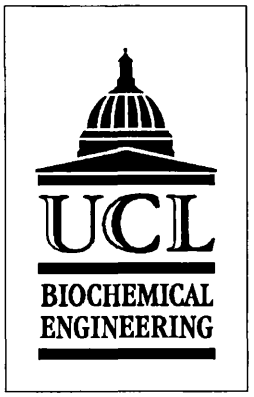
FACTORS AFFECTING THE PRODUCTION OF A SINGLE-CHAIN ANTIBODY FRAGMENT (SCFV) BY *ASPERGILLUS AWAMORI* IN STIRRED TANK REACTORS.



A thesis submitted to the University of London for the degree of Doctor of Philosophy

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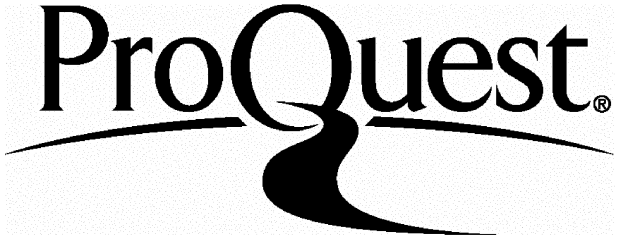

ABSTRACT

Recombinant antibodies have in recent years proved their potential to be an important class of therapeutics. Potential applications demand large doses for administration and therefore production of antibody fragments in microbial or fungal systems has become important.

In this study, a recombinant strain of *Aspergillus awamori*, producing anti-lysozyme scFv under the control of the xylanase promoter, was used. A fermentation strategy was created for the production of the antibody fragments by a fed-batch process, based on the investigation of parameters which affected production, namely growth medium composition, induction regime and protease production. Experiments with the time of induction showed that the optimum results are achieved when induction is started in the late exponential phase (21 hours after inoculation) improving the titre of the product from 14.5 mg L^{-1}, obtained in the early exponential phase (7 hours after inoculation) to 16.2 mg L^{-1}. A 100% increase of the carbon (fructose) and nitrogen (ammonium sulphate) sources in the growth medium resulted in an increase in product concentration from 16.2 mg L^{-1} to 108.9 mg L^{-1} and an increase in maximum dry cell weight from 7.5 g L^{-1} to 11.5 g L^{-1}. A 50 % reduction in the concentration of the inducer resulted in an increase in the specific product yield from 10 mg g^{-1} dry cell weight to 12 mg g^{-1}. Proteolytic enzymes were produced during the fermentation up to concentrations equivalent to 1.4 g L^{-1} trypsin but storage experiments showed that they had no detrimental effect on the concentration of the antibody fragment. The process was scaled-up to 75 L where maximum scFv concentration reached 160 mg L^{-1} after 42 hours of fermentation. Specific yield and specific productivity were increased by 100 % and 50 % respectively, while volumetric productivity decreased by 500 %. Pellet dimensions were the same. A comparison between production of the same scFv antibody fragment in *A. awamori* and *E. coli* was carried out.

ACKNOWLEDGEMENTS

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Most of all, I would like to thank my family for their continued support during my career as a student. Without them this thesis would not be possible.

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List of Abbreviations

CER: CO_2 Evolution Rate.

CEST: Centre for Exploitation of Science and Technology.

C_H: Constant Heavy Chain.

C_L: Constant Light Chain.

CS: Cleavage Sequence.

DCW: Dry Cell Weight.

DEA: Diethanolamine.

DNS: Dinitrosalicylic Acid.

dO2: Dissolved Oxygen Tension.

DOT: Dissolved Oxygen Tension.

ELISA: Enzyme-Linked Immunosorbent Assay.

Fc: Crystallizable Fragment.

FDA: Food and Drug Administration.

FP: Fungal Promoter.

FTS: Fungal Transcription Stop.

G-6-P: Glucose 6-Phosphate.

GLA: Glucoamylase.

GRAS: Generally Regarded as Safe.

HCR: Heterologous Coding Region.

IC: Inorganic Carbon.

IgG: Immunoglobin G.

IPTG: Isopropyl-β-D-thiogalactoside:

k_La: Volumetric mass transfer coefficient.

LB Medium: Luria-Bertani Medium.

NA: Nutrient Agar.

NAD: Nicotinamide Adenine Nucleotide.

NADH: Reduced form of NAD.

NDIR: Non-Dispersive Infra Red

OUR: Oxygen Uptake Rate.

PA: Pellet Area.

PBS: Phosphate Buffered Saline.

PBSTA: Phosphate Buffered Saline with Tween 20 and Sodium Azide.

PD: Pellet Diameter.

PDA: Potato Dextrose Agar.

PMSF: Phenylmethanesulfonyl fluoride.

pNPP: p-Nitrophenyl Phosphate.

rpm: Revolutions Per Minute.

rhLIF: Recombinant Human Leukaemia Inhibitory Factor.

RQ: Respiratory Quotient.

RTDAS: Real Time Data Acquisition Systems.

ScFv: Single-chain Antibody Fragment.

SDS-PAGE: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis.

SF: Shaken Flask.

SP: Stabilizing Protein.

SS: Signal Sequence.

TC: Total Carbon.

TCA: Trichloroacetic Acid.

TEMED: $NNN^{1}N^{1}$-Tetramethylethylenediamine.

$\mathbf{V_H}$: Variable Heavy Chain.

$\mathbf{V_L}$: Variable Light Chain.

1. INTRODUCTION

1.1 ANTIBODIES AND ANTIBODY FRAGMENTS. STRUCTURE AND FUNCTION.

Antibodies are soluble proteins produced by plasma cells in response to the presence of a foreign substance. They are the recognition elements of the humoral response system (*humor*: Latin for liquid) (Stryer, 1995).

Antibodies are of great importance both in the medical and industrial fields. Their high affinity and specificity make them highly suitable and attractive for a range of applications. However, applications outside medical care have been limited by the high, often prohibitive cost. The increase in commercial demand for such products has given rise to a need for an efficient and low cost process for their production. The "traditional" and more common way for the production of monoclonal antibodies, mammalian cell culture, is too complicated and uneconomical for large-scale operations. Microbial cell cultures, where growth can be performed by well-established fermentation processes, have been used as alternative systems.

Recombinant DNA technology has made possible the expression of antibody fragments from microorganisms; the antibody fragment gene is genetically engineered into the host organism and then the organism is grown in a fermenter vessel. Organisms that have been used for the expression of antibody fragments include *Escherichia coli, Saccharomyces cerevisiae* and *Pichia pastoris*. The choice of host has significant effect on the productivity of the process and the nature, number and complexity of the isolation and purification unit operations required for the recovery of the desired antibody fragment.

Early work in this field involved the use of *E. coli* as the host microorganism and the expression of the antibody fragments as inclusion bodies. The major disadvantage of such a process in large scale is the complexity of the downstream processing, specifically the renaturation of the protein. Recent work

performed at University College London involved the production of antibody fragments in the periplasm of *E. coli* (Harrison, 1995). The concentration of the scFv obtained from this process reached 200 mg L^{-1}. Periplasmic expression makes the recovery of the product simpler but is still labour intensive. Therefore, a system that secretes the desired product into the culture medium is more advantageous. Filamentous fungi have been shown to secrete high levels of homologous proteins (Jeenes *et al.*, 1991). However, secretion levels of heterologous proteins are much lower and consequently current research is focused in enhancing the secretion levels of such proteins (van den Hondel *et al.*, 1992). The main aim of this project was to investigate the factors that affect the production of a single-chain antibody fragment (scFv) from *Aspergillus awamori*.

1.1.1 Structure of Antibodies and Antibody Fragments.

Antibodies consist of structurally similar functional domains that can fold independently. Every molecule of the Immunoglobulin G (IgG) class consists of a pair of identical light and a pair of identical heavy chains. Disulphide bonds and van der Waals forces hold these constituents together, forming a 'Y' shape. The whole antibody is divided in two regions; the constant and the variable. The amino terminal of the heavy and light (H and L) chains is characterized by a region in which the amino acid sequence is variable (V_L and V_H). The antigen specificity of the antibody is confined to this region; it is the antigen-binding site.

Each variable region folds into a nine-stranded β-pleated sheet formed by two layers of antiparallel β-pleated sheets. Three and a half of these strands form the outside layer of each subunit, and the remainder five and a half form the inside layer. The strands in every layer are extensively hydrogen bonded to each other and the two subunits are held together by means of a hydrophobic interface region that extends to the inside β-sheet layers. The surface of the variable region that comes in contact with the antigen is composed of six amino acid loops, which are positioned along a structural framework region (Better and Weickmann, 1993).

The rest of the structure of the antibody consists of a relatively constant amino acid sequence and is referred to as the constant region (C_L and C_H) The heavy part of this region (C_H) is further subdivided into three structurally distinct regions referred to as C_{H1}, C_{H2} and C_{H3}. The constant region that forms the stem of the antibody is called the crystallizable fragment (Fc) and is the site with which the antibody can bind to a cell.

Antibody fragments are formed by enzymatic cleavage of the antibody. Fab is the antigen-binding fragment, formed when the region between C_{H1} and C_{H2}, the hinge region, is cleaved by papain. Fab′ is the same except that it also contains one or more of the cysteine residues that are present in the hinge region. The Fv fragment is derived from the cleavage by papain between the constant and variable regions of the Fab fragment. It consists of only variable domains of heavy and light chains bonded together by hydrophobic interactions. Finally, the single chain antibody fragment (scFv) is a fragment consisting of a single V_H and V_L chain connected by a short polypeptide linker. All of the above fragments have been expressed as functional molecules using genetic engineering. An overview of the antibody structure can be seen in figure 1.1 below.

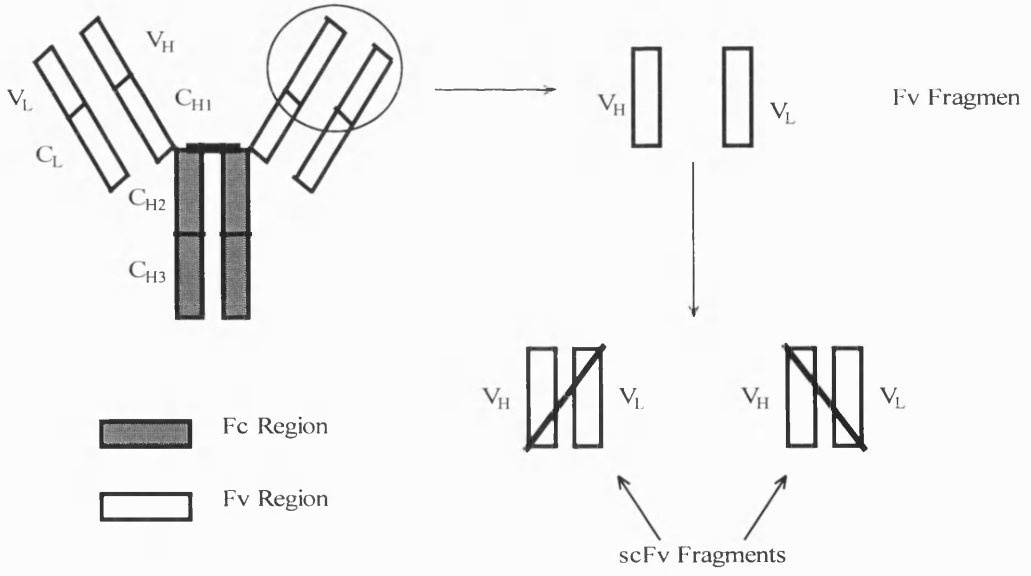


Fig. 1.1 Schematic view of an antibody molecule, showing the different antibody fragments.

1.1.2 Applications of Antibody Fragments in Medicine and Industry.

In the medical field, antibody fragments consisting only of the binding site are used for the diagnosis and treatment of cancer and autoimmune diseases. They are linked to a drug, a bacterial toxin or a tumor-imaging agent, their small size allowing rapid arrival at the targeted tissue (Colcher *et al.*, 1990; Reiter and Pastan, 1998). Their reduced size may also allow the penetration of dense tumor tissue and the generation of reduced antigenicity (Plückthun, 1991). It is estimated by CEST (UK Centre for Exploitation of Science and Technology) that the global market value for antibody fragments will be around US$ 6 billion by the end of the century (Harrison and Keshavarz-Moore, 1995). A more recent application of antibodies and antibody fragments is in oral care and hygiene. Studies have shown that the oral cavity is colonized by more than 300 different bacteria (Marcotte and Lavoie, 1998). Most of these oral bacteria posses specific cell-surface associated macromolecules (adhesins) that allow them to adhere to different oral microhabitats including tooth and mucosal surfaces. The colonization of these surfaces by bacteria is the first step to dental carries. Studies using polyclonal or monoclonal antibodies specific to adhesins have shown a notable reduction in adhesion of oral bacteria. Therefore, antibody fragments could be applied to oral care products, helping in this way to reduce the incidence of dental carries (passive immunization) (Frenken *et al.*, 1998).

The specific binding ability of the antibody fragments has made them attractive to industry as well; in immunoaffinity purification columns, they bind to the internal pores of the matrix, allowing in this way a greater surface area for purification and consequently increasing the capacity of the column (Berry *et al.*, 1991). Also, antibody fragments have been used in biosensors. In addition, an scFv has been used, immobilized on polystyrene beads, to effectively remove a herbicide (paraquat) from water samples (Graham *et al.*, 1995).

1.2 Expression Systems for Heterologous Proteins.

The development of genetic engineering has made possible the expression of heterologous proteins in various systems including bacteria, fungi and plants. *E. coli* is a genetically versatile microorganism, in which recombinant DNA technology has been well established. The fast growth rate, the simple fermentation and the degree of safety are additional reasons that make it attractive as a host for the large-scale production of heterologous proteins (Plückthun, 1991). This is the reason why *E. coli* was one of the first microorganisms to be used for the expression of antibody fragments; Fv (Skerra and Plückthun, 1988), scFv (Bird *et al.*, 1988) and Fab (Better *et al*, 1988; Carter *et al.*, 1992) fragment production was first described more than 10 years ago. ScFv fragments have been engineered in *E. coli* as expression vectors and expressed as single proteins, (Bird *et al.*, 1988; Billiad *et al.*, 1995) or as fusion proteins (Clark and Freedman, 1994).

However, *E. coli* has several disadvantages as well; pyrogen and endotoxin production, improper folding of the protein and protein insolubility, inability to perform post-translational modifications (glycosylation) and the fact that there is a risk of infection as it is part of the microflora of the gastrointestinal tract are some drawbacks that have resulted in research for the expression of heterologous proteins in general, and consequently antibody fragments, into other hosts.

Alternative hosts for the expression of scFv fragments include plant, yeast, insect, bacterial (other than *E. coli*) and fungal cell systems. The yeast *Pichia pastoris* has been used for the expression of monoclonal rabbit scFv fragments, specific for recombinant human leukaemia inhibitory factor (rhLIF) (Ridder *et al.*, 1995). It was selected as a host due to the fact that it combines a fast growth rate with the features of a eukaryotic expression system (ability to perform post-translational modifications). High levels of pure and functional antibody fragment were recovered (100 mg L^{-1}) from the supernatant by one-step affinity chromatography. The same microorganism (*P. pastoris*), has more recently been used for the expression of an scFv against *Pseudomonas aeruginosa* lipoprotein I

(Cupit *et al.*, 1999). *Saccharomyces cerevisiae* has been used for the expression of functional IgM Fab fragments (Edqvist *et al.*, 1991). Finally, the Fv domain of a monoclonal antibody against hen egg-white lysozyme has been expressed in the filamentous Gram-positive bacterium *Streptomyces lividans* (Ueda *et al.*, 1993). The antibody fragment was isolated from the culture supernatant by a two-step purification procedure (affinity chromatography and gel filtration) with a yield of 1 $\mu g\ ml^{-1}$.

Other hosts referred to in literature include viruses and plants. Recombinant baculovirus was used to infect insect cells that in turn secreted high levels of the desired glycosylated mouse monoclonal IgG antibody fragment that is directed against lipoprotein I of *Pseudomonas aeruginosa* (zu Putlitz *et al.*, 1990). Complementary DNAs derived from a mouse hybridoma mRNA have been used to transform tobacco leaf segments (Hiatt *et al.*, 1989). Plants expressing single γ- or κ-immunoglobulin chains were crossed to yield progeny in which both chains were expressed simultaneously and produced functional antibodies that accumulated to 1.3% of the total leaf protein. Finally, Fecker *et al.* (1996) reported the expression of scFv in *Nicotiana benthamiana.*

Filamentous fungal systems have the ability to secrete very large quantities of a wide range of homologous proteins; yields of 20 $g\ L^{-1}$ in culture media have been reported for glucoamylase from *Aspergillus niger* (Finkelstein, 1987). This makes them attractive hosts for the production of heterologous products including antibiotics, organic acids, enzymes, polysaccharides, foods and beverages (Jeenes *et al.*, 1991). To date, a number of filamentous fungal strains and their products are being used in the food processing industry and have achieved a GRAS (Generally Regarded As Safe) status by the food and drug administration (FDA) (Gouka *et al.*, 1997). However, the secreted yields of heterologous proteins have seldom reached the levels of homologous proteins (Archer *et al.*, 1994). Research has shown that this is mainly due to problems occurring at the secretory pathway (MacKenzie *et al.*, 1993). Nevertheless, filamentous fungi still remain very attractive hosts for the expression of heterologous proteins due to the fact that

there is a well-developed technology for the growth of filamentous fungi and the recovery of their products. Filamentous fungi have also been used for the production of antibody fragments. *Trichoderma reesei* was successfully used to express a Fab fragment at levels up to 150 mg L^{-1} (Nyyssönen *et al.*, 1993).

1.3 *ASPERGILLUS* AND ITS USE IN INDUSTRY.

Aspergillus is a member of the Ascomycotina group, class Plectomycetes, order Eurotiales. It is a non-ascocarpic species. Some strains are pathogenic to plants, especially in the tropics (Webster, 1980). Its name is derived from the Latin *aspergillum*, which is mop for the distribution of holy water.

Species of the genus *Aspergillus* are, together with those from the genus *Trichoderma*, the ones used most extensively in the large-scale production of proteins. Initially, work on heterologous protein production in *Aspergilli* started with *A. nidulans*: this species has been studied since the early 1940's. In recent years, other species have been exploited and they are being used since higher expression levels can be achieved. These species are primarily *A. niger, A. oryzae* and *A. awamori*. Table 1.1 on the next page summarizes the major heterologous proteins produced from *Aspergillus* species, while Table 1.2 on the following page gives a summary of the homologous enzymes produced by the same species.

