale production is to establish a reliable and continuous source of transgenic plants. This source can be modelled on current agricultural seed product development methods, or the producer may instead choose to utilise a seed bank system analogous to the Master Cell Bank (MCB) and Working Cell Bank (WCB) used for cell line characterisation in the production of pharmaceuticals. In both situations, highly characterised transgenic plants are relied upon to produce progeny for the production of seed for the manufacture of product which would meet the established acceptance criteria.

10.4.2 Field production procedures

The major regulatory issues involved in transgenic plant production in the open system are concerned with the controllable procedures which are primarily related to crop isolation, agricultural practices e.g. application of fertiliser, pesticides and water, and crop handling. These issues are discussed previously. This section provides

information on the regulation of crop development, and the validation of consistent growing conditions.

10.4.2.a Product identity preservation with scale-up of crop production

The production of transgenic plants during the development phase is frequently in contained facilities, such as greenhouses or growth chambers. Production operations in these confined facilities must be in accordance with the correct regulations (e.g. USDA APHIS regulations in 7 CFR 340), and the physical surroundings of the plants should be described in detail. As plant production moves from the control of the greenhouse to external areas, the selected plant lines from the nursery, for further development, must be validated to ensure that only the intended genetic sequences are present. A precise description of the Identity Preservation procedures employed must be provided to the regulatory agencies (Emlay, 2002).

10.4.2.b Limited control of the open system

Agricultural variables become a major factor, and are inevitable in the production of vast quantities of crops, due to production on different sites and in different seasons. Reduction of these variables to the maximum degree possible, by the application of the same procedures to the same product, is an essential regulatory requirement. Metabolic changes that can affect drug safety are more likely to develop in open field systems than in controlled fermentation systems (Crosby, 2003). However, the regulatory agencies must accept that there is a middle ground for plant production between complete control and no control. The controllable factors include the designated land, plot sizes, spatial and temporal separation for containment, type of fertilisers used and the chemicals used for pest control. The variation lies in the environmental conditions, which, subjected to potentially large variations between growing regions and seasons, can impact upon water and fertiliser usage, and chemical applications for the control of pests. Record keeping and absolute compliance with labels will be a crucial aspect of production.

10.4.3 Pre-processing in a controlled environment

Pre-processing, or upstream processing, is a critical stage in the manufacturing process, which from a regulatory perspective, involves consideration of the form of

the product that will enter the cGMP facility for the production of finished product. Where large volumes of plant material are involved, the initial extraction of the biologic under sterile conditions may not be practical. Under these circumstances, the initial processing facilities should achieve levels of cleanliness required for initial food processing, in order to minimise increases in contaminant types and levels. Using transgenic corn as an example, and within the context of this research, the dry-milling and degerming of corn seeds for the production of product-rich endosperm fraction could be considered as an extension to the product targeting to the seed. Just as seed was separated from the cob using standard agricultural machinery, so it is also unlikely that the separation of germ from the endosperm would necessitate the building of processing facilities any different to those currently used in the dry-milling and degerming of non-modified seed for the production of food grade materials. However, the extraction process would produce a concentrated mixture of corn proteins and the intended product, and so this step would take place in a sterile environment similar to the standards used in food production. Dry-milling followed by extraction in a sterile environment may serve as the bridge in the manufacturing process between field production and cGMP procedures. Although the point of transfer into a cGMP facility has been speculated upon here, the exact stage at which cGMP procedures ought to be applied remains unspecified. This is discussed in the following section.

10.4.4 Production in a cGMP facility

Production of the finished product in a cGMP facility has been the subject of much debate. The cause of ambiguity is in the identification of a stage in the production and processing of pharmaceutical-expressing plants at which cGMP procedures should be applied. Up until this point, all stages of production of pharmaceutical proteins produced by traditional methods i.e. cell culture, have been conducted under cGMP procedures in closed systems. The successful commercialisation of plant-produced pharmaceuticals depends, to a great extent, upon the point within the process at which cGMP procedures must first be applied. One possible solution to this conundrum could be to follow precedents established by the production of pharmaceutical products from naturally occurring plants, such as bark from *Taxus*

brevifolia processed under cGMP conditions to produce Taxol (Emlay, 2002). Most importantly, however, is that a highly purified pharmaceutical produced in the cGMP facility is identical to the approved product, and consistent between batches. The same quality assurance procedures can be applied to the finished product in the same manner as they are currently applied with traditionally produced pharmaceutical products (Miele, 1997).