HETEROLOGOUS PROTEINS	SOURCE SPECIES	EXPRESSION HOST	PROMOTER	YIELD	REFERENCE
Chymosin	Cow	*A. nidulans, A. awamori, A. oryzae*	*glaA*	140 μg L^{-1} (*A. awamori*), 98 μg g^{-1} DCW (*A. nidulans*)	Cullen *et al.*, 1987; Ward *et al.*, 1990
Interferon α2	Human	*A. nidulans*	*alcA, glaA*	1mg L^{-1}	Gwynne *et al.*, 1987
Epidermal Growth Factor	Human	*A. nidulans*	*alcA*	N/A	Gwynne *et al.*, 1987
Growth hormone	Human	*A. nidulans*	*alcA*	N/A	Devchand and Gwynne, 1991
Interleukin 6	Human	*A. nidulans*	*alcA*	34-54 mg L^{-1}, 25 ng ml^{-1}	Carrez *et al.*, 1990; Yadwad *et al.*, 1996
Lactoferrin	Human	*A. oryzae*	*amyA*	25 mg L^{-1}	Ward *et al.*, 1992
Tissue Plasminogen Activator	Human	*A. nidulans*	*alcC, tpiA, adhA*	1 mg L^{-1}, 0.1 mg L^{-1}, 1 mg L^{-1}	Upshall *et al.*, 1987
Egg-White Lysozyme	Chicken	*A. niger*	*glaA, gpdA*	14 mg g^{-1} 12mg L^{-1}	Archer *et al.*, 1990; Archer *et al.*, 1995
Granulocyte-Macrophage Colony Stimulating Factor	Human	*A. nidulans*	*adhA*	1 mg L^{-1}	Upshall *et al.*, 1991
Aspartyl-protease	*Rhizomucor miehei*	*A. oryzae*	*amyA*	>3 g L^{-1}	Christensen *et al.*, 1988
Glucoamylase	*A. niger*	*A. nidulans*	*glaA*	20-40 μg ml^{-1}	Punt *et al.*, 1991
Endotoxin subunit B	*E. coli*	*A. nidulans*	*amdS*	24 g g^{-1}	Turnbull *et al.*, 1989
β-Galactosidase	*E. coli*	*A. niger*	*gpdA*	23,300 U mg^{-1} protein	Punt *et al.*, 1991
β-Glucuronidase	*E.coli*	*A. niger*	*gpdA*	192,000 U g^{-1} mycelium	Punt *et al.*, 1991
Endoglucanase	*Cellulomonas fimi*	*A. nidulans*	*alcA, glaA*	20 mg L^{-1}	Gwynne *et al.*, 1987
Bm86 (gut cell surface)	*Boophilus microplus*	*A. nidulans*	*amdS*	24 g g^{-1} (wet weight)	Turnbull *et al.*, 1990

Table 1.1 Heterologous proteins expressed in *Aspergillus* (Partly adapted from Davies, 1994).

ENZYME	SPECIES	USE OF ENZYME
α- & β-Amylase	*A. oryzae, A. niger*	Starch liquefaction
Glucoamylase	*A. oryzae, A. niger*	Glucose syrup production
Cellulase	*A. oryzae, A. niger*	Fruit and vegetable processing
α- & β-Galactosidase	*A. oryzae, A. niger*	Oligosaccharide (and lactose) hydrolysis
β-Glucanase	*A. oryzae, A. niger*	Plant extracts flavours
Glucose oxidase	*A. niger*	Anti-oxidant, preservative
Inulinase	*Aspergillus*	Production of sweeteners
Xylanase	*A. awamori*	Cellulose hydrolysis
Lipase	*Aspergillus*	Leather and wool processing, dairy products, waste treatment
Pectinase	*Aspergillus*	Fruit juice clarification, extraction of coffee and spices
Protease	*Aspergillus*	Meat and fish processing, cheese manufacture
Ribonuclease	*A. oryzae*	Flavouring agent production

Table 1.2 Homologous enzymes from *Aspergillus* (adapted by Jeenes *et al.*, 1991).

The features that make *Aspergillus* such an attractive host include acceptance by industry, long history of safe usage, good protein secretion, well characterized genetics (*A. nidulans*), relatively rapid growth on simple, inexpensive media, FDA approved (*A. niger*), well established fermentation and product recovery strategies.

1.3.1 Basic Features of *Aspergillus* Expression and Secretion Systems.

As Already mentioned above, the *Aspergillus* species that have been developed as hosts for the expression of heterologous proteins are: *A. niger, A.*

nidulans, A. oryzae and *A. awamori* (also found in literature as *A. niger* var. *awamori*). All the systems that are presently in use achieve stable heterologous gene expression through the integration of a vector molecule into random sites throughout the genome. Vectors are essentially standard bacterial plasmids that act as amplification and delivery systems for the heterologous gene expression construct. A separate plasmid is often used as a selectable marker and therefore cotransformation is employed (Davies, 1994).

In the case of intracellular gene expression, the heterologous gene fragment, which is usually derived from cDNA, is flanked upstream by a DNA fragment containing *Aspergillus* transcription and translation initiation signals, and downstream by an *Aspergillus* transcriptional terminator. When secretion of the product is desired, as it is in most cases, the promoter DNA fragment includes the secretion signal sequence of an *Aspergillus* gene. Until recent years, all constructs used direct fusion of the desired portion of the heterologous coding region to the *Aspergillus* signal sequence. Now, it has been shown that the fusion of the heterologous coding region with the entire coding region of an *Aspergillus* gene encoding a product that is well secreted (glucoamylase in *A. niger*), results in much higher yields of the product of choice (Davies, 1994).

Cleavage of the heterologous protein from the carrier can be achieved by several mechanisms. These include self-cleavage (prochymosin), cleavage by the *Aspergillus* equivalent of yeast KEX-2 endopeptidase, or by commercially available endopeptidases such as Factor Xa; the design of the construct reflecting the planned cleavage mechanism (Davies, 1994).

The most widely used technique for transformation is the protoplast-based technique that was initially used for yeast transformation. Protoplasts are produced via enzymatic digestion of the cell walls of germinating conidia or young mycelia in the presence of osmotic stabilizers (KCl, NaCl, sucrose or sorbitol). Enzyme preparations are used for the digestion (e.g. Novozyme 234). Polyethylene glycol and $CaCl_2$ are then used to facilitate transfer of DNA across

the plasma membrane and finally, the transformed cells are placed on selective media where they regenerate cell walls and produce sporulating colonies (Jeenes *et al.*, 1991). The transformants are then assayed for expression and the structure of the integrated vector is assessed in genomic blots.

Yields of several grams of fungal protein per litre of fermentation broth have been achieved using *Aspergillus* strains as hosts for transformation with constructs of the type shown in fig. 1.2, panel C. Over 3 g L^{-1} of *Rhizomucor miehei* aspartic protease were obtained from *A. oryzae* and 2-5 g L^{-1} of *A. niger* glucoamylase were secreted from *A. nidulans*. Vertebrate proteins expressed in *Aspergillus* grown in shake flasks, using the same construct have been in the range of 0.1 to 10 mg L^{-1}, regardless of the *Aspergillus* species used as host. The primary factor affecting the yield is the sequence and the structure of the heterologous mRNA and protein, hence the productivity of a particular secreted vertebrate protein may be 10 to 1000 times lower than that of a given secreted ascomycete fungal protein (Davies, 1994).

Using the integration construct fusion protein D, shown in fig. 1.2, a major increase in the yields was achieved; 20 to 100 times. Bovine chymosin secreted from *A. awamori* gave titres of 150 mg L^{-1} when grown in 50 ml shake flasks. That was a 10-fold improvement on the previous transformant with the same promoter (*A. niger gla*A), and 100-fold on the previous transformant (Ward, 1991).

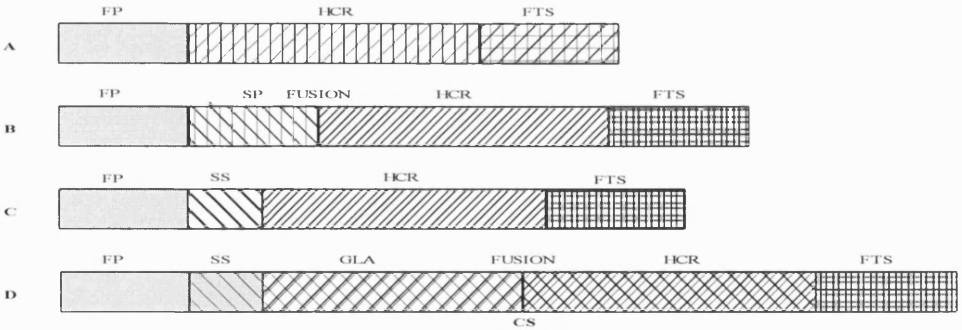


Fig. 1.2 Expression cassette constructs commonly used in *Aspergillus* (Davies, 1994). A and B are for intracellular synthesis and C and D for secretion of heterologous proteins. FP, fungal promoter; HCR, heterologous coding region; FTS, fungal transcription stop; SP, stabilizing protein; SS, signal sequence; GLA, glucoamylase or equivalent secreted filamentous fungal protein; CS, cleavage sequence.

1.3.2 Morphology of *Aspergillus*.

Aspergillus, similarly to other filamentous microorganisms, can be grown either as freely dispersed mycelia or as aggregated forms (clumps or pellets). Studies have shown that the type of morphology is a major factor affecting the yield of metabolites. Freely dispersed hyphal elements can increase the viscosity of the medium and thus cause gas-liquid mass transfer and mixing problems. Pellets have the advantage of low viscosity and good mass transfer but can show reduced growth due to nutrient (Metz and Kossen, 1977) and oxygen (Kobayashi *et al.*, 1973) limitations in the dense core of the mycelial aggregate. In addition, Wösten *et al.* (1991) have demonstrated that glucoamylase and other proteins are being secreted from the tips of growing hyphae of *A. niger*. More recently, Gordon *et al.* (2000), have constructed a glucoamylase::green fluorescent protein fusion in order to study *in vivo* protein secretion in *A. niger*. They reported that fluorescence was most intense at the apices of the hyphae, suggesting that protein secretion occurs at hyphal tips. It is therefore obvious that the development of the largest possible number of growing hyphal tips without the problems associated with the free mycelia are two conditions essential for high protein titres in the culture. These two conditions can be met when the microorganism is grown in the form of very small (diameter < 1.0 mm) pellets, where the small volume of the pellet ensures both a higher number of tips per culture volume and the reduction of nutrient limitation. Hotop *et al.* (1993) have shown that by reducing the pellet diameter from 1.0 mm to 0.6 mm, the product concentration from a *Penicillium chrysogenum* culture doubled.

In another study, Martin and Bushell (1996) suggested that the product (erythromycin) was produced at a fixed distance from the growing hyphal tip and that there is a critical hyphal particle diameter, below which the particle is incapable of producing any product. They tested this hypothesis in antibiotic producing *Saccharopolyspora erythraea* cultures.

In a recent paper, Johansen *et al.* (1998) investigated the influence of morphology on the formation of the extracellular lipase, cutinase in *A. awamori*. They concluded that morphological differences did not affect significantly the product formation, meaning that structural features are not important. They also stated that the primary effect of morphology on product formation is due to viscosity.

A number of factors affect the formation of pellets and generally the morphology of filamentous fermentations. They include shear stress, inoculum size, nutrition, pH, temperature and carbon/nitrogen ratio (Braun and Vecht-Lifshitz, 1991; Glazebrook *et al.*, 1992). Inoculum size, nutrition and carbon and nitrogen concentration are discussed in section 1.4.2 of this report.

Shear stress is a major factor affecting the morphology of filamentous microorganisms. Shear stress in a fermenter is mainly caused by agitation. Mitard and Riba (1988), in a study they performed with *A. niger* ATCC 26036, concluded that at shear stress values of 5 Pa or greater, pellet morphology disappeared. In addition, various studies performed with *P. chrysogenum* (Van Suijdam and Metz, 1981, Smith *et al.*, 1990, Makagiansar *et al.*, 1993) have demonstrated that an increase in shear stress resulted in a decrease in mean hyphal length. Tamura *et al.* (1997), also showed that increasing the shear stress in a *Streptomyces fradiae* fermentation producing tylosin, led to changes in morphology; at high shear stress, more than 400 rpm agitation rate, filamentous morphology comprised more than 50 % of the total, while at lower stress (less than 400 rpm), pellets formed more than 50 % of the total morphology. They stated that when mycelia and pellet areas were more than 4.0 mm^2, the production rate of tylosin decreased drastically. Finally, Fujita *et al.* (1994) proposed a theoretical model for the formation of pellets from *A. niger*, based on shear stress, where the average diameter of pellets and the number of pellets formed per unit volume are linearly related to shear stress.

1.3.2.1 Image analysis.

Image analysis is defined as the extraction of information from a picture by a computer (Thomas, 1992). The first step is image capture, usually from a television camera mounted on an optical microscope or, alternatively, from another video source or an electron microscope. Then the computer extracts or enhances features of interest, and the subsequent measurements are processed with the aid of specialized software. Its main advantage is that repetitive measurements can be automated and therefore processed faster and be more reproducible than measurements based on human observations. Current applications of image analysis in the field of biotechnology include quantification of growth and cellular viability, biomass determination, microbial morphology and motility and modeling of mycelial morphology and metabolism. Vecht-Lifshitz and Ison (1992) present a comprehensive review of the present and future uses of image analysis in biotechnology. Thomas and Paul (1996) provide a review of the current uses of the technique, while Cox *et al.* (1998) review the uses of image analysis in the investigation of the morphology of filamentous microorganisms. Also, a review of the hardware and software available has been presented by DeYoung (1988), together with tips for buyers of such systems.

Image analysis will be used in this project in order to investigate changes in morphology during the *A. awamori* fermentation.

1.3.3 Proteases.

The problem of proteolytic degradation of heterologous proteins expressed in *Aspergillus* is evident in the literature (van den Hondel *et al.*, 1992; Archer and Peberdy, 1997; van den Hombergh *et al.*,1997). Yields of hen egg-white lysozyme and porcine pancreatic phospholipase A_2, expressed and produced in *A. niger* have been affected by high levels of extracellular proteases (Archer *et al.*, 1992). Classical mutagenesis and gene disruption techniques have been suggested as possible solutions to the problem. Mattern *et al.* (1992) managed to obtain

mutants of *A. niger* deficient in extracellular proteases by using a series of *in vivo* and *in vitro* UV mutagenesis and gene replacement techniques. They found that degradation of secreted heterologous protein was reduced, when using a mutant with only 1-2 % of the original protease activity, but was not completely eliminated. Also, Broekhuijsen *et al.* (1993) concluded that elimination of aspergillopepsin had a significant beneficial effect on the amount of human interleukin-6 produced by *A. niger*.

1.4 Fermentation Strategy.

1.4.1 Fermenter Vessels.

Commonly to most other filamentous fungi, *Aspergillus* can be grown in three main types of bioreactors: solid state, stirred tank and air-lift. Table 1.3 below summarizes the major advantages and disadvantages of each type of vessel.

Reactor Type	Advantages	Disadvantages
Solid State	◆Low energy requirements. ◆Inexpensive raw materials.	◆Limitation in nutrients and oxygen transfer. ◆Control of fermentation variables is problematic.
Stirred Tank	◆Very well studied and established. ◆Ability to control fermentation parameters easily.	◆Presence of high shear regions. ◆High energy requirements.
Air-lift	◆Simple construction, no moving parts. ◆Well-defined flow pattern; ease of scale-up. ◆Absence of high shear regions in the bulk liquid. ◆Low energy requirements. ◆High oxygen absorption efficiency.	◆Microorganisms encounter changing conditions. ◆Poor oxygen transfer in viscous cultures.

Table 1.3 Advantages and disadvantages of three different bioreactor types.

A large proportion of the processes that employ *Aspergillus* for the production of a product are performed in mechanically agitated vessels. There are

a number of reports that cite the use of air-lift bioreactors for the production of organic acids by *Aspergillus* fermentation. Okabe *et al.* (1993) used a 3 L air-lift fermenter for the production of itaconic acid by *Aspergillus terreus*. They achieved a maximum acid concentration of 44 g L^{-1}, a result that was similar to other processes using stirred tank vessels of the same volume. Similarly, when Głuszcz and Michalski (1994) used a pilot scale (200 L) external loop air-lift vessel for the production of citric acid by *A. niger*, they obtained high levels of product (90 g L^{-1}). Additionally, in a comparative study between air-lift and stirred bioreactors, Träger *et al.* (1989) concluded that gluconic acid yields by *A. niger*, at laboratory scale (4 L and 10 L respectively) were not notably different between the two types of vessel. Similar were the results of an earlier study by Barker and Worgan (1981) who obtained similar values of biomass yields of *A. oryzae* in 7.5 L and 100 L air-lift fermenters and in a 3.5 L stirred tank reactor.

Since nearly all processes that involve the production of recombinant proteins from *Aspergillus*, are performed with the use of stirred tank reactors, further discussion will focus on this type of vessel.

1.4.2 Inoculum.

Common to any other fermentation process, the inoculum used for an *Aspergillus* fermentation must satisfy a number of conditions (Stanbury *et al.*, 1995);

a. It must be in a healthy, active state in order to minimize the length of the lag phase of the subsequent fermentation.
b. It must be available in sufficiently large volume to provide an inoculum of optimum size.
c. It must be in a suitable morphological form.
d. It must be free of contamination.
e. It must retain its product-forming capabilities.

The inoculum used can be either conidial suspension or vegetative mycelia. Conidiospores are first allowed to germinate in shake flasks and then the suspension is used to inoculate the fermentation medium. Age and condition of the spore inoculum, medium composition and spore concentration are factors affecting the degree of germination in *Aspergillus*. It has been suggested that the size of the inoculum can affect the yield of the secreted protein (Archer *et al.*, 1990). Vegetative mycelial inocula have been used to achieve rapid increase in fungal biomass in fermenters and to overcome problems associated with conidiospores (Jeenes *et al.*, 1991).

Studies have shown that spore inoculum levels affect irreversibly mycelial growth, morphology and productivity. Smith and Calam (1980) investigated the effect of inoculum in the production of penicillin and griseofulvin by *P. chrysogenum* and *P. patulum*, respectively. They concluded that the kind of inoculum used had a significant effect in product yield. Tucker and Thomas (1992), studied the effect of spore inoculum concentration on the eventual morphology of an industrial *P. chrysogenum* strain grown in shaken flasks. There appeared to be a transition in morphology from pellets to dispersed mycelia at inocula concentrations above 10^5 spores L^{-1}. In a more recent study, Tucker and Thomas (1994) compared the effect that the inoculum size had on the morphology of the same industrial strain of *P. chrysogenum* grown in shaken flasks and in a 6 L stirred tank reactor. They concluded that there was no sharp transition from the pelleted growth to the more dispersed forms in the reactor, with relatively small changes occurring in the mycelial morphology in that type of vessel. Since mycelial morphology is of great importance for the production of heterologous products from filamentous fungi, the level of the inoculum (which is a factor affecting morphology) is in turn a significant factor in the fermentation. In a recent study, Paul *et al.* (1999) have used *A. niger* fermentations to study and quantify the effects of inoculum morphology and fermentation conditions. They used image analysis to characterize morphology and HPLC to measure the concentration of citric acid produced. They concluded that initial inoculum morphology does have a notable effect on the subsequent culture morphology and

citric acid productivity of the fermentation. More specifically, they achieved a relatively high yield of citric acid (0.74 g g^{-1} glucose) when they inoculated the medium with dispersed mycelia. When they used inoculum in the form of pellets, the yield was lower (0.49 g g^{-1} glucose and 0.58 g g^{-1} glucose).

1.4.3 Medium Composition.

The medium used for the fermentation of *Aspergillus*, and any other microorganism in general, must ideally meet as many of the following criteria as possible (Stanbury *et al.*, 1995);

a. It should help produce the maximum yield of product per gram of the substrate used.
b. It should aid the production of maximum product concentration.
c. It should permit for the maximum rate of product formation.
d. There should be the minimum yield of unwanted products.
e. It should not cause problems during media making and sterilization.
f. It should be of consistent quality and readily available.
g. It should cause minimal problems in other aspects of the process, mainly extraction, purification and waste treatment.

In the case of *Aspergillus*, as in most microorganisms, the composition of the growth medium can have a dramatic effect on the concentration of the product. The choice of the carbon source is dependent on factors such as the type of promoter used. Carbon sources used in *Aspergillus* fermentations vary in composition from complex to totally defined. Examples of complex carbon sources include ball-milled oat straw for the production of xylanase and β-xylosidase by *A. awamori* (Smith and Wood 1991), sugar beet slices for the production of polygalacturonase by *A. niger* (Hermersdörfer *et al.*, 1987) and cassava flour for the production of glucoamylase by *A. awamori* (Chiquetto *et al.*, 1992; Queiroz *et al.*, 1997). Defined carbon sources are being used mainly for the laboratory-scale production of recombinant proteins. They include maltose (Ward

et al., 1990), fructose (Yadwad *et al.*, 1996) and in most of the other cases, glucose.

The choice of the nitrogen source has also great effect on the level of product secretion; Smith and Wood (1991) reported that production of a xylan-degrading enzyme by *A. awamori* is affected by the nature and the concentration of the nitrogen source. Nitrogen sources commonly used include corn steep liquor, yeast extract, urea, peptone, ammonium chloride and sodium nitrate. Ammonium sulphate is also used as a low cost nitrogen source, mainly for the production of glucoamylase (Ariff and Webb, 1992). When ammonium nitrate is used as the source for nitrogen it can also affect the morphology of the microorganism (Joung and Blaskovitz, 1985); depending on the concentration of ammonium nitrate, three different morphologies were observed: **a**. vegetative mycelia, when ammonium nitrate concentration was 2 g L^{-1}; **b**. complete natural morphology comprising of fruiting bodies and mycelia, when ammonium nitrate concentration was 0.5 g L^{-1}; **c**. short, branched and bent mycelia with bulbous and horny masses, at ammonium nitrate concentration of 8 g L^{-1}. It has also been reported that urea can be the ideal nitrogen source for the production of glucoamylase, since it gave enzyme activity values that were 40 % higher than those of ammonium sulphate (Chiquetto *et al.*, 1992). Finally, soya milk has been used as a supplementary nitrogen source and has been sown to increase productivity, possibly due to a reduction in protease levels (Ward *et al.*, 1990; Jeenes *et al.*, 1991).

Studies have been conducted in order to assay the effects of different carbon and nitrogen sources on product yields and to develop an optimized medium for *Aspergillus*. Ariff and Webb (1992) studied the effect of the carbon and nitrogen source concentration on glucoamylase production by *A. awamori*. They used potato starch and maltodextrin and ammonium sulphate and yeast extract as carbon and nitrogen sources, respectively. They concluded that maximum enzyme activity was achieved at 5 % w/v potato starch and 4 % w/v maltodextrin for the carbon sources, and for the nitrogen, the maximum value of enzyme activity was observed at 0.75 % w/v ammonium sulphate. They also

stated that addition of 0.1 % w/v of yeast extract to the medium favoured both growth and enzyme production.

At a more recent study, Žužek *et al.* (1996) optimized a fermentation medium for the growth and production of mevinolin by *A. terreus*, using a modified method of genetic algorithms. Ten different medium components were selected for medium optimization. These were glucose, glycerol, KH_2PO_4, sodium acetate, tomato paste, dry yeast, $FeSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, corn steep liquor and oat meal. They report that in four generations and 47 performed experiments, they improved productivity by nearly three times.

1.4.4 Mode of Fermentation.

There are three major types of fermentation: batch, continuous and fed-batch. Batch culture is a closed system which contains an initial, and therefore limited, amount of nutrients, the culture goes through a number of stages and finally, as nutrients are spent, the microorganism growth rate decreases the process is stopped and the product recovered. Exponential growth in a batch culture may be prolonged by the addition of fresh medium to the vessel. If an equal volume of the culture is simultaneously removed from the vessel, then the process is termed as continuous. The third type of fermentation, simple fed-batch, is a culture to which medium is added continuously or sequentially, without the removal of broth.

The fed-batch mode has the advantage of controlling the levels of the carbon source and other nutrients in the medium and in this way minimizing the production of metabolic by-products, which inhibit growth and product formation. Feed can be added to the vessel in an "on" and "off" manner. It can also be added either at a constant, a linear or at an exponential rate. The latter is more commonly employed in bacterial cultures, where the rate of growth is high. Such a feed rate could raise problems with *Aspergillus* fermentations; its relatively low growth rate would mean that nutrient concentration would increase rapidly with time,

resulting in possible substrate inhibition. Aguilar and Huitron (1986), report that there is a 40 % and 60 % increase in the activity of pectinases produced by *Aspergillus* sp., when fed-batch culture with recirculation of medium was used. Dawson *et al.* (1988) applied a nitrogen limited fed-batch regime in the fermentation of *A. niger* and reported a 100 % increase in citric acid production. Imai *et al.* (1994) used specific glucose consumption rate for the control and optimization of the feed rate in a glucoamylase producing culture of *A. niger*; this resulted in a 34 % increase in product, compared to the constant rate feeding. Chen (1995), showed that fed-batch fermentation of *A. japonicus* producing β-fructofuranosidase, resulted in a 20 % increase in the maximal enzyme production, compared to the batch culture of the same organism. Finally, Novak *et al.* (1997) reported a 37 % increase in the yield of lovastatin from *A. terreus*, when a repeated fed-batch process was employed.

1.4.5 Induction Strategy.

The majority of proteins of industrial interest produced by microorganisms are inducible. Since the reduced cell growth that generally accompanies constitutive expression leads to lower yields of the target protein, a regulatable promoter system is employed. Such a system provides the ability to "turn on" the expression of a foreign gene by varying an environmental factor or the concentration of a particular component in the growth medium (Carter *et al*, 1992).

Donovan *et al.* (1996) provide an extensive review examining the factors that influence the expression of foreign proteins in *E. coli* under the control of the *lac* and *tac* promoters. They examined the influence of IPTG (isopropyl-β-D-thiogalactoside) concentration, the growth medium composition, the culture temperature, the time point at which induction started and the duration of the induction phase. They concluded that all of the above factors have an influence on the product concentration.

1.5 Scale in Relation to Fermentation.

All fermentation processes start in the laboratory and if they are successful end in the production plant. During the transfer of a process from laboratory to production scale, many problems may be encountered. The studies at different scales of operation provide information to overcome such problems. Laboratory, pilot and production are the three scales of fermentation. Broadly, they range in volume from a few milliliters to 10-20 L, 20-10,000 L and 1,000-1,000,000 L respectively. The boundaries between the scales are indistinct, however each possesses certain unique characteristics.

1.5.1 Laboratory Scale.

Development at this scale revolves around the identification of possible products and the microorganisms that produce them: a process called screening. Equipment used is of small volume, ranging from test tubes and shake flasks to small fermenters. Usually, the equipment used at this stage has little resemblance to the production equipment. The main objective is to make the process work.

1.5.2 Pilot Scale.

Since many man-years are required to develop a new process, companies cannot risk financial loss due to failure at the production scale. This necessitates trials before the production plant is built. These trials are carried out in the pilot scale. Pilot scale is the most complex of the three scales of fermentation as it has many roles to fulfill. It has a number of objectives which all focus in the determination of the optimal operating conditions for the process. The fermenters used at this scale are either of geometric similarity to that of the production scale or of variable geometry aiming to cover the range of commonly encountered production vessel geometries.

1.5.3 Production Scale.

The optimized process is scaled-up from the pilot to production scale. The objective of this scale is the production of the desired product in sufficient quantities at a minimum cost. Vessel volume can range from a few hundred litres for high value/low volume products to several cubic meters for low value/high volume products.

1.5.4 Scale-up.

The problems encountered during the scale-up of a process fall into three main categories: inoculum development, medium sterilization and aeration/agitation (Banks, 1979). From these, problems arising from the latter category are the more difficult to overcome. Several strategies have been adopted for scaling-up aeration and agitation. The more commonly used are those on the basis of constant measured volumetric mass transfer coefficient (k_La), constant power consumption per unit volume, constant impeller tip speed and constant mixing time. The advantages and disadvantages of these methods, the calculations involved have as well as other aspects of fermentation scale-up been extensively discussed in a number of reviews in the literature (Charles, 1985a, b; Smart, 1991; Kossen, 1992).

For this project it was decided to perform scale-up of the fermentation process by keeping constant the energy per unit volume. In this way, the shear stress encountered by the microorganism would not increase and oxygen limitation problems encountered at high energy inputs would be avoided. A similar approach was used for the scale-up of an *A. awamori* fermentation process by Cui *et al.* (1998). Their objective was to collect essential information for *A. awamori* fermentations; time profiles, kinetics, morphology effect of operation parameters were amongst the parameters they studied. Although a study has been presented for the large-scale production of antibody fragments by *A. awamori*

(Frenken *et al.*, 1998), no systematic approach to the scale-up and the factors affecting the fermentation have been given.

1.6 Downstream Processing.

The choice of the recovery system for heterologous proteins secreted in the medium, and generally any other microbial product, is based on the following criteria (Stanbury *et al.*, 1995);

a. Concentration of the product in the fermentation broth.
b. Physical and chemical properties of the product.
c. Intended use of the product.
d. Minimum acceptable standard of purity.
e. Impurities in the fermentation broth.
f. Price of the product in the market.

The first stage of the recovery involves the removal of the biomass. The microbial cells have to be removed under contained conditions and then killed, or killed in the fermentation vessel prior to separation. Since the product is likely to be negatively affected by the deactivation procedure, the former is more commonly used. The size of hyphae eliminates the use of a disc-stack centrifuge and at the same time, traditional equipment used for the separation of fungal cells, such as the rotary vacuum filter, is not usable due to the fact that it cannot be contained. A containable basket centrifuge could be the ideal option. Microfiltration is then employed to achieve product concentration in the supernatant.

1.7 AIMS AND OBJECTIVES.

Although *Aspergillus* has been used as host for heterologous proteins, little data is published on the production of these proteins. Particularly, a minimal amount of information is available for antibody fragment production. The challenge, therefore, in this study has been to define a production strategy to allow evaluation of secretion of scFv fragments in *A. awamori* against production in *E. coli*, a well-established and studied system. The significance of this project is the fact that a novel product (scFv antibody fragment) is produced in a novel way (using *Aspergillus*). The aim is to devise a successful fermentation strategy for the production of scFv antibody fragments in *A. awamori*. In order to do so, several parameters affecting the production of scFv antibody fragments were investigated. The emphasis has been to study the effects of induction time and mode of fermentation, growth medium composition, inducer concentration, proteases and scale of operation. Optimal integration of these parameters will be important in the elucidation of a complete and efficient process for antibody fragment production.

2. MATERIALS AND METHODS

All reagents and chemicals were purchased from BDH chemicals (Merck Ltd., Lutterworth, Leicestershire, UK) and SIGMA (SIGMA Chemical Co., St. Louis, MO, USA), unless otherwise stated.

2.1 Organism and Inoculum Preparation.

Aspergillus awamori strain AXCL 415gs#25 was used for the project. It was a kind donation by the Unilever Research Laboratorium, Holland. The microorganism was stored as 1 ml glycerol stocks at –70°C. The stocks were replaced every three months. The spore inoculum for the fermentations was prepared by growing the microorganism on Potato Dextrose Agar (PDA). For ease of spore harvest, an H-bond membrane (Amersham International) was placed on the surface of each plate. Sterile swabs were used to inoculate five plates for every fermentation run. The plates were incubated at 28°C for 5 days. On the fifth day, the membranes containing the spores were removed, placed aseptically into 100 ml of physiological saline containing 1.5-2.0 mm diameter glass beads (0.9 g NaCl, 0.1 ml Tween 80, 5 g glass beads) and shaken vigorously by hand in order to remove the fungal spores. Spores were counted using a haemocytometer; 100 µl of the spore containing saline were diluted in 100 ml of distilled water and a 10 µl aliquot from the diluted spore solution was pipetted on to the haemocytometer. A 2 L baffled shake flask was then inoculated with 0.9-1.2x10^7 spores ml^{-1}. The shake flask contained 500 ml of germination medium (1 g KH_2PO_4, 3 g $NaNO_3$, 0.5 g $MgSO_4 \cdot 7H_2O$ and 0.88 g yeast extract (DIFCO Laboratories, Detroit, MI, USA), in 500 ml of distilled water). The shake flask was then placed into an orbital incubator and was incubated at 37 °C, 150 rpm for 7 hours.

2.2 Fed-batch Fermentation.

The first three fermentations were performed in an Inceltech 7 L LH Series 210 glass bioreactor (Inceltech, Reading, UK), containing three sets of 70 mm diameter, six-bladed, Rushton turbine impellers. On-line variables, such as pH,

dissolved oxygen tension (DOT) and temperature, were measured and controlled by Turnbull Control Systems (TCS) instrumentation (Worthing, UK). The pH and DOT probes were calibrated prior to every fermentation. Fermenter exit gases were monitored by a VG Gas Analysis Prima 60 mass spectrometer (Cheshire, UK), which measures inlet and outlet concentrations of nitrogen, oxygen, argon and carbon dioxide, enabling the determination of oxygen uptake and carbon dioxide evolution rates (OUR & CER) and also the respiratory quotient (RQ). The software package Real Time Data Acquisition Systems (RTDAS) (Acquisition Systems, Fleet, UK) logged data from the mass spectrometer and the fermenter TCS unit. The formulae used by the software to calculate these values are presented in Appendix I.

2.2.1 Initial Medium.

The composition of the medium used for these fermentations was as follows: 2 g L^{-1} KH_2PO_4, 6 g L^{-1} $NaNO_3$, 1 g L^{-1} $MgSO_4 \cdot 7H_2O$ and 1.77 g L^{-1} yeast extract. This medium will be called "initial medium" (A1). This medium (3.3 L) was sterilised in the fermenter for 30 minutes at 121°C. After sterilization, the vessel was inoculated with 300 ml of the seed culture from the shake flask, giving a final fermenter concentration of $1x10^6$ spores ml^{-1}. Process parameters were: temperature set-point of 30°C, pH 6.0 (maintained by addition of 4M NaOH and 1M orthophosphate), air-flow rate 3 L min^{-1}, stirrer speed 400 rpm. DOT levels were kept above 30% air saturation by adjustment of the stirrer speed. Seven hours after inoculation, a constant, linear feed (A2) was commenced through a 2 mm diameter tube, with the use of a 2 rpm peristaltic pump (Watson-Marlow, Falmouth, Cornwall, UK), calibrated for that feed. It consisted of the following: 6.67 g L^{-1} fructose, 53.3 g L^{-1} xylose (inducer), 12.5 g L^{-1} KH_2PO_4, and 6.67 g L^{-1} yeast extract. Xylose and fructose were sterilised separately from the other two ingredients of the feed, at 121°C for 20 min and were mixed aseptically afterwards. This was done in order to avoid caramelization. Feeding rate was 0.252 ml min^{-1} and the total volume added was 900 ml. Samples for biomass and antibody fragment analysis were taken every

three to four hours during working hours and also immediately before the initiation of the feeding. The running time for the fermentation was 65 to 70 hours.

Subsequent laboratory scale fermentations were all performed in a 7 L Applikon stainless steel vessel (Applikon, Schiedam, Holland), containing 3 sets of 60 mm diameter, six-bladed, Rushton turbine impellers. On-line variables were measured and controlled by Metler Toledo instrumentation (Metler Toledo GA, Greinfensee, Switzerland). Data from the mass spectrometer and the fermenter were logged into the BioXpert software package (BioExpert Ltd., UK). The two initial fermentation runs performed in this vessel used the same medium (A1) and feed composition (A2) and were carried out under the conditions described above. They were used as control fermentations.

2.2.2 Old Medium.

Further fermentations that were performed in the Applikon 7 L vessel employed a growth medium (B1) and a feed (B2) with different compositions. The ingredients of the batch growth medium were as follows: 1 g L^{-1} KH_2PO_4, 19.8 g L^{-1} $NaNO_3$, 3.3 g L^{-1} $MgSO_4 \cdot 7H_2O$, 3.33 g L^{-1} $(NH_4)_2SO_4$, 2 g L^{-1} yeast extract and 8 g L^{-1} fructose. This medium will be termed as "old medium". All the medium components were sterilized in the fermenter at 121 °C for 20 minutes, except fructose that was sterilized separately to avoid caramelization. Process parameters were: temperature 30 °C, stirrer speed 500 rpm, pH 6.0 (maintained by addition of 4M NaOH and 1M orthophosphate) and air-flow rate 3 L min^{-1}. DOT levels were kept above 30 % by adjustment of the stirrer speed. Seven hours after inoculation, a constant, linear feed was commenced through a 2 mm diameter tube, with the use of 10 rpm peristaltic pump (Applikon, Schiedam, Holland). It consisted of the following: 32.3 g L^{-1} fructose, 6.15 g L^{-1} KH_2PO_4 and 6.8 g L^{-1} yeast extract. Feeding rate was 0.252 ml min^{-1} and the total volume added was 900 ml. Xylose, the inducer, was fed separately, with the aid of a 32 rpm peristaltic pump (Watson-Marlow, Falmouth, Cornwall, UK), at times stated in the "Results" and "Discussion" sections.

The concentration of xylose was 63.8 g L^{-1} and the total volume added was 750 ml. Samples for biomass and scFv analysis were taken every 3 to 4 hours during working hours and also immediately before the initiation of the feeding. The running time for these fermentations was 65 to 70 hours.

2.2.3 New Medium.

The experiments carried out with altered medium composition, known as "new medium", had double the concentration of fructose and ammonium sulphate in the medium (C1) and the feed (C2) and induction started 21 hours after inoculation. Finally, last fermentations in the small scale as well the runs in the large scale used a further "improved medium" (D1) and feed (D2) with half the concentration of xylose (compared to C2) and a step feed of fructose. Table 2.1 summarizes compositions of the different growth media used in this study.

Component ($g\ L^{-1}$)	**"INITIAL"**		**"OLD"**		**"NEW"**		**"IMPROVED"**	
	A1	**A2**	**B1**	**B2**	**C1**	**C2**	**D1**	**D2**
Fructose	-	6.67	8	32.3	16	64.6	16	84.6
Xylose	-	53.3	-	63.8	-	63.8	-	32
KH_2PO_4	2	12.5	1	6.15	1	6.15	1	6.15
$NaNO_3$	6	-	19.8	-	19.8	-	19.8	-
$MgSO_4 \cdot 7H_2O$	1	-	3.3	-	3.3	-	3.3	-
Yeast extract	1.77	6.67	2	6.8	2	6.8	2	6.8
$(NH_4)_2SO_4$	-	-	3.33	-	6.66	-	6.66	-

Table 2.1 Summary of different fermentation media used.

2.3 SCALE-UP.

The fermenter vessel used for the pilot scale runs was a 75 L total (50 L working) volume, Inceltech LH HI-CAT (Inceltech, Reading, UK) made of stainless steel. The dimensions of the vessel and its specification are presented in Appendix II, together with those of the Applikon vessel. The calculations used for the scale-up were those described by Banks (1979) for geometrically similar vessels. The formulas used were: $N_2=N_1(V_1/V_2)^{2/9}$ and $Q_2=Q_1(V_2/V_1)^{2/3}$ (where N=stirrer speed, V=volume and Q=air-flow rate, index 1 is for small scale and index 2 is for large scale).

Substituting the values for an initial stirrer speed of 700 rpm and aeration of 3 L min^{-1} in the laboratory scale it was found that the initial stirrer speed for the pilot scale would have to be 420 rpm and the air-flow rate 14 L min^{-1}. The growth medium used was the medium described previously with half the concentration of xylose and a step-feed of fructose (D1 and D2). Induction was started at the end of the exponential phase of growth (21 hours after inoculation). The volumes of feed and inducer were adjusted for the large scale. The feed and the inducer were added with the aid of two 32 rpm peristaltic pumps (Watson-Marlow, Falmouth, Cornwall, UK). The pH was controlled to 6.0 by addition of 4M NaOH and 1M orthophosphate. Samples for biomass and scFv analysis were taken every 3 to 4 hours during working hours. The duration of the fermentations was 68 hours.

2.4 BIOMASS ESTIMATION.

2.4.1 Dry Weights.

A. awamori biomass was calculated as grams Dry Cell Weight (DCW) per litre of fermentation broth . Duplicates of 10 ml samples were filtered through pre-dried, pre-weighed, 4.7 cm diameter, 0.2 μm glass microfibre GF/A filters (Whatman International, Maidstone, UK). The filters were dried for 24 hours in an oven, at 100°C. Biomass was calculated after reweighing the filters, by subtracting the filter

weight from the final weight and multiplying that value by 100 to calculate the weight in g L^{-1}.