10.4.5 Disposal of transgenic plants

Disposal of transgenic plants should be in a manner consistent with good agricultural practices and in accordance with USDA and Animal and Plant Health Inspection Service (APHIS) regulations governing the production of GMOs. Alternatively, if the manufacturer intends to market the by-products of transgenic plant processing for human or livestock feed, then the Centre for Food Safety and Applied Nutrition (CFSAN) or the Centre for Veterinary Medicine (CVM), respectively, would coordinate the appropriate food and feed safety assessments of these products.

10.5 Summary

The introduction of a part of a pharmaceutical production system into the environment presents novel issues to regulatory agencies. Although practices exist for the containment of value enhanced crops, the extent of containment must be significantly improved to demonstrate complete containment and product identity preservation on the level required by the appropriate regulatory agencies. The regulations controlling the production of pharmaceutical proteins in crops are evolving as more information is gathered. It is the responsibility of the manufacturing company to provide documentation to the regulatory agencies supporting crop production in accordance with their standards, to keep up to date with the guidelines provided by the agencies, and to take any necessary action, whether documented within the regulatory guidelines or not, to assure complete containment and the highest quality of product manufacture.

Appendix A

(i) Minimum corn sample for oil analysis

The minimum mass of sample required for the reliable determination of sample oil content was calculated according to the minimum required concentration of oil in hexane solution, and the mass of sample which contained sufficient oil to achieve this concentration. Without a standard procedure to follow for corn oil extraction using this method, and without any information regarding the hybrid 8366 seed oil content before experimentation, oil was extracted from an arbitrary mass of ground whole corn seeds (20g) and volume of hexane (60ml). The mass of oil extracted per gram of ground seed (0.032g), measured using a 1ml sample of extract, fell well above minimum concentration for assay reliability (i.e. 5g/L). The minimum mass of samples required for oil analysis was then calculated based on this oil content of ground hybrid 8366 seed:

Seed oil	=	0.032g/g
Minimum [Oil]	=	5g/L or 0.005g/1ml
Excess oil per gram	=	0.032/0.005
	=	6.4 (i.e. 6.4 times too much oil in a 1g seed sample)
Minimum mass	=	1/6.4
	=	0.156g

(ii) Oil content expression and calculation

Oil content of the degermer waste stream was expressed as a percentage by mass of the waste stream (%, g/g) (1), which was then used to calculate the total oil mass contained in this stream (2). This mass of waste stream oil was then expressed as a percentage of the total oil collected in each degermer run (3) i.e. oil recovery. Sample calculations are shown below:

Mass of sample collected on sieve 4.0:	0.8	(g)
Extraction sample mass:	0.2	(g)
Hexane volume:	1.0	(ml)
Extract solution volume:	0.75	(ml)
Oil residue:	0.024	(g)

Mass of oil in 1ml hexane mixture: (0.024/0.75)	=	0.032g
(1) Oil fraction (%, g/g) of sample: (0.032/0.2)×100	=	16.0%
(2) Total oil in sieve 4.0 fraction: (0.16×0.8)	=	0.128g
(Total oil detected = 0.4g)		
(3) % of total seed oil on sieve 4.0: (0.128/0.4)×100	=	32%

Appendix B

Oil assay criteria

The method of factorial experiment design was used to investigate the impact of sample mass to hexane volume ration, and mixing time, on the extent of oil extraction. The data was analysed using Design Expert 5 software. The effect of these three factors upon the oil content (%, g/g) of each sample taken from the same batch of ground seed, and the oil concentration in hexane solution, are illustrated in Figure B.1 and Figure B.2, respectively. In the production of these two figures, the mixing time factor was fixed at 5.5 minutes.