2.4.2 Moisture analyser.

A halogen moisture analyser was additionally used for the estimation of the dry biomass. The instrument used was a Metler Toledo, model HG 53 (Metler Toledo GA, Greinfensee, Switzerland). It provided a quick (20 min) and accurate (within 5 % of dry weights) method for the measurement of the dry biomass. The instrument operates by determining the weight of the wet sample at the start by means of an internal electronic balance. Then the sample is quickly heated by the halogen dryer unit to a set temperature (95 °C) to remove the moisture. On completion of drying, the dry weight of the sample is displayed as the final result. This instrument was compared to data obtained from the conventional method for the estimation of the dry weight and the results are shown in the "Results" section of this report. Comparison of the results obtained from this method with those from the traditional method used for biomass estimation (as described above), found them to be within 5 % with each other.

2.4.3 On-line Biomass Monitor.

An *in-situ*, steam sterilizable capacitance probe was used in a few fermentation runs, in order to provide an on-line measurement of the biomass. The instrument used was the Biomass Monitor, model 214 (Aber Instruments Ltd., Aberystwyth, UK). Measurements were performed with the instrument set at single frequency mode, high range. The low pass filter was set at 5 sec and the frequency at 0.6 MHz. Data from the Biomass Monitor were collected in a laptop computer, through a Picologger (analogue-digital converter). On-line data were displayed on the computer screen with the aid of Pico software (Pico Technology Ltd., Cambridgeshire, UK).

All biological cellular systems consist of a "poorly conducting" lipid membrane, separating the conducting cytoplasm from the conducting extracellular milieu (including cell wall). At low frequencies this membrane behaves as a capacitor and becomes charged. The biomass probe records this capacitance. The output signal from a capacitance biomass monitor is directly proportional to the membrane-enclosed volume fraction of the microbial culture. The advantage of this method is the fact that because only cells with intact membranes alter the capacitance measured by the instrument, only viable cell mass is measured.

Harris *et al.* (1987) provide a general outline and description of the biomass monitor principle and demonstrate the linearity of capacitance with yeast biomass. The capacitance biomass monitor has been since used for the biomass measurement of a wide range of microbial fermentations. These include *Saccharomyces cerevisiae, Pichia pastoris* and *Streptomyces virginiae* (Fehrenbach, *et al.*, 1992), *Saccharomyces uvarum (carlsbergensis)* and lager yeast (Austin *et al.*, 1994) and *Saccharopolyspora erythrea* (Sarra *et al.*, 1996). It has also been used for mouse fibroblasts fermentations (Davey *et al.*, 1988), for solid substrate fermentation of *Rhizopus oligosporus* (Davey *et al.*, 1991) and for the determination of biomass in plant cell cultures (Markx *et al.*, 1991).

2.5 ELISA QUANTIFICATION OF SCFV.

Enzyme-Linked Immunosorbent Assay (ELISA) combines the specificity of antibodies with the sensitivity of spectrophotometric enzyme assays by using antibodies or antigens conjugated to an easily assayed enzyme, and also provides a high turnover number. The ELISA method used for the quantification of the scFv antibody fragments is often termed as the *indirect* ELISA. The specific antigen is coated to a solid phase (nylon pegs) and is reacted with the putative antiserum. Any specific antibody molecule binds to the antigen and all other material is washed away. Exposure of the complex to enzyme-labelled anti-immunoglobulin antibody results in binding to any specific antibody molecule adsorbed from the original serum. The

complex is washed and the substrate for the enzyme added, resulting in activity proportional to the amount of specific antibody in the original serum. Figure 2.1 provides a schematic representation of the ELISA method employed for the scFv assay.

The fermentation samples were filtered through low protein binding syringe filters (Sartorius) and kept refrigerated. The assays were performed at the end of the fermentation. The use of such filters was adopted after it was shown that the glass microfibre filters used up to that point retained some of the product. It was found that they retained almost 50 % of the scFv, depending on the duration and force of the filtration.

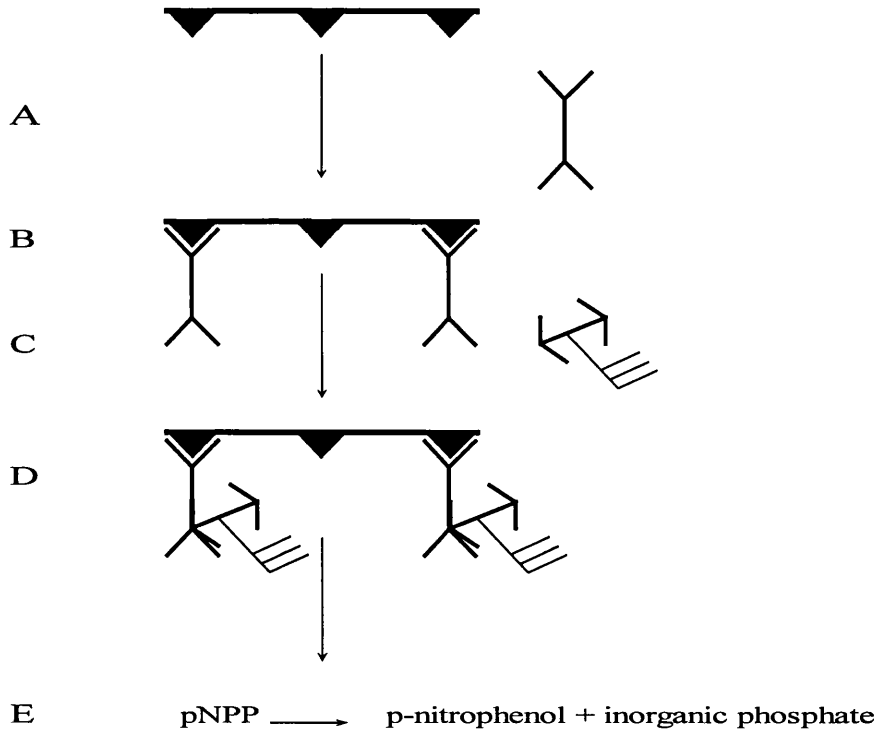


Fig. 2.1 Schematic representation of the indirect ELISA method. (Adapted from Burrin, 1992) **A**: Specific antigen is covalently attached to the solid phase. Antibody fragment added, incubated and washed. **B**: Specific antibodies bound. **C**: Enzyme-labelled anti-immunoglobulin (goat anti-rabbit IgG alkaline phosphatase conjugate) added, incubated and washed. **D**: Enzyme-labelled anti-immunoglobulin bound. **E**: Enzyme substrate (pNPP) added and incubated. Absorbance reading recorded.

ELISAs were performed in flat-bottomed, 96-well, microtitre plates. The antigen (lysozyme) was bound to nylon pegs designed to fit the wells of the microtitre plates. These nylon pegs were kindly provided by Unilever Research, Colworth, UK. Before use the pegs were rehydrated for 10 min in distilled water. Affinity purified Fv fragment was diluted to the following standards in PBSTA (PBS, 0.15% Tween 20, 0.02% Sodium Azide)(in μg L^{-1}): 1,000, 500, 250, 100, 50, 25, 12.5, 10, 5, 2.5 and 0. Triplicates (200 μl) of each standard and of every sample were dispensed onto the plates. The nylon pegs were placed in the wells and left to incubate for 30 min at room temperature. After incubation, the pegs were removed and washed in distilled water. Rabbit polyclonal antibody raised against the Fv fragment (kind donation from Unilever Research, Colworth, UK) was diluted 1:2000 in PBSTA and 200 μl were added to the wells of new plates. The nylon pegs were again placed in the wells and were left to incubate for a further 30 min at room temperature. At the end of the second incubation, the pegs were rewashed. Goat anti-rabbit IgG, alkaline phosphatase conjugate (200 μl), diluted 1:1000 in PBSTA was added into the wells of new plates. The pegs were placed onto the wells again and incubated under the same conditions. Finally the pegs were rewashed and placed onto new plates containing 1 mg ml^{-1} p-nitrophenyl phosphate, as the substrate, dissolved in 1M diethanolamine (DEA) buffer at pH 9.8. Colour development was measured by a plate reader at the wavelength of 405 nm. The plates were left to incubate at room temperature up to the point that the 10 μg ml^{-1} standard reached an absorbance reading of approximately 1.0. At that moment the absorbance of all the standards and the samples was recorded and the assay was completed. Concentration of the scFv was determined by a standard curve created by with the values obtained from the ELISA. A sample calculation of scFv from the standard curve is presented in Appendix III. The results are averages of triplicate samples which varied within 10 %.

2.6 Image Analysis.

In order to measure changes in the morphology of the microorganism during the course of the fermentations, an image analysis system was used. The system used

was a Magiscan MD system by Joyce Loebl (Slough, UK), running general purpose image analysis software (GENIAS). The system is attached to a Polyvar microscope (Slough, UK), set for bright field illumination, capable for automatic motion. A video camera attached to the microscope sends signal of the field of view to the image analysis system, where it is digitised and processed.

The *A. awamori* samples were diluted 1/10. A volume (50 µl) of the diluted sample was spread onto a microscope slide and left to air-dry overnight. When dry and fixed, the sample was stained with methylene blue as described by Adams and Thomas (1987) (0.3 g methylene blue, 30ml of 95 % ethyl alcohol in 100 ml water). After final air-drying, the microscope slide was run through the system. The microscope was set to x100 magnification and the image analysis to semi-automatic mode. Individual pellets were picked and measured. At least 35-40 pellets were measured from every sample.

2.7 Carbon Analysis.

The rate at which the carbon source is consumed is an important parameter in a fermentation process. It allows for the optimization of the growth medium composition. The consumption of carbon and, as a result, the carbon left in the fermentation broth, were measured using a Shimadzu (Milton Keynes, UK) TOC 5050 carbon analyser.

The instrument consists of a total carbon (TC) combustion tube that is heated to 680 °C and filled with oxidation catalyst. Carrier gas in the form of high purity air is supplied into this tube at a flow rate of 150 ml min^{-1} and moistened by a humidifier. The sample is introduced by an injector into the combustion tube and the total carbon is combusted to CO_2. The carrier gas, which contains the combustion product, flows through an inorganic carbon (IC) reaction vessel and is cooled and dried by a dehumidifier. It is then sent through a halogen scrubber into a sample cell set in a non-dispersive infra red (NDIR) gas analyser, where the CO_2 is detected. The

NDIR outputs an analogue signal, which generates a peak, the area of which is calculated by a computer. The peak area is proportional to the TC concentration in the sample. The TC concentration in the sample is determined by comparing the peak area to that of a calibration curve produced in advance, using a standard solution.

2.8 Fructose Assay.

To monitor the consumption of fructose, a fructose assay kit by Sigma was used. The assay works on the principle that fructose is phosphorylated by ATP in a reaction catalysed by hexokinase. Fructose 6-phosphate is then converted to glucose 6-phosphate (G-6-P) by phosphoglucose isomerase. G-6-P is then oxidised to 6-phosphogluconate in the presence of NAD in a reaction catalysed by G-6-P dehydrogenase. During this oxidation an equimolar amount of NAD is reduced to NADH. The consequent increase in absorbance at 340 nm is directly proportional to the concentration of fructose. The assay is performed as described in the Sigma technical bulletin (1996), by mixing 20 µl of phosphoglucose isomerase with 100 µl of the sample and 2 ml of the glucose assay reagent provided. The sample is incubated for 15 minutes at room temperature and its absorbance is measured at 340 nm.

2.9 Total Reducing Sugars Assay.

In order to calculate the amount of xylose used from the organism the dinitrosalicylic acid (DNS) reagent assay was performed as described by Miller (1959). Equal volumes (0.5 ml) of sample and DNS reagent were mixed in a test tube and heated in a boiling water bath for 5 minutes. Then the test tubes were cooled and 4 ml of distilled water were added. The absorbance was read at 540 nm and the reducing sugar concentration was calculated from a standard curve (Appendix IV).

DNS reagent assay measures the amount of reducing sugars in a sample. Reducing sugars are those containing a free aldehyde or keto group. Both fructose

and xylose are reducing sugars, containing a keto and an aldehyde group respectively. Therefore, by measuring the amount of fructose left in the sample using the fructose assay described before and by subtracting that value from the DNS reagent assay result, the amount of xylose in the sample was calculated.

2.10 Protease Assay.

In order to establish the presence of proteolytic enzymes in the fermentation broth, casein resorufin-labelled was used as a universal protease substrate (Boehringer Manheim). This preparation is a general substrate for proteases and is particularly suitable for the detection of traces of protease activity. By treating the substrate with the fermentation broth containing proteases, resorufin-labelled peptides were released. These peptides cannot be precipitated by trichloroacetic acid. Their concentration in the supernatant is proportional to the proteolytic activity present.

The substrate solution (50 µl) was initially incubated at 37 °C for 15 minutes in the presence of culture supernatant (100 µl) and incubation buffer (0.2 M Tris-HCl, pH 7.8 and 0.02 M $CaCl_2$, 50 µl). The reaction was stopped by addition of 480 µl of 5 % trichloroacetic acid (TCA) in redistilled water. The solution was incubated at 37 °C for 10 minutes and then centrifuged for 5 minutes at 13000 rpm. A volume of the supernatant (400 µl) was mixed with 600 µl of assay buffer (0.5 M Tris-HCl, pH 8.8) and the absorbance was read at 574 nm against a blank containing water instead of the culture supernatant. Results were converted to mg L^{-1}, trypsin equivalent, with the aid of a calibration curve constructed using trypsin standard (Appendix V).

2.11 Effect of Proteases.

In order to establish the possible effect of the proteolytic enzymes on the product, a storage experiment was designed. Aliquots (300 µl) of pure scFv (88 mg L^{-1}) were spiked with 100 µl of a) buffer, b) sterile fermentation medium, c) fermentation broth containing 700 mg L^{-1} of proteases and 40 mg L^{-1} scFv and

d) fermentation broth and 1 mg L^{-1} PMSF as protease inhibitor. The samples were incubated at 4 °C and 30 °C, in order to simulate the storage and fermentation temperatures respectively, for 0, 1, 2, 3, 4, 24, 48 and 72 hours. After incubation, ELISA was performed on the samples in order to establish any decrease in scFv concentration.

2.12 SDS-PAGE Analysis of scFv Presence.

Presence of the product was analysed by SDS-PAGE gel, according to the method of Laemmli (1970). This analytical method separates protein by molecular weight. Sodium Dodecyl Sulphate (SDS) is a surfactant that denatures the proteins and remains bound, producing an overwhelming negative charge. Unlike native electrophoresis, separation by SDS-PAGE is entirely due to molecular weight and not the protein charge.

In order to achieve efficient separation of scFv fragment discontinuous gel was used, hence there was a stacking gel placed on top of the separating gel. The stack enables dilute proteins to be separated with good resolution, as they are concentrated whilst migrating through this zone. Efficient separation was obtained with 12.5 % acrylamide gel.

Samples were prepared by diluting them 1:1 in sample buffer (60 mM Tris-HCl pH 6.8, 10 % Glycerol, 2 % SDS (GibcoBRL, Life Technologies Inc., Paisley, Scotland, UK), 0.001 % Bromophenol Blue and 5 % β-mercaptoethanol). Then denatured by boiling for 3 minutes. SDS-PAGE was performed using a dual min-slab gel system (Atto System AE 6400, supplied by Genetic Research Instruments Ltd., Essex, UK). The glass plates were cleaned using Industrial Methylated Spirits (IMS), gloves were worn to prevent contamination with external proteins. Once cleaned they were set up as in the manual.

The separating gel was prepared as table 2.1. Gloves were worn at all times as most of the reagents are toxic. Separating gel was degassed to remove any air bubbles, by passing it through a 0.2 μm polysulfone syringe filter (Whatman). Polymerization of the gel was initiated with the addition of 225 μl of freshly made 10 % ammonium persulphate and 15 μl of NNN^1N^1-Tetramethylethylenediamine (TEMED). The gel was carefully mixed whilst avoiding bubbles, then poured into the space between the two plates and allowed to set. Water was poured on to the setting gel to prevent it from drying out.

Reagent	Separating gel (ml)	Stacking gel (ml)
Acrylamide-bisacrylamide (30:0.8)	6.25	1.25
Tris-HCl 3.0M (pH 8.8)	1.875	-
Tris-HCl 0.5M (pH 6.8)	-	2.5
10% SDS	0.15	0.1
Distilled Water	6.125	5.0

Table 2.2 Protocol for SDS-PAGE gels (adapted from Hames, 1990).

Once the separating gel had set, the water was poured off. The stack was prepared as in table 2.1 and degassed as before. Polymerization was initiated by the addition of 150 μl of 10 % ammonium persulphate and 15 μl of TEMED. Again the gel was carefully mixed avoiding bubbles and then poured on top of the separating gel. A comb was carefully inserted between the two plates, and the gel was then left to set. Once the gel had set, the comb was carefully removed, so not to destroy the formed wells. The glass plates were placed in the electrophoresis tank. To the tank 1/10 dilution of reservoir buffer (0.25 M Tris, 1.92 M glycine, 1 % SDS, pH 8.3) was added. Wells were flushed with reservoir buffer to remove any residue gel.

Appropriate volumes (10 μl) of the denatured samples were loaded to the wells. To the first two wells, a low molecular weight marker (Pharmacia) and scFv

standard were added respectively. The tank was connected to a power supply. Separation through the stack was at 64 V and 30 mA (30 min). Once the samples had reached the separating gel, signaled by the formation of a single blue line across the gel, the power was increased to 120 V and 50 mA. The separation was run for a further hour or until the blue line reached the bottom of the plate.

Gels were stained using Coomassie brilliant blue (Bio-Rad Laboratories Ltd., Hemel Hempstead, Herefordshire, UK.). Gels were stained by gently agitating overnight in staining solution (coomassie brilliant blue R-250 (1 g L^{-1}) in 500 ml water, 400 ml methanol, 100 ml acetic acid). Then the gels were transferred into destain solution (25 % methanol, 10 % acetic acid in distilled water) and continued agitation until protein bands were revealed.

2.13 CONTAMINATION.

Contamination problems were encountered during a number of fermentations. In order to trace the origin of the contaminant, a series of procedures were followed. Initially, the fermenter was checked by pressurising it and recording the drop in pressure over a period of time. Also, after sterilising it with commissioning medium, it was run for 60 hours and % DOT, OUR and CER were recorded. Finally, samples were taken and checked for sterility at various stages (after sterilisation, after inoculation, during the fermentation), by plating them on nutrient agar and incubating them at 30 $^{\circ}C$. The Inceltech LH 7 L fermenter was checked in this way and it was found that the main seal needed changing. In the meantime, a new fermenter had become operable (Applikon 7 L) and it was used for subsequent fermentations. Contamination was also a problem in the first runs with this fermenter. The difference was that the contaminant was evident 24 hours later than in the previous vessel.

The same checks were performed in this fermenter and when it was established that it was sterilising properly and was holding pressure, attention was focused on the inoculum. When the glycerol stock used for the inoculum was plated

on nutrient agar using a loop there was no foreign growth. However, when a swab was used, foreign colonies appeared on the plate, suggesting that the contaminant was present in the inoculum in very small numbers. They were flat and grey in colour. These were isolated, Gram-stained and examined microscopically. It was revealed that they were Gram-positive, spore-forming rods, possibly *Bacillus*. Going back to the master seed stock in silica beads supplied by the industrial partner, it was found that it was also contaminated with the same microorganism. It was therefore decided to decontaminate the strain. This was done by picking single colonies of *Aspergillus* from a "contaminated" culture and plating them on PDA agar containing a mixture of antibiotics (50 µl ml^{-1} kanamycin, 50 µl ml^{-1} gentamicin, 100 µl ml^{-1} tetracycline and 50 µl ml^{-1} neomycin, added in the agar after sterilisation and when it was at 50 $^{\circ}C$). These antibiotics were effective against *Bacillus* sp. but would not affect *Aspergillus*. Single colonies were then picked and grown in shake flasks containing Luria-Betani (LB) medium (10 g L^{-1} bactotryptone, 5 g L^{-1} yeast extract and 10 g L^{-1} NaCl), to test for contamination. This procedure was repeated three times, each time taking single *A. awamori* pellets from the shake flask with the LB medium and growing them on agar. After the third time that the agar plates and the shaken flasks cultures with the LB medium were free of contamination, it was concluded that the stock was clean and new glycerol stocks were prepared. Before performing a fermentation with the new stocks, a series of shake flask fermentations were performed and ELISAs' were carried out in order to test for loss of product formation ability. There was none detected. After successfully decontaminating the strain all the PDA agar plates used for growth of the organism contained the aforementioned antibiotics as a precautionary measure.

3. RESULTS

The total number of fermentation runs performed was in excess of thirty. However, mainly due to contamination problems, the number of runs that were usable were less than twenty. Table 3.1 below summarises the fermentations described in this report. The remainder of the fermentation runs are presented in table A6.1 in Appendix VI. An effort was made to duplicate every fermentation run in order to check for reproducibility. Runs are not shown in chronological order.

FERMENTATION CODE	MEDIUM AND FEED CODES	INDUCTION TIME (H AFTER INOC.)	FERMENTER USED	REMARKS
F1	A1/A2	7	7 L Applikon	Trial
F2	A1/A2	7	7 L Applikon	Trial
F3	B1/B2	7	7 L Applikon	
F4	B1/B2	7	7 L Applikon	Duplicate
F5	B1/B2	0	7 L Applikon	
F6	B1/B2	21	7 L Applikon	
F7	B1/B2	21	7 L Applikon	Duplicate
F8	B1/B2	42	7 L Applikon	
F9	C1/C2	21	7 L Applikon	
F10	C1/C2	21	7 L Applikon	Duplicate
F11	D1/D2	21	7 L Applikon	
F12	D1/D2	21	75 L LH	
F13	C1/C2	21	7 L Applikon	On-line Biomass Monitor
F14	B1/B2	7	7 L Applikon	Contaminated

Table 3.1 Description of fermentation runs.

3.1 Initial Fermentations.

As stated in section 2.2.1 of this report, the majority of fermentations were performed in the 7 L Applikon vessel. The first run in this vessel (F1) was a short one (45 hours), the aim was to become familiar with the new fermenter and its operation. The vessel was not logged to the RTDAS system during the first run and therefore no on-line data was available for that fermentation.

Although the following fermentation performed in this vessel (F2) had only partial on-line data and no % DOT and stirrer speed profiles were available, CER, OUR and RQ profiles were produced, together with the off-line estimation of biomass and scFv levels. This and the previous fermentation were performed using the initial growth medium and feed (A1 and A2).

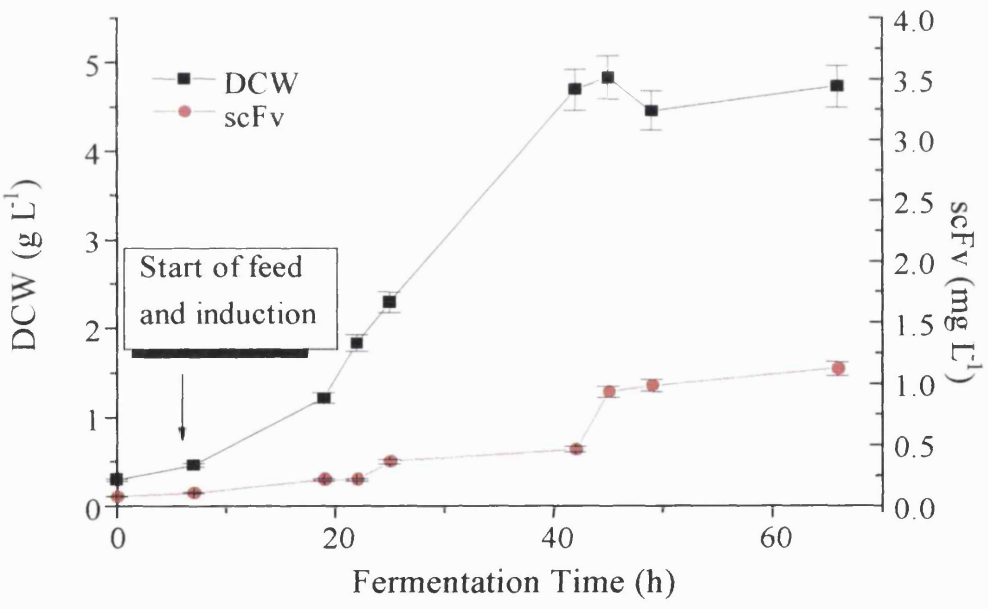


Fig. 3.1 Biomass and scFv estimation for F2.

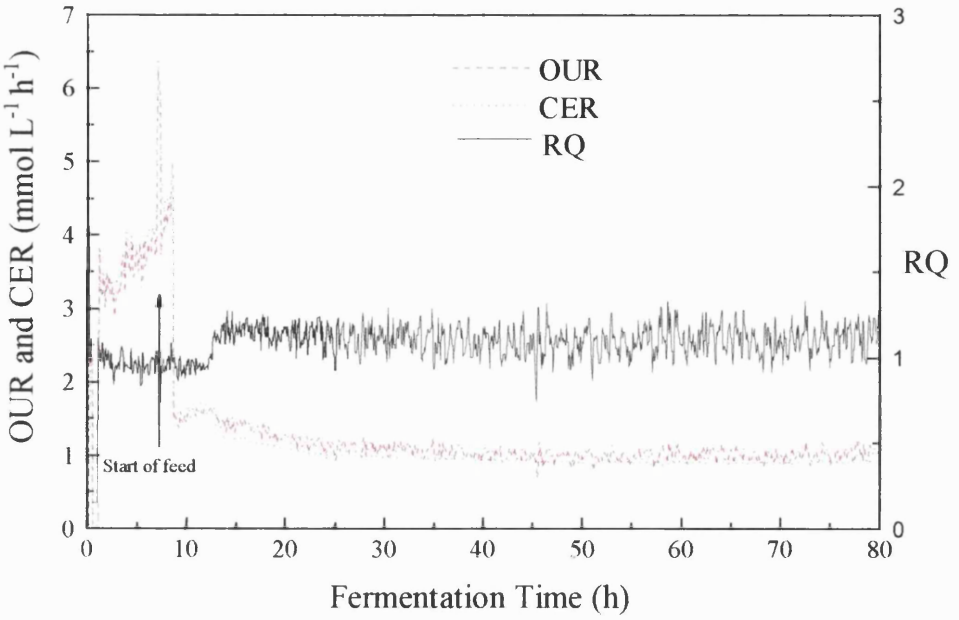


Fig 3.2 Respiration profile for F2.

These data provide the first indication for the production of the antibody fragment from *A. awamori*. The level produced was 1.5 mg L^{-1}. This value is much lower than the values obtained up to that point by other researchers from either *E. coli* or *A. awamori* fermentations (Boulding, 1996, Harrison, 1996). Biomass levels were comparable at approximately 5 g L^{-1} DCW.

The graphs on the following page show the profiles for the first fermentation performed with the "old medium" and feed (B1 and B2). This fermentation was induced 7 hours after inoculation, following the original protocol. The main difference of this medium from A1/A2 is that it contains a major carbon source (fructose) at the start of the fermentation. It also contains 2 g L^{-1} $(NH_4)_2SO_4$ and the concentration of fructose in the feed is higher (32.3 g L^{-1} compared to 6.67 g L^{-1} previously).

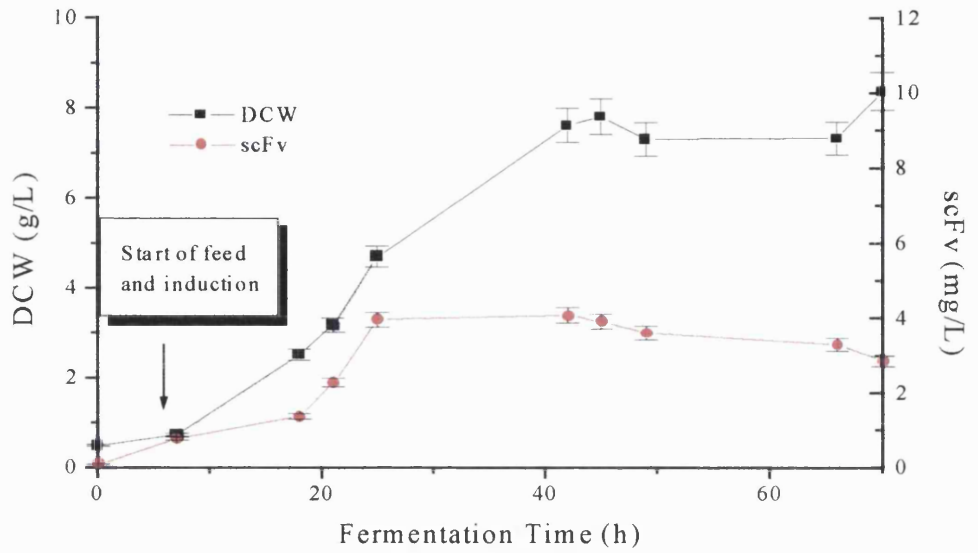


Fig. 3.3 Biomass and scFv estimation for F3.

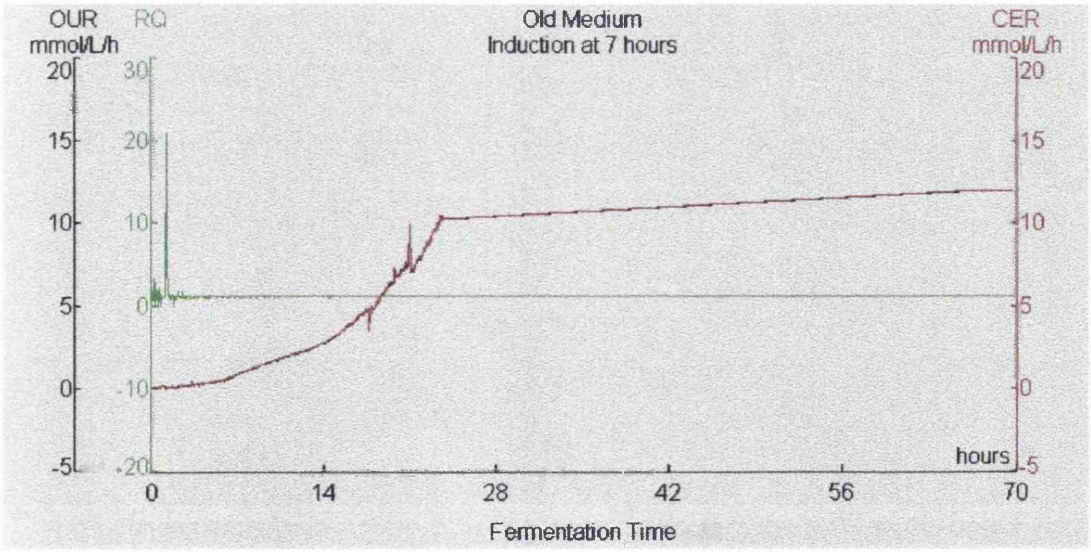


Fig. 3.4 Respiration profile for F3.

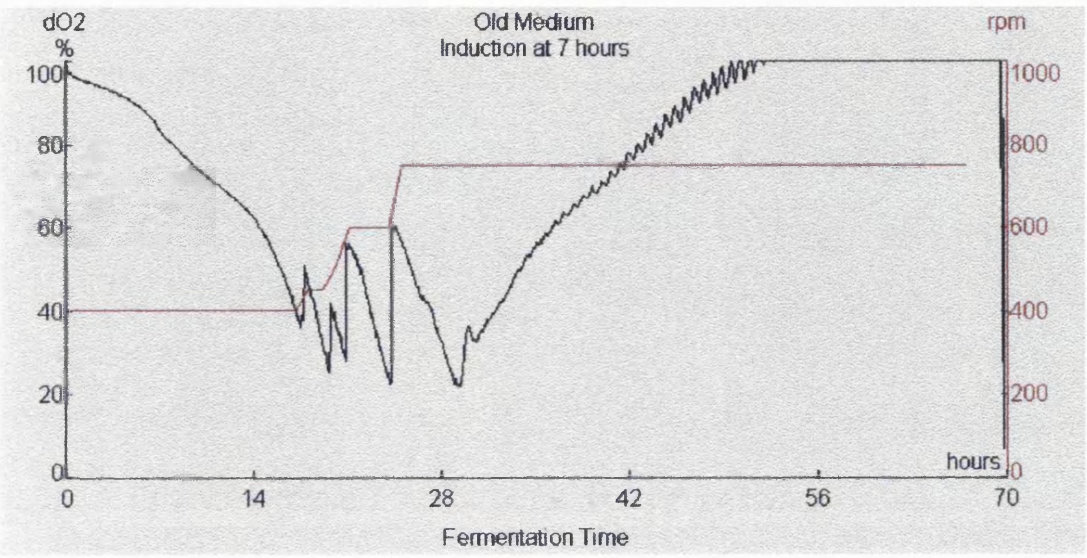


Fig. 3.5 DOT and agitation profiles for F3.

The presence of fructose from the start of the fermentation resulted in a sharp drop in the % DOT values from the beginning of the run. There was also an increase in biomass levels to 8 g L^{-1} DCW. This was followed by an increase in the scFv levels to 4 mg L^{-1} at 42 hours after inoculation. Finally, the diameter of the mycelial clumps was visibly smaller than those obtained with medium A1/A2 (Table 3.2, p. 83).

The following fermentation was performed using the same medium (B1/B2) and under the same conditions and had as an aim to investigate the reproducibility of the results that were obtained with the enhanced medium. The profiles from this fermentation are shown below.

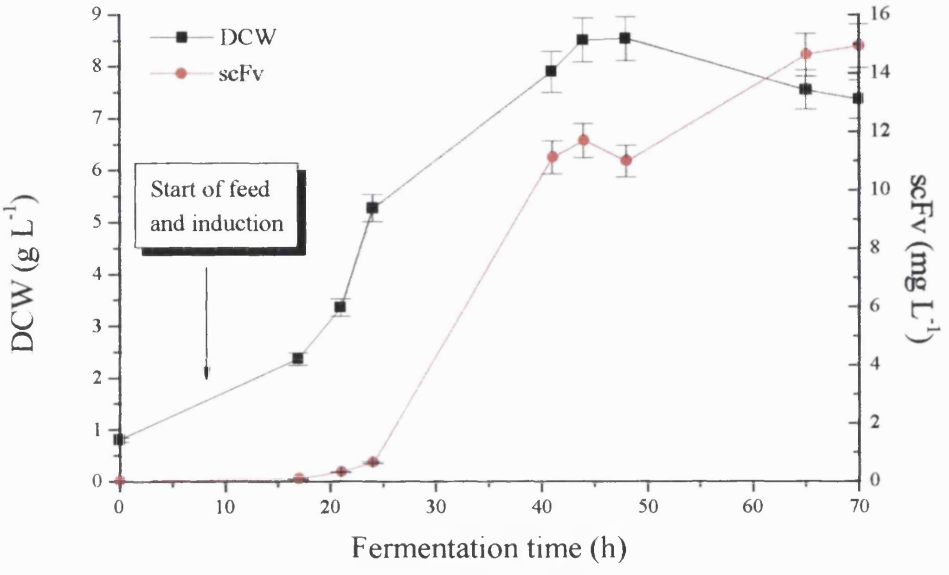


Fig. 3.6 Biomass and scFv estimation for F4.

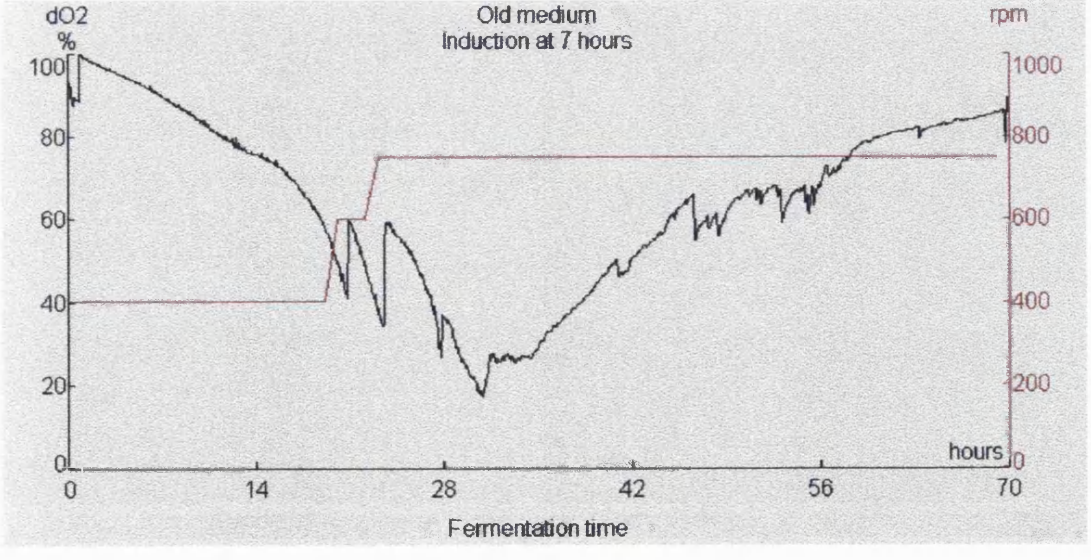


Fig. 3.7 DOT and agitation profile for F4.

The graphs obtained from this fermentation show that the two runs have given reproducible results with respect to the DOT profile and the biomass. Maximum biomass was again 8.5 g L^{-1} DCW. Oxygen saturation levels also started decreasing from the start of the fermentation. However, scFv concentration was found to be 14.8 mg L^{-1}. The increase in the product concentration as compared to the previous run is due to the acquisition and use of the correct standard for the ELISA. It was therefore decided that the values obtained from this run would be used as representative for this set of conditions. Respiration profiles for this run were not included due to the fact that the mass spectrometer was not in operation at the time.

3.2 Induction Time.

3.2.1 Induction at 0 hours.

The next fermentation employed the same growth medium (B1/B2) but a different induction strategy; the inducer was fed to the culture from the start through a separate port, as described in the 'Materials and Methods' section of this report. The profiles from this fermentation are provided on the following pages.

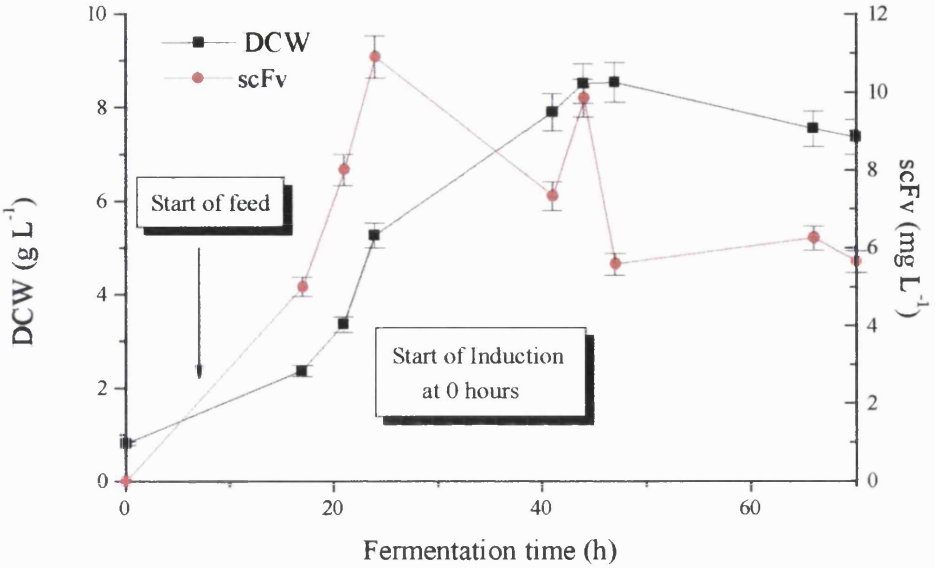


Fig. 3.8 Biomass and scFv estimation for the run induced 0 hours after inoculation (F5).