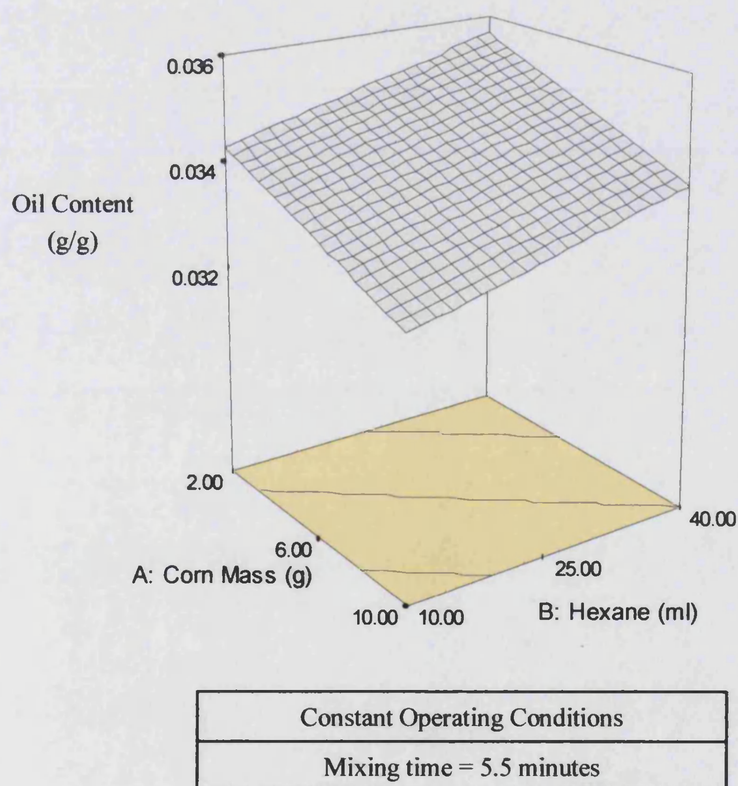
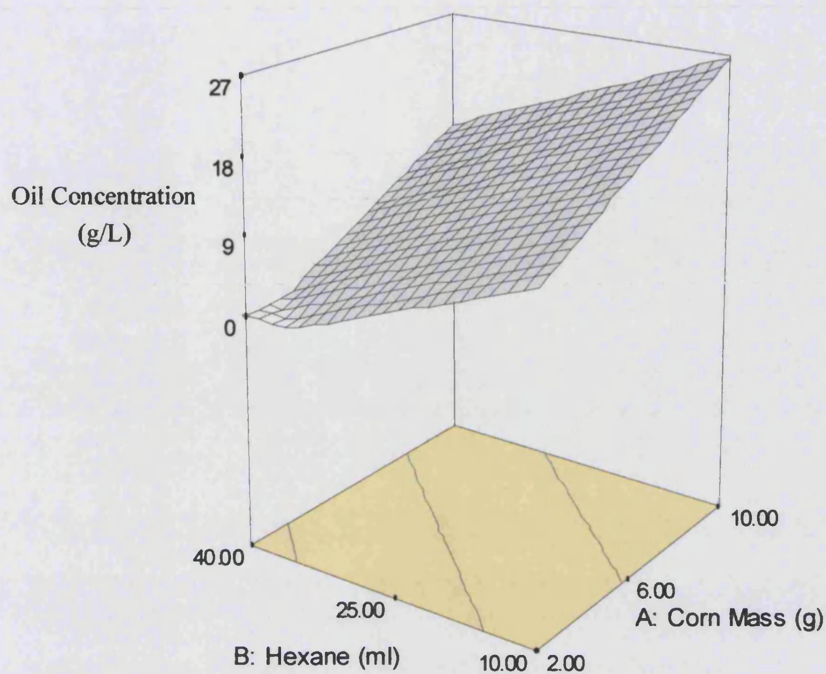


Figure B.1 Response surface illustrating the improved oil extraction with increasing solvent to sample extraction ratio



Constant Operating Conditions
Mixing time = 5.5 minutes

Figure B.2 Response surface illustrating the improved oil assay reliability with decreasing solvent to sample extraction ratio