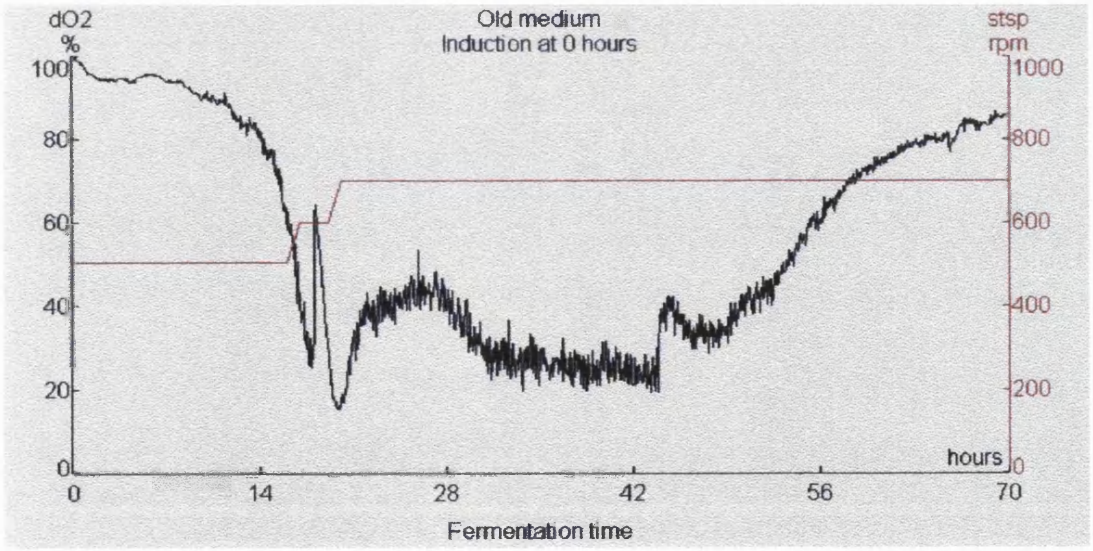


Fig. 3.9 DOT and agitation profile for the run induced 0 hours after inoculation (F5).

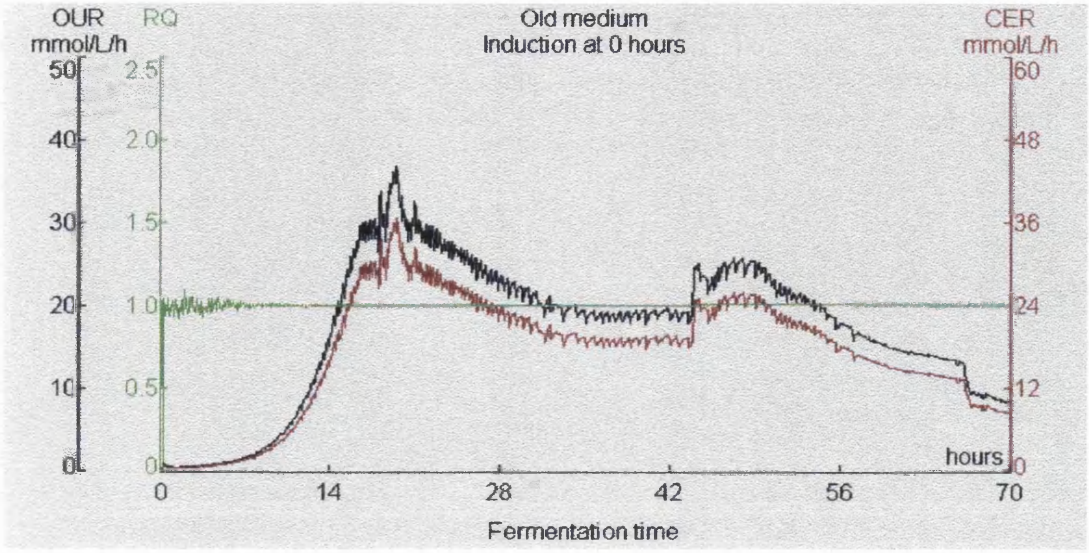


Fig. 3.10 Respiration profile for the run induced 0 hours after inoculation (F5).

The main observation from this run was that the organism entered the stationary phase several hours earlier than the other fermentations, a fact that is visible from the respiration profile. It was also noted that the concentration of the product peaked at a much earlier stage of the fermentation as compared to the other induction times. Maximum scFv concentration observed was 11 mg L^{-1} at 21 hours after the start of the fermentation. Biomass was again 8.5 g L^{-1}.

3.2.2 Induction at 21 hours.

The next selected time of induction was at the middle of the exponential phase of growth, 21 hours after inoculation. Research has shown that when the expression of the product is low, the yield is maximized by inducing expression throughout the entire growth phase (Donovan *et al.*, 1996). The results from this fermentation are shown below.

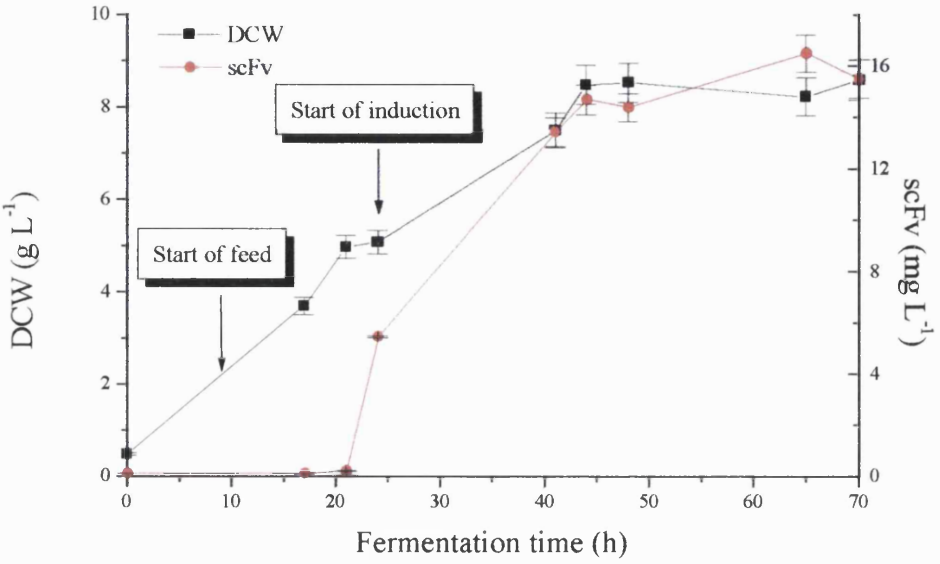


Fig. 3.11 Biomass and scFv estimation for the run induced 21 hours after inoculation (F6).

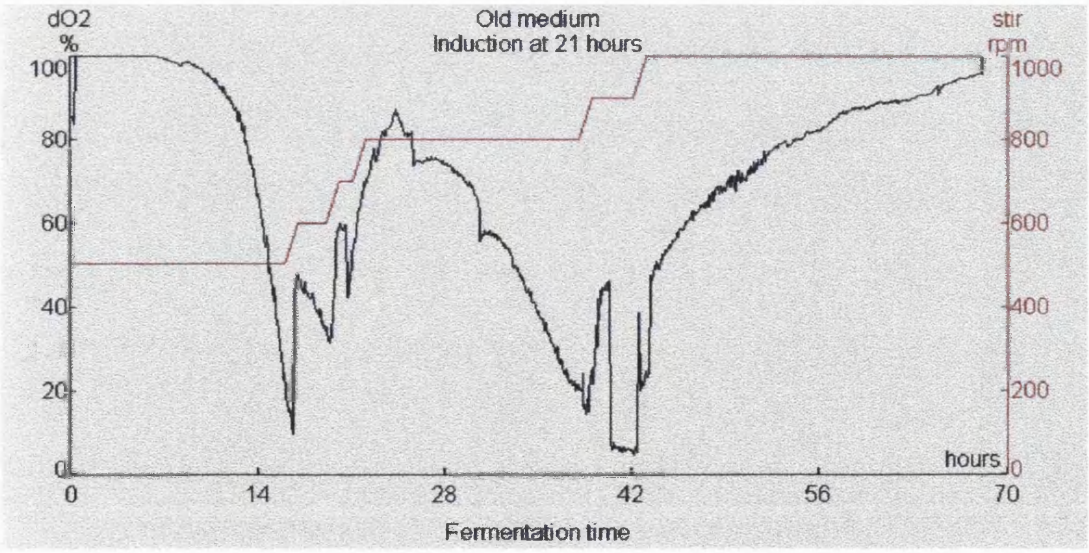


Fig. 3.12 DOT and agitation profile for the run induced 21 hours after inoculation (F6).

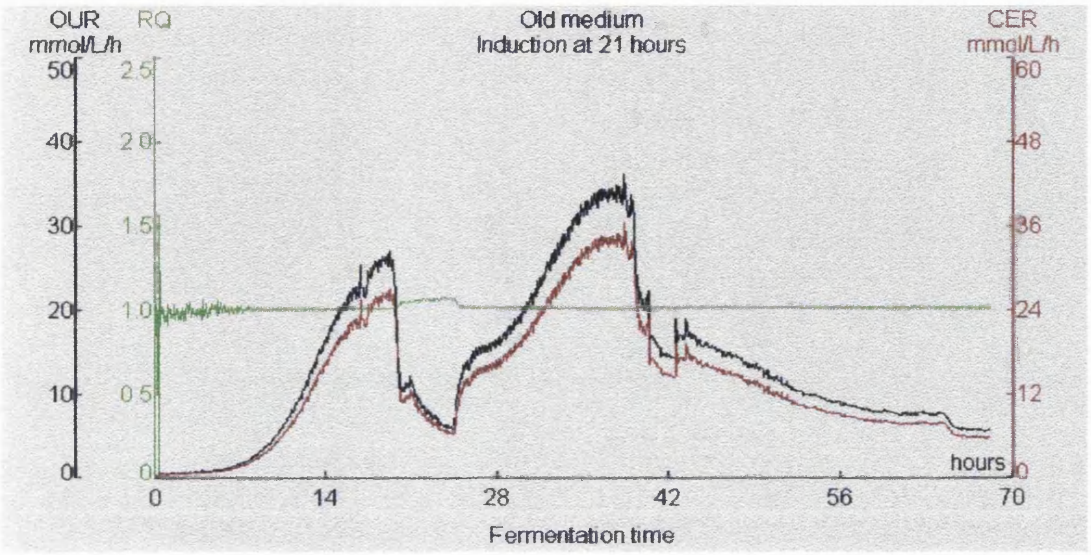


Fig. 3.13 Respiration profile for the run induced 21 hours after inoculation (F6).

Inducing the culture 21 hours after inoculation increased the concentration of the product from 14.8 mg L^{-1} to 16.5 mg L^{-1} after 48 hours of fermentation. In addition, the maximum scFv concentration was obtained at an earlier stage of the fermentation, as compared to the other induction times. This is beneficial as it means that the fermentation can be shortened, making in this way the upstream part of the process more economical. The fermentation employing the 21 hours induction time was repeated in order to establish the results. Data for this fermentation is shown on the following pages.

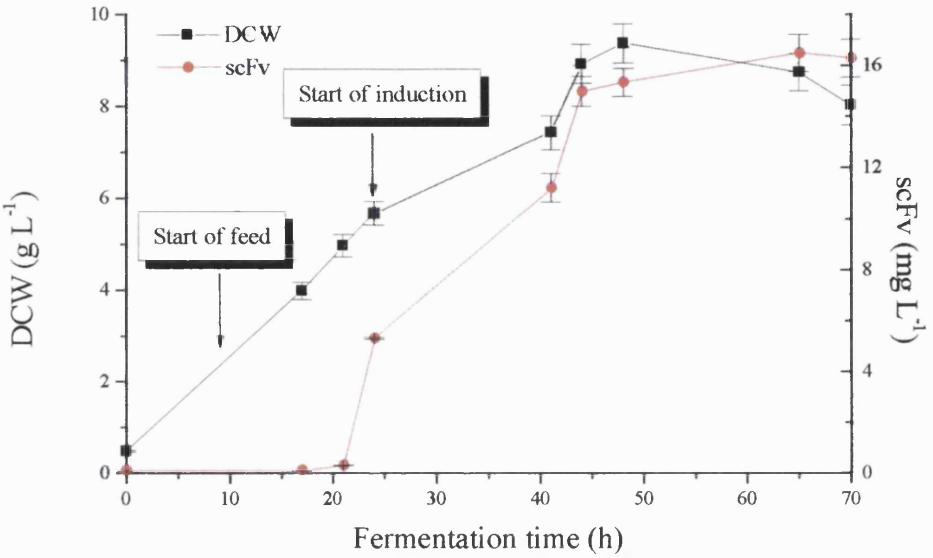


Fig. 3.14 Biomass and scFv estimation for the duplicate run induced 21 hours after inoculation (F7).

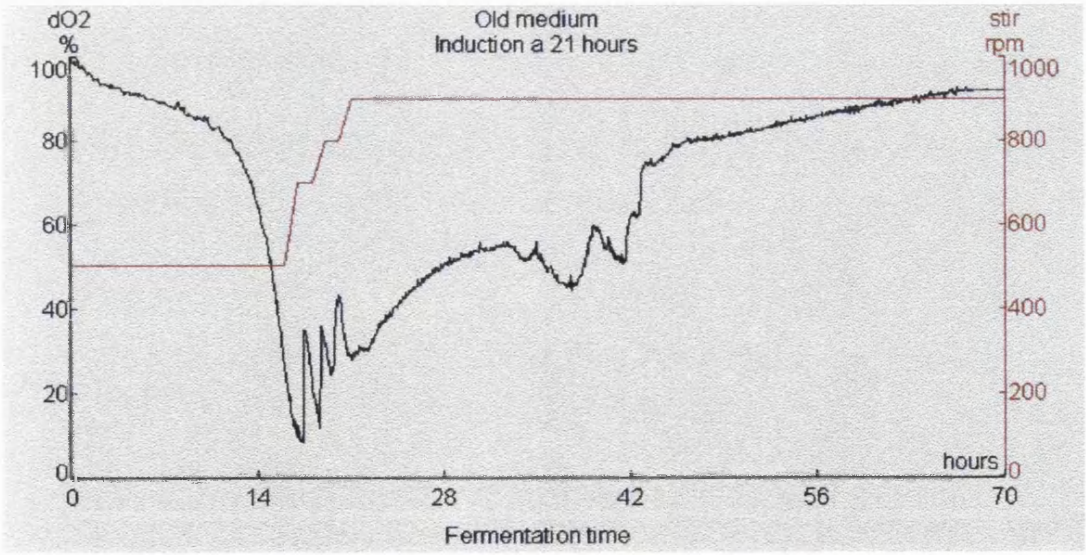


Fig. 3.15 DOT and agitation profile for the duplicate run induced 21 hours after inoculation (F7).

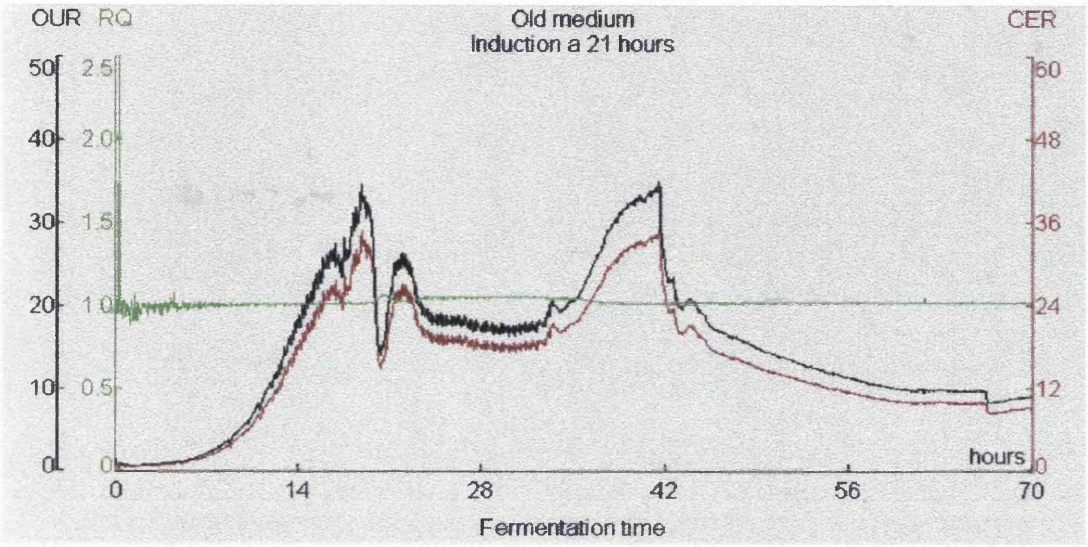


Fig. 3.16 Respiration profile for the duplicate run induced 21 hours after inoculation (F7).

The duplicate fermentation followed the same trend as the first one. Product concentration was more than the other induction times and it was also observed earlier. It is noted that OUR and CER traces follow a trend; there is a peak initially and then, immediately after the point of induction, a second peak. This leads to the possibility that xylose is used by the microorganism for growth as well as for induction.

3.2.3 Induction at 42 hours.

The last time for induction experiments chosen was at the beginning of the stationary phase, 42 hours after inoculation. Results from these experiments are shown below.

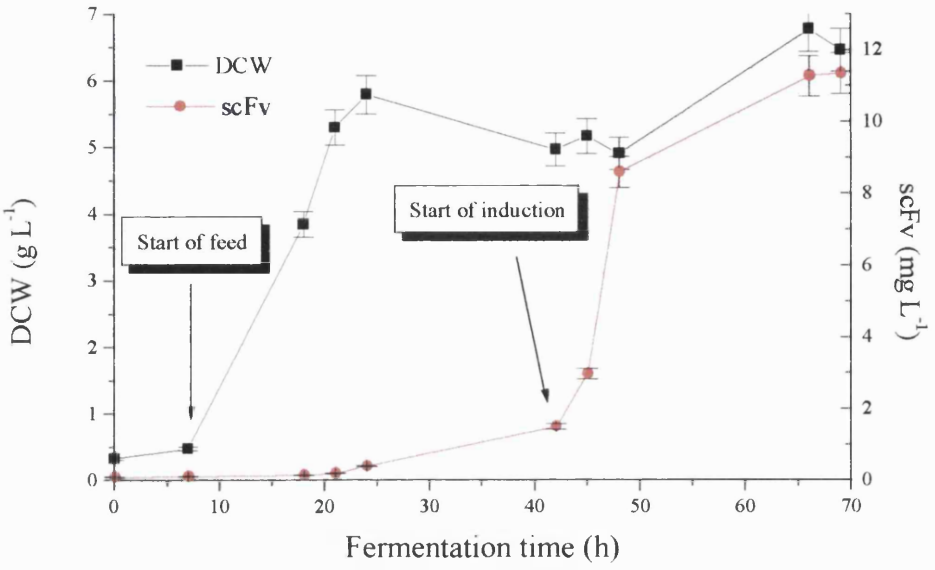


Fig. 3.17 Biomass and scFv estimation for F8.

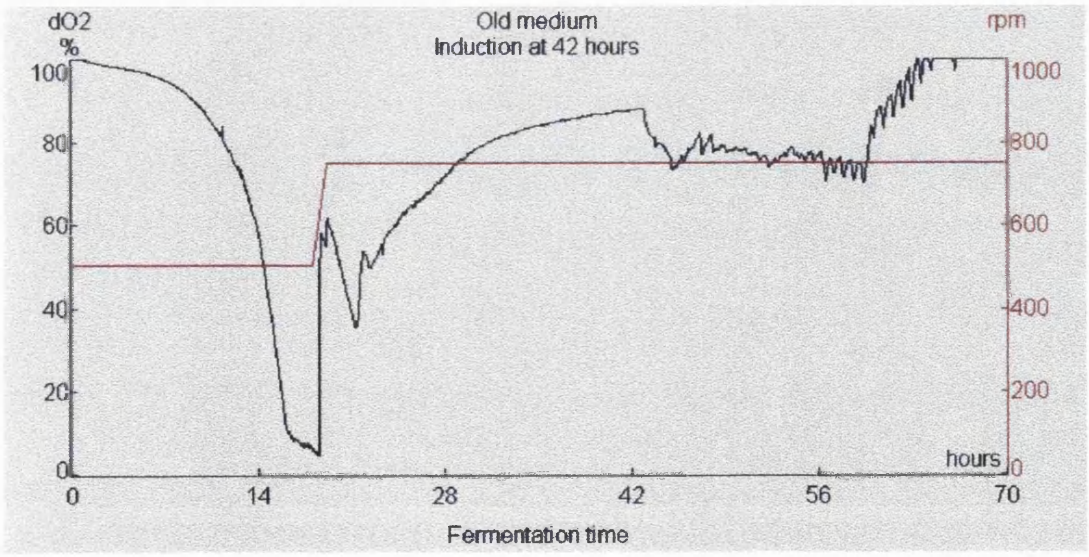


Fig. 3.18 DOT and agitation profile for F8.

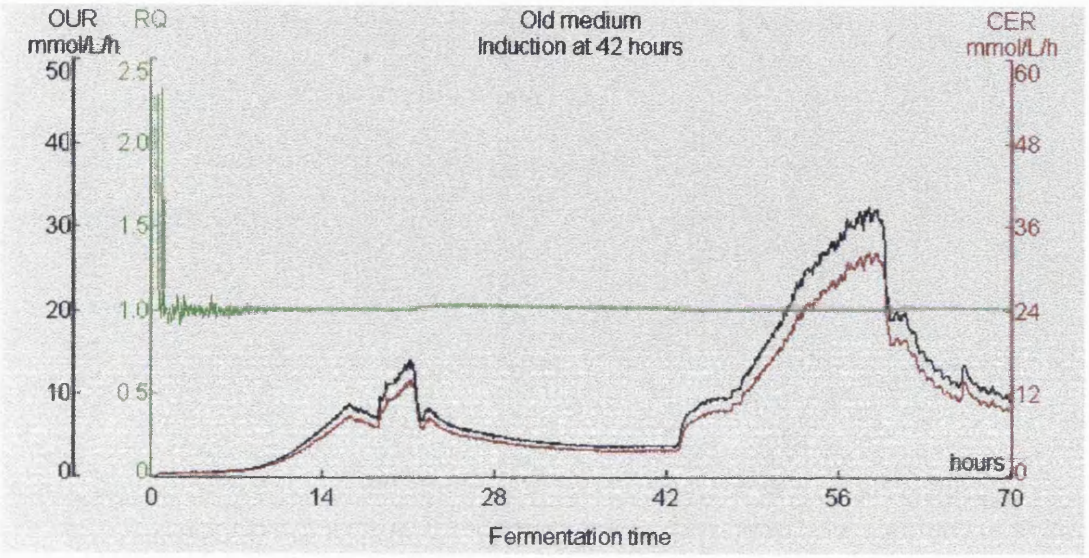


Fig. 3.19 Respiration profile for F8.

During this fermentation, the respiration profiles show two distinct peaks. One at the beginning of the fermentation (20 h), as in previous runs, and one after the start of induction (56 h). The same trend is shown by the biomass data; it increases up to 22 hours and then again after 45 hours, shortly after induction. Both biomass and scFv titres were less in comparison with the two previous runs, the concentration of the scFv antibody fragment reaching no more than 11.5 mg L^{-1}. Maximum product concentration was observed at a later stage of the fermentation, 69 hours after inoculation. The respiration profile and the biomass data suggest that *Aspergillus* was still growing at the end of the fermentation, though at a decreasing rate.

Based on the findings of the experiments with varying induction times, it was decided that the best results were obtained when the culture was induced at the middle of the exponential phase, 21 hours after inoculation and therefore that strategy was used for subsequent fermentations. Figure 3.20 summarizes the results from the different induction times.

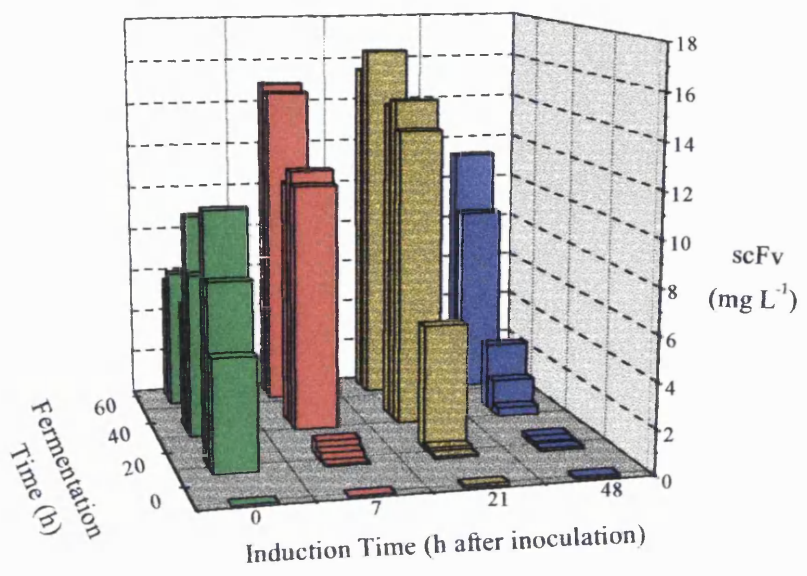


Fig. 3.20 Product concentration from fermentations with different times of induction initiation.

3.3 New Medium.

The composition of the growth medium can affect dramatically the concentration of the product. It was decided to perform a series of shaken flask fermentations with varying concentrations of the main carbon and nitrogen sources, fructose and ammonium sulphate, respectively. The results of this experiment are summarized in figure 3.21. For this and the other shaken flask experiments, a set of 14 identical, 500 ml, baffled flasks was used.

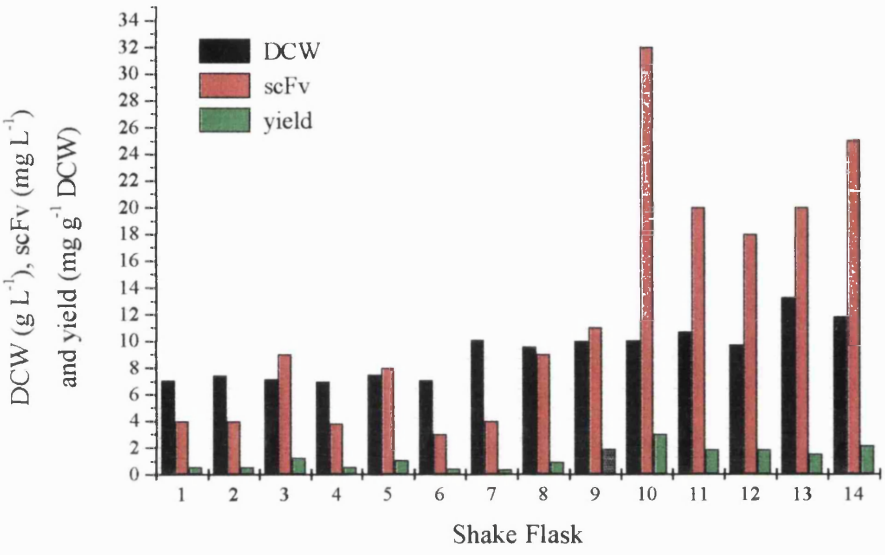


Fig. 3.21 Effect of fructose and ammonium sulphate concentrations on product yield (F34). 1-3 control; 4-6, **2*** fructose conc., **1*** ammonium sulphate conc.; 7-9, **1*** fructose conc., **2*** ammonium sulphate conc.; 10-12, **2*** fructose conc., **2*** ammonium sulphate conc.; 13-14, **3*** fructose conc., **3*** ammonium sulphate conc.

This experiment showed that when the concentration of both components was increased there was an increase in both dry biomass and scFv concentration. In order to confirm these results and also to decide which combination to scale-up to 7 L, the experiment was performed again using the double and triple concentrations of the two components. The results are shown in figure 3.22. There was a 300 % increase in titres and yield when the concentration of the two components was doubled. However, in the shaken flasks containing triple the concentration of these elements, no further enhancement of the product titre was detected. Based on these findings, the medium with double the concentrations of fructose and ammonium sulphate was scaled-up to 7 L fermentations, in order to confirm the results in the controlled environment of the reactor and compare them with the previous runs.

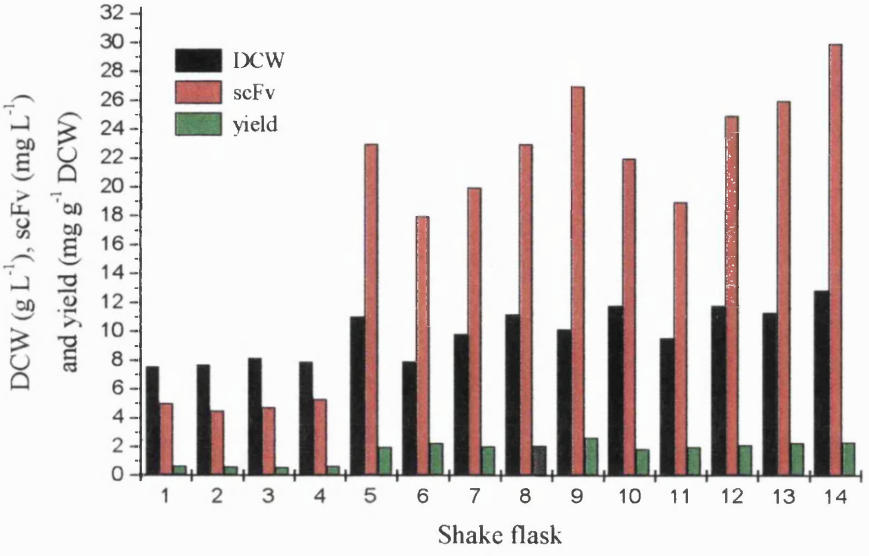


Fig. 3.22 Effect of fructose and ammonium sulphate concentrations on product yield (F35). 1-4 control; 5-9, **2*** fructose conc., **2*** ammonium sulphate conc.; 10-14, **3*** fructose conc., **3*** ammonium sulphate conc.

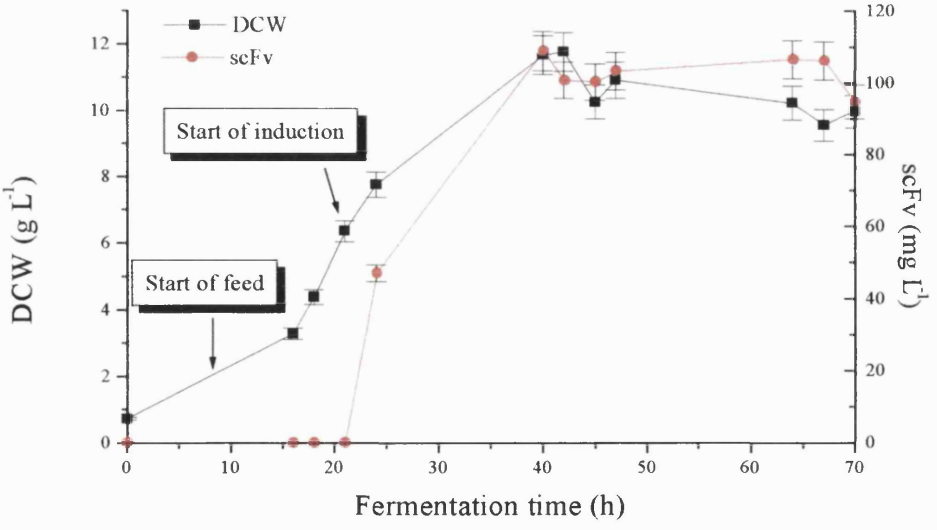


Fig. 3.23 Dry biomass and scFv estimation for the fermentation employing the "new medium" (C1/C2) (F9).

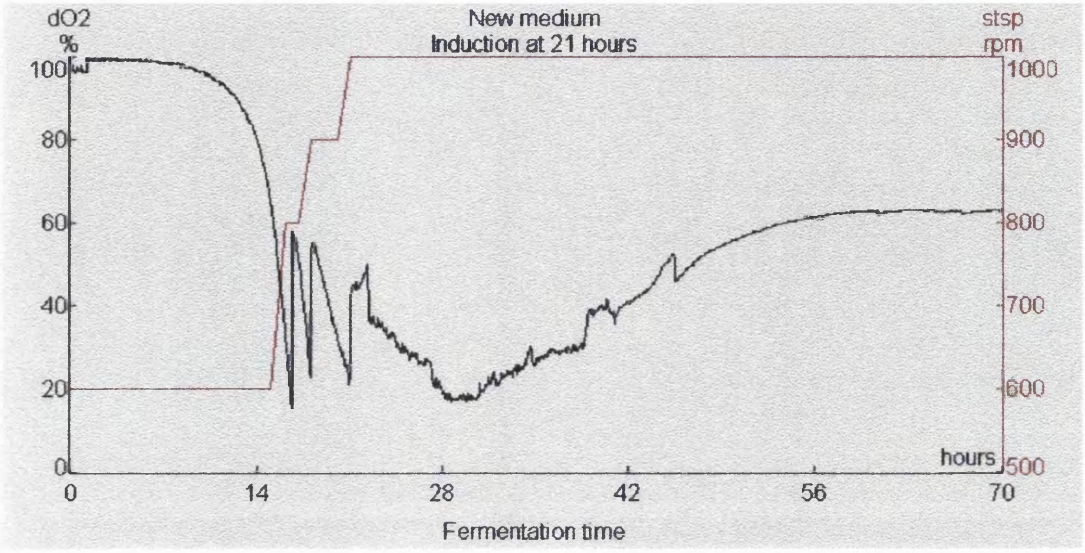


Fig. 3.24 DOT and agitation profile of the fermentation using the "new medium" (C1/C2) (F9).

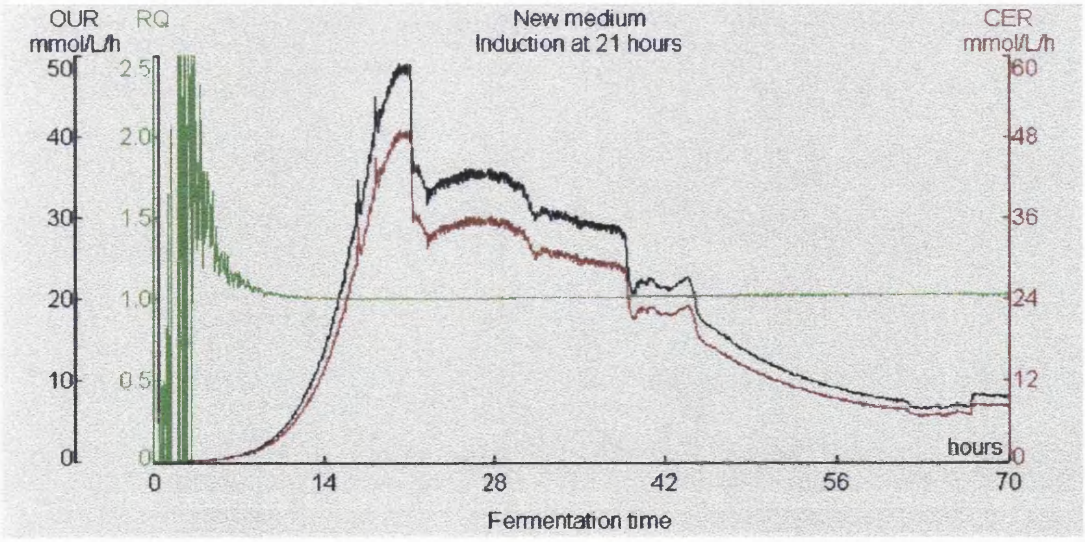


Fig. 3.25 Respiration profile of a fermentation using the "new medium" (C1/C2) (F9).

Figures 3.23, 3.24 and 3.25 summarize the results of the fermentation performed in the 7 L Applikon fermenter, using the "new medium" and feed (C1/C2).

Compared to the fermentations performed using the "old medium" (B1/B2), dry biomass was increased by 50 % to 12 g L^{-1} and scFv reached 105 mg L^{-1}, an increase of more than 750 % compared to runs F6 and F7. OUR and CER values were also increased to a maximum of 50 mmoles L^{-1} h^{-1}, indicating higher biomass production. As before, in order to verify the results, the fermentation was repeated. The results of the duplicate fermentation are shown in figures 3.26, 3.27 and 3.28.

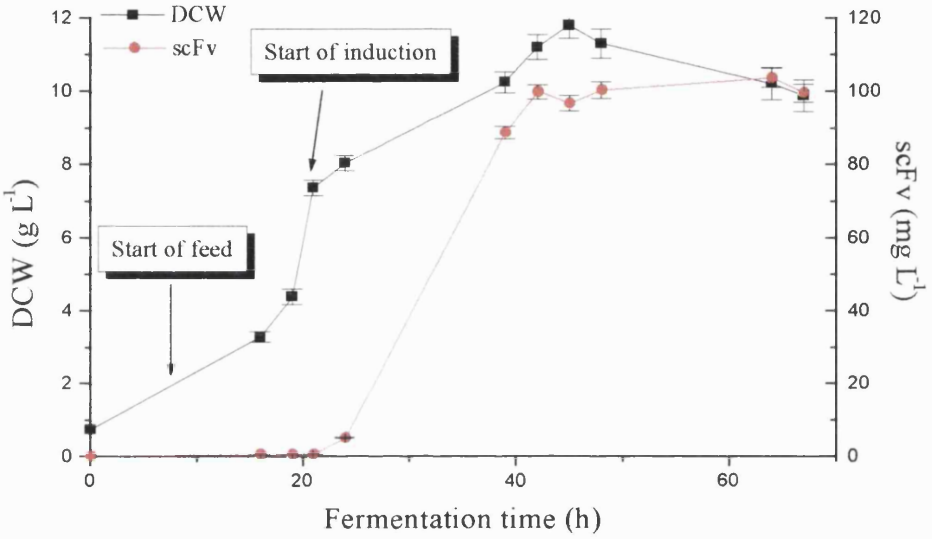


Fig. 3.26 Dry biomass and scFv concentration from second fermentation with "new medium" (C1/C2) (F10).

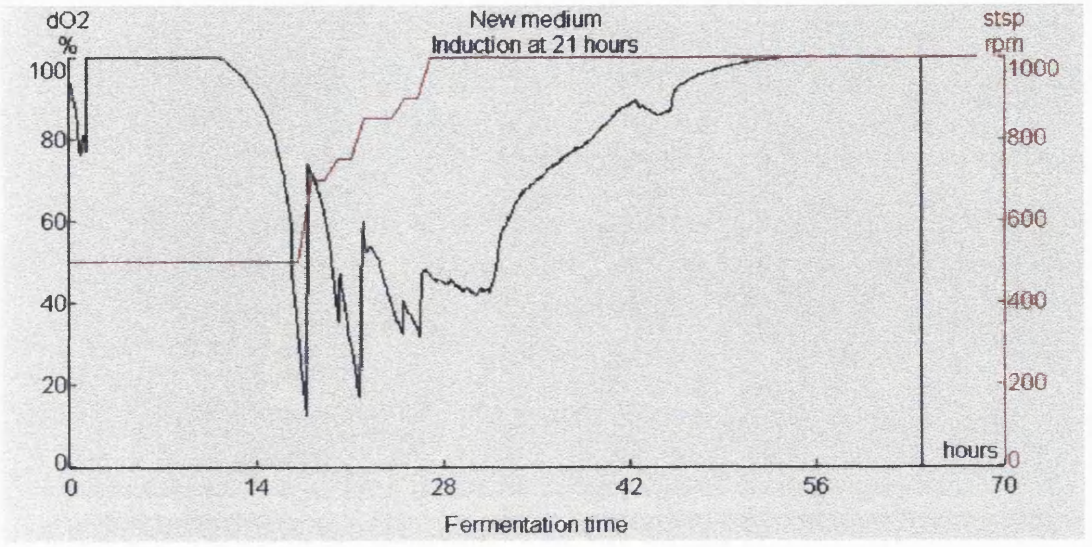


Fig. 3.27 DOT and agitation profile of the second fermentation using the "new medium" (C1/C2) (F9).