Appendix C

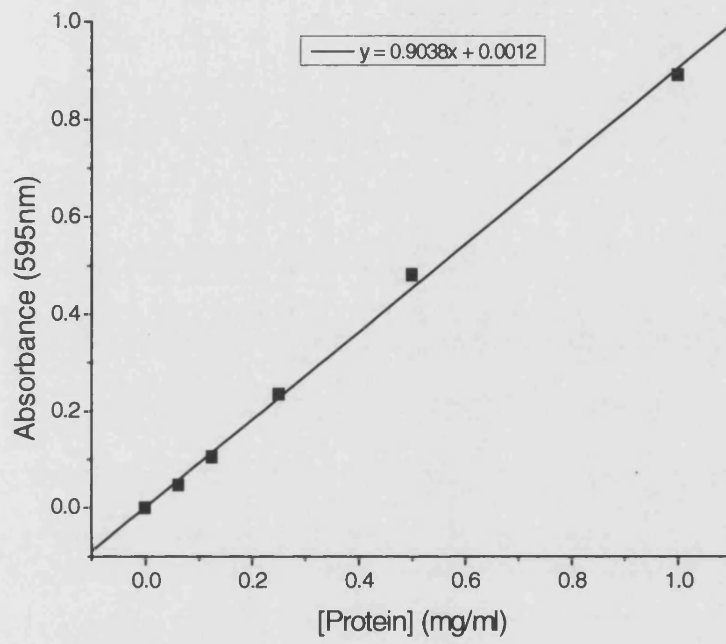


Figure C.1 Protein assay standard curve

Appendix D

Standard dilutions

1. Dilution 1: 10 μl Synagis added to 99μl blocking buffer
2. Dilution 2: 10μl Dilution 1 added to 990 μl blocking buffer (100 ng/ml solution)
3. Standard dilution: Blocking buffer added to Dilution 2 as follows:

[Standard] (ng/ml)	Dilution 2 (μl)	Sample diluent (μl)
25	250	750
20	200	800
15	150	850
10	100	900
5	50	950
2	20	980
1	10	990
Blank	0	1000

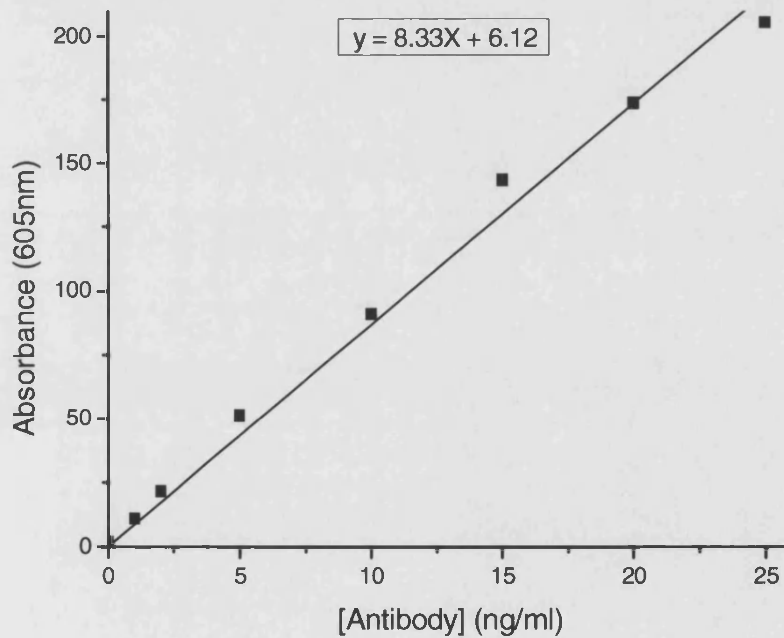


Figure D.1 Elisa standard curve for antibody measurement

Appendix E

Appendix E(i)

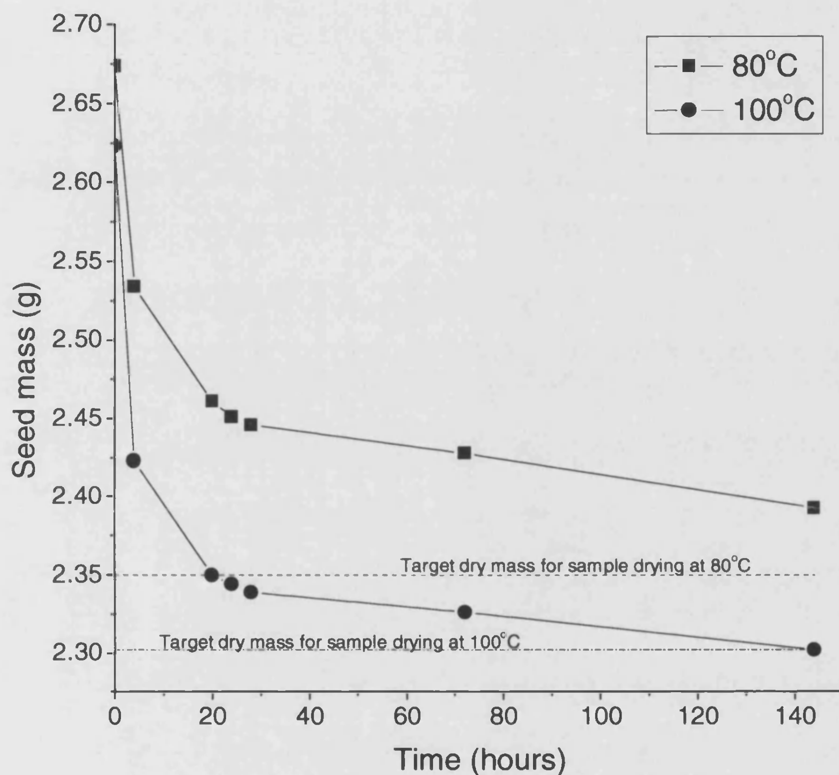


Figure E.1 Time and temperatures required to achieve seed dry mass based on the initial (delivery) seed moisture content of 12%.

Appendix E(ii)

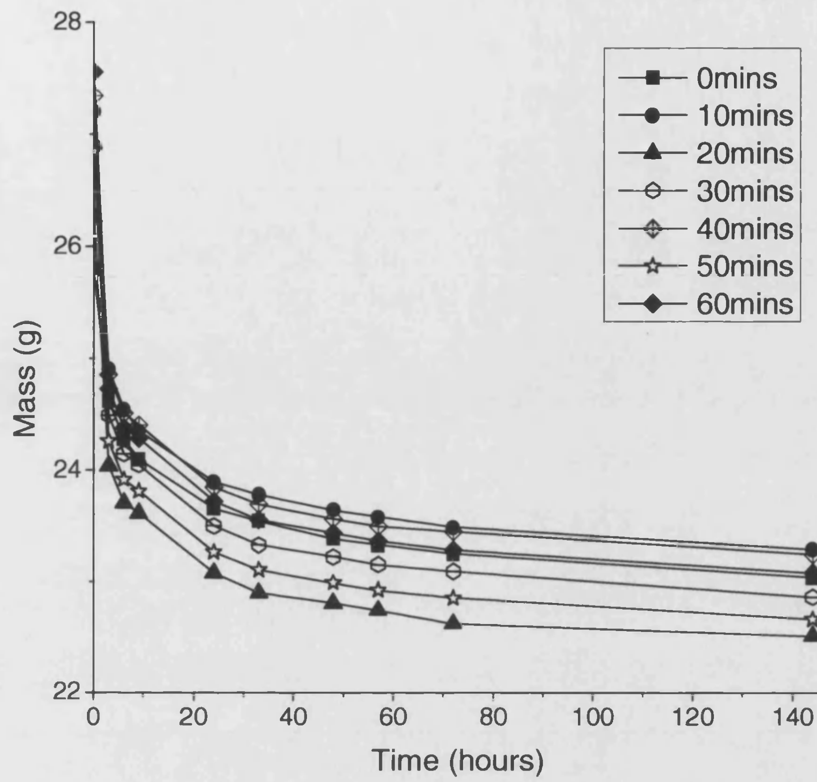


Figure E.2 Graph illustrating that the initial seed moisture content had no effect on the final seed mass after drying at 80°C for 6 days.

Appendix F

Feeder design results

Table F.1 Variation in seed feed flow rate with hook agitation, using different power inputs and agitator positions relative to the hopper constriction (triplicate experiments)

HOOK POSITION	POWER INPUT (VOLTS)	FLOW RATE (KG/HOUR)	ERROR (%)
Above constriction	3	0.15	25.07
	6	0.49	19.51
	9	**0.90**	**23.58**
	12	**1.15**	**19.41**
Inside constriction	3	0.43	28.67
	6	0.82	27.34
	9	1.39	24.69
	12	1.61	10.71
Below constriction	3	0.42	24.50
	6	**1.04**	**19.25**
	9	1.46	17.13
	12	1.50	7.83

Table F.2 Variation in seed flow rate using alternative agitator designs, outlet pipe sizes and power inputs (trplicate experimets)

	PIPE SIZE (MM)	POWER INPUT (VOLTS)	FLOW RATE (KG/HOUR)	ERROR (%)
Screw lift	1.3	3	0.36	23.8
		6	**1.15**	**6.5**
		9	1.59	10.2
	1.4	3	0.00	0
		6	1.65	1.3
		9	2.36	5.8
	1.5	3	2.15	6.8
		6	2.88	0
		9	2.02	18.6
Screw press	1.3	3	0.60	10.9
		6	1.54	4.4
		9	1.79	3.5
	1.4	3	0.73	7.5
		6	**0.96**	**13.6**
		9	**0.92**	**4.7**
	1.5	3	1.34	2.2
		6	2.42	6.2
		9	2.88	0.0

Appendix G

High quality transgenic seed degerming

The products of degerming hybrid 8366 seed were analysed in order to ascertain the degree of cross contamination of germ in the product (i.e. endosperm) stream and endosperm in the waste (i.e. germ) stream. These levels of contamination were calculated based on the mass and oil content of manually fractionated seed components, and the mass and oil content of product and waste streams based on perfect separation in the small-scale degerming process. The higher oil content of the product stream indicated the presence of germ, and using the known concentration of oil in endosperm and germ, it was possible to calculate an approximate mass of germ in product stream. Degerming of high quality transgenic seed would yield a waste stream mass approximating that achieved by processing hybrid 8366 seed. After subtracting the known mass of germ in the product stream, it was possible to calculate the mass of endosperm in the waste stream.

The methodology used for the calculation of high quality transgenic seed product and waste streams, produced using the small-scale degerming process, and based on transgenic seed (HVY2) data and high quality seed (hybrid 8366) processing data, is described below:

8366 seed oil contained in the endosperm = 15%
8366 seed oil contained in the germ = 85%
8366 product stream oil content = 32.5%

Germ contribution to product stream oil content = 32.5 - 15
= 17.5%

Proportion of germ in product stream = 17.5 / 85
= 20.59%

HVY2 seed data:
Germ antibody content = 1.264ug/g
Endosperm antibody content = 5.742ug/g

8366 Data

Germ mass	= 0.0287g
Endosperm mass	= 0.2074g
Waste stream mass	= 1.2011g
Product stream mass	= 11.0048g

Mass Balance

Mass of germ in the waste stream	= (1-20.59) ×50×0.0287 = 1.1396g
Mass of germ in product stream	= 50×0.0287-1.1396 = 0.2954g
Mass of endosperm in waste stream	= 1.2011-1.1396 = 0.0615g
Mass of endosperm in product stream	= 11.0048-0.2954 = 10.7094g

Antibody content of product and waste streams

Antibody content in waste stream germ	= 1.1396g×1.264μg/g = 1.44μg
Antibody content in waste stream endosperm	= 0.0615g×5.742μg/g 0.35ug
Antibody content of product stream germ	= 0.2954g×1.264μg/g = 0.37μg
Antibody content in product stream endosperm	= 10.7094g×5.742μg/g = 61.49μg

Antibody recovery (i.e. percentage of antibody in the product stream)

= (61.49+0.37) / (61.49+0.37+1.44+0.35) × 100
= 97.18%

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