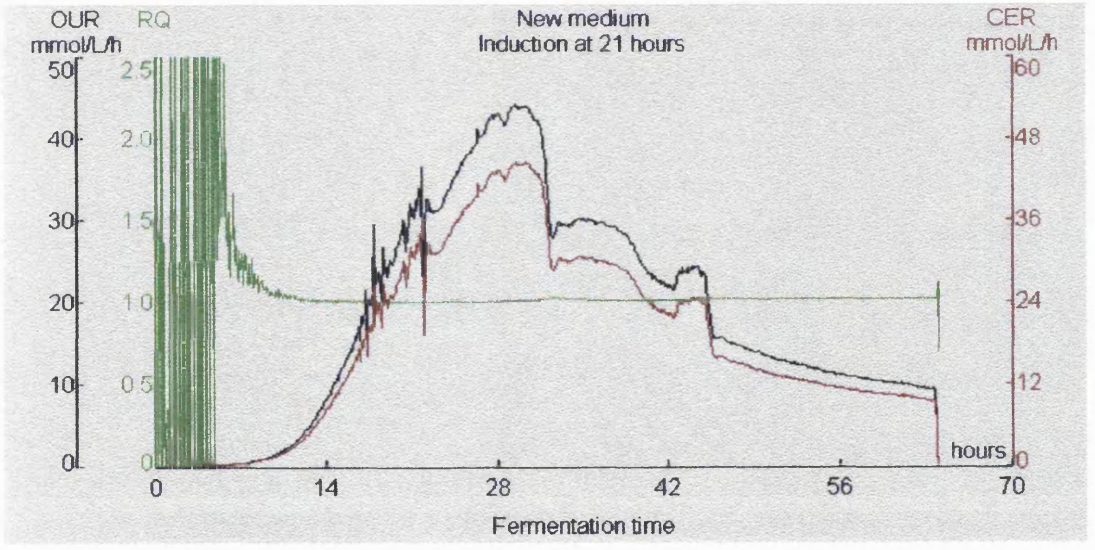


Fig. 3.28 Respiration profile from second fermentation with "new medium" (C1/C2) (F10).

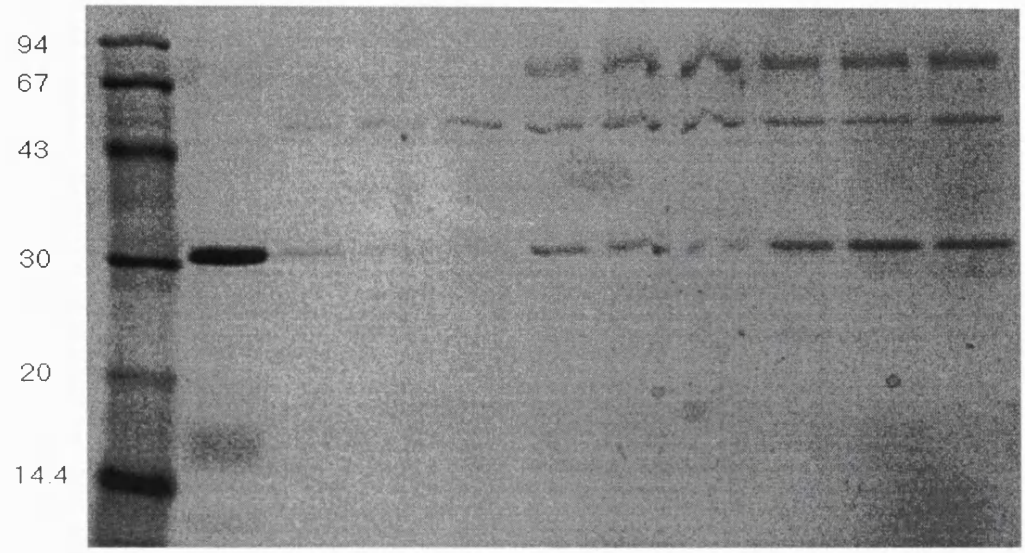


Fig. 3.29 SDS-PAGE gel of a fermentation using the "new medium" (C1/C2) (F10). Bands (from left to right): Low molecular weight marker, scFv standard, 16 h, 21 h, 24 h, 40 h, 43 h, 45 h, 48 h, 64 h, 68 h. The protein evident at the top of the gel is glucoamylase. Molecular weights of the marker are in kDa, as shown on the left hand side of the gel.

The SDS-PAGE gel confirmed the production of scFv.

The second fermentation using the "new medium" (C1/C2) confirmed the results of the first one with respect to dry biomass, scFv, DOT and respiration profiles. It was therefore decided to use the new medium for further fermentations. Figures 3.30 and 3.31 show a comparison between the results obtained from the "old" and "new" growth media.

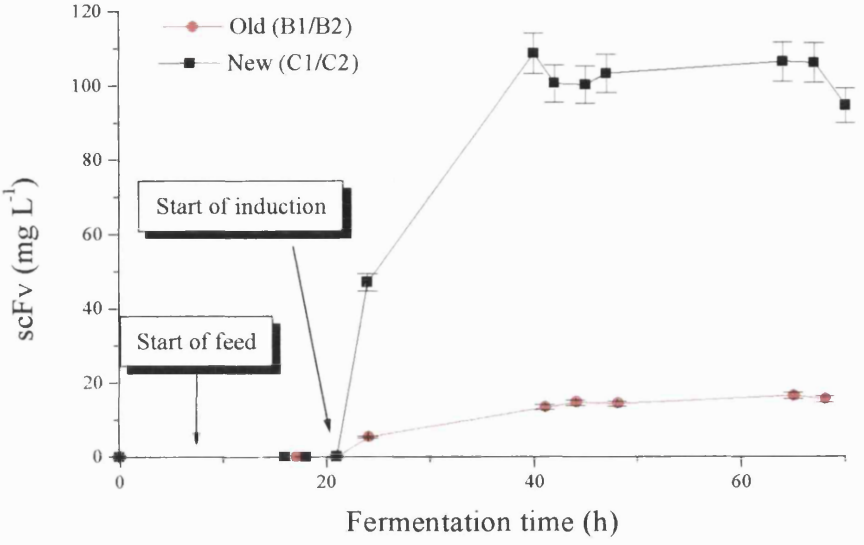


Fig. 3.30 Comparison of scFv concentration from the two different growth media (runs F6 and F9).

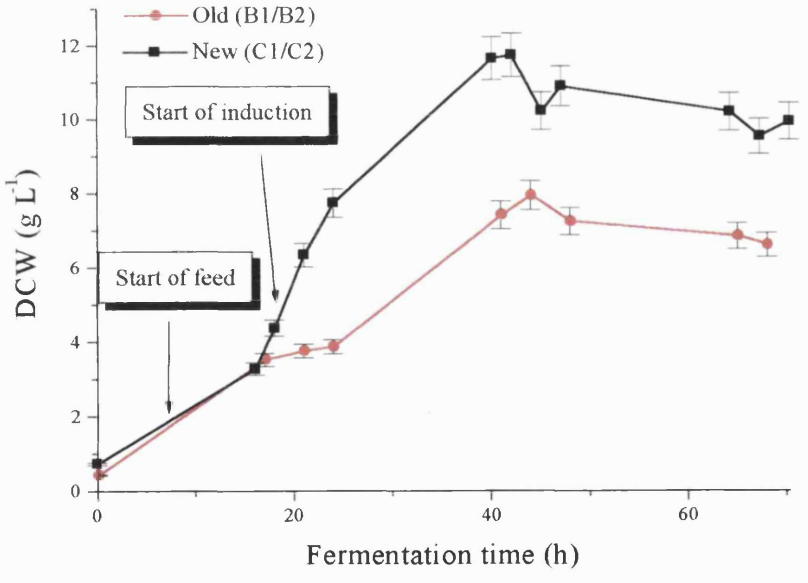


Fig. 3.31 Dry cell weight comparison between the two different growth media (runs F6 and F9).

3.4 CARBON CONSUMPTION.

To investigate whether there is residual carbon in the fermentation when the new medium was used, an enzymatic fructose assay and a carbon analyzer were used to analyze fermentation samples. The results are summarized in figure 3.32.

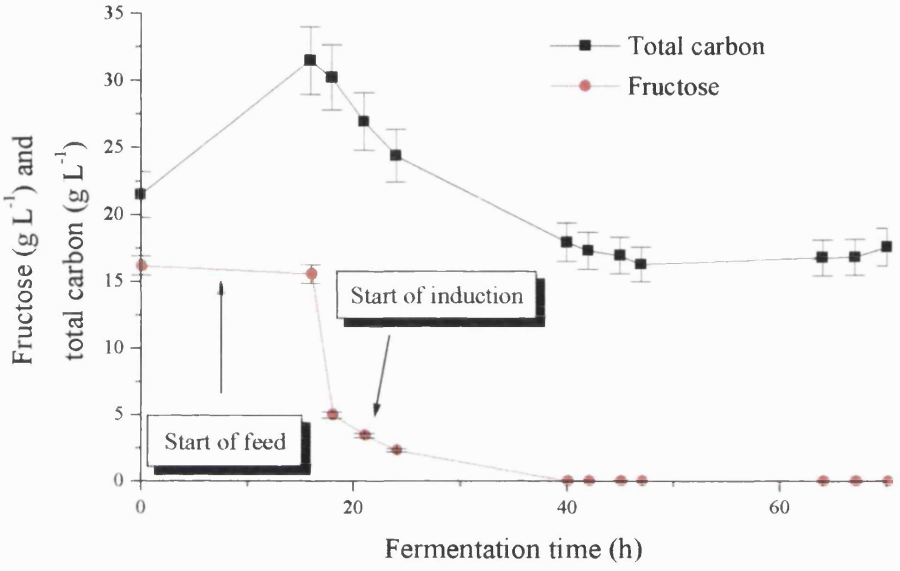


Fig. 3.32 Carbon consumption from fermentation using the "new medium" (C1/C2).

From the graph it can be seen that the fructose is totally consumed after 30 hours of fermentation. Also from the results from the carbon analyzer, it is obvious that there is some carbon left in the medium. A small proportion of this (less than 5 %) is from the yeast extract, but the majority may be attributed to the xylose used as the inducer. A DNS test confirmed the presence of a carbon source in the form of a reducing sugar, probably xylose. This lead to an experiment to see whether the concentration of the antibody would remain the same when a reduced concentration of xylose is used in the medium.

3.5 INDUCER CONCENTRATION.

To investigate the effect of the inducer concentration on the product formation, shaken flask experiments were carried out with varying concentrations of xylose in the medium (from 0 g L^{-1} to 64 g L^{-1}). The results are shown in figure 3.33.

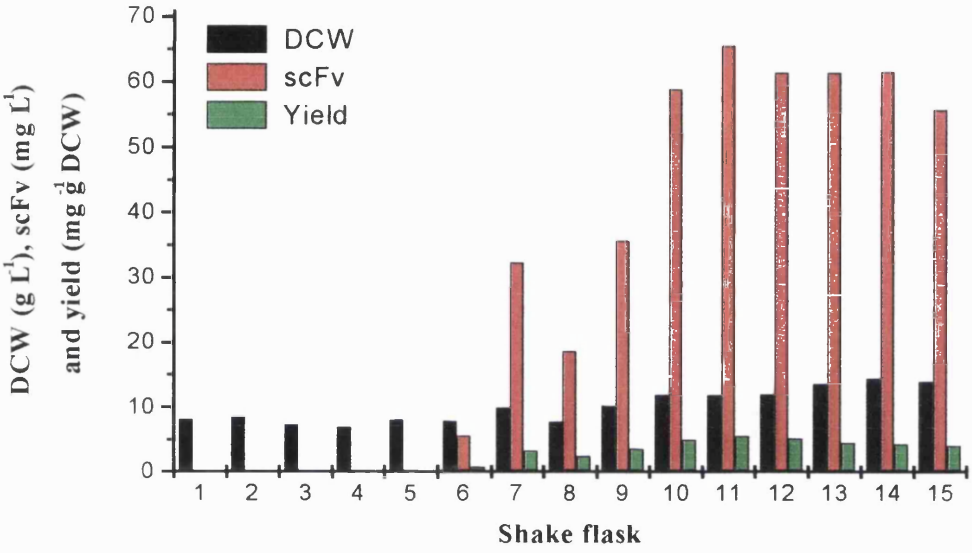


Fig. 3.33. Effect of inducer concentration on product yield (F36). 1-3 control (no xylose); 4-6, **1** g L^{-1} xylose; 7-9, **16** g L^{-1} xylose; 10-12, **32** g L^{-1} xylose and 13-15, **64** g L^{-1} xylose.

It was evident that there was no significant difference in the titres of the product when xylose concentration was halved, although the biomass concentration showed a 10 % reduction. This together with the findings of the previous experiments with fructose consumption led to the design of a new fermentation process where half the original xylose concentration was used and a step-feed was employed. The results of this fermentation are shown in fig. 3.34.

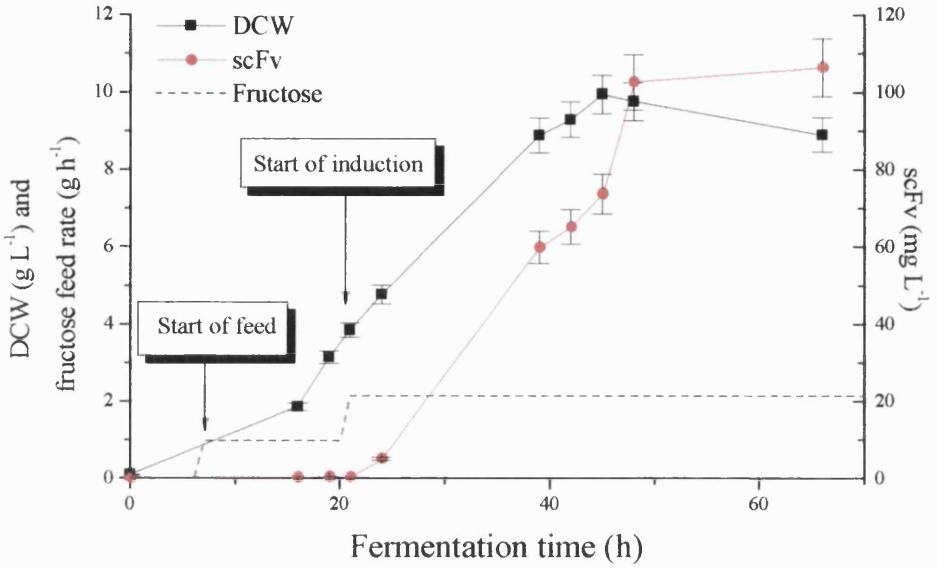


Fig. 3.34 Biomass and product concentration from a fermentation (F11) using half the original xylose concentration and a step-feed of fructose (medium D1/D2).

The concentration of the scFv remained the same as before (with the constant feed and the higher inducer concentration).

3.6 SCALE-UP.

The improved fermentation strategy consisting of the "improved medium" (D1/D2), step-feeding, induction at the middle of the exponential phase hours and half the initial concentration of inducer, was employed in scale-up studies to investigate its efficiency at a larger scale and identify possible problems and limitations. Scale-up calculations were made on the basis of keeping constant the power consumption per unit volume. The calculations performed are shown in the "Materials and Methods" section. Figures 3.35, 3.36, 3.37 and 3.38 summarize the findings of the pilot scale run.

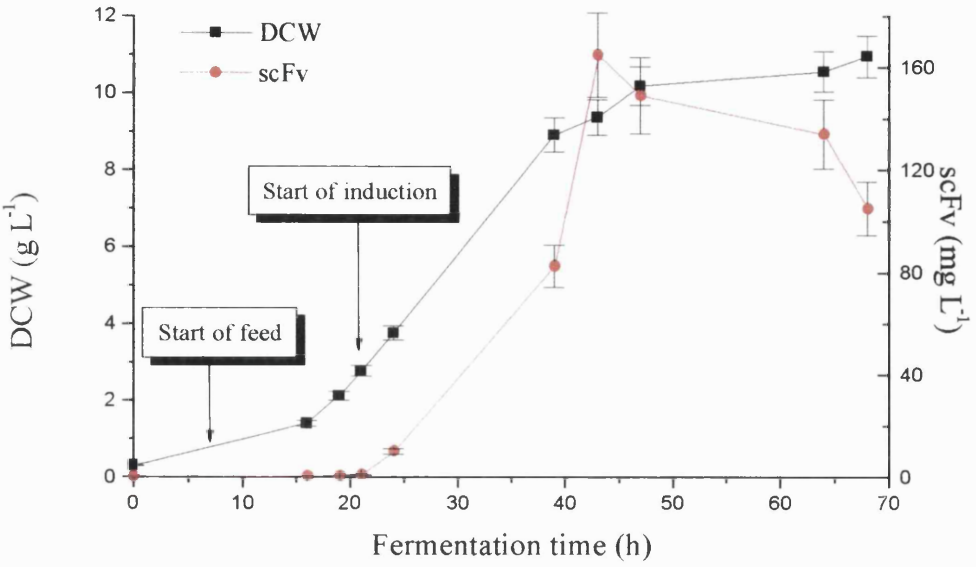


Fig. 3.35 Dry biomass and scFv concentrations from pilot scale fermentation with medium D1/D2 (F12).

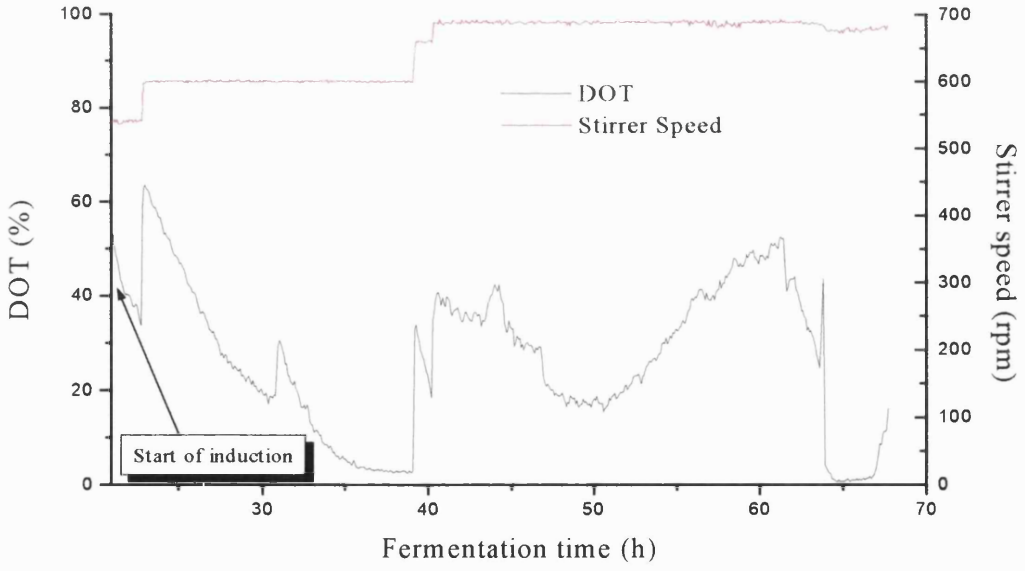


Fig. 3.36 DOT and agitation profiles from pilot scale fermentation with medium D1/D2 (F12). Missing data at the beginning of the fermentation is due to the malfunction of the data logging software.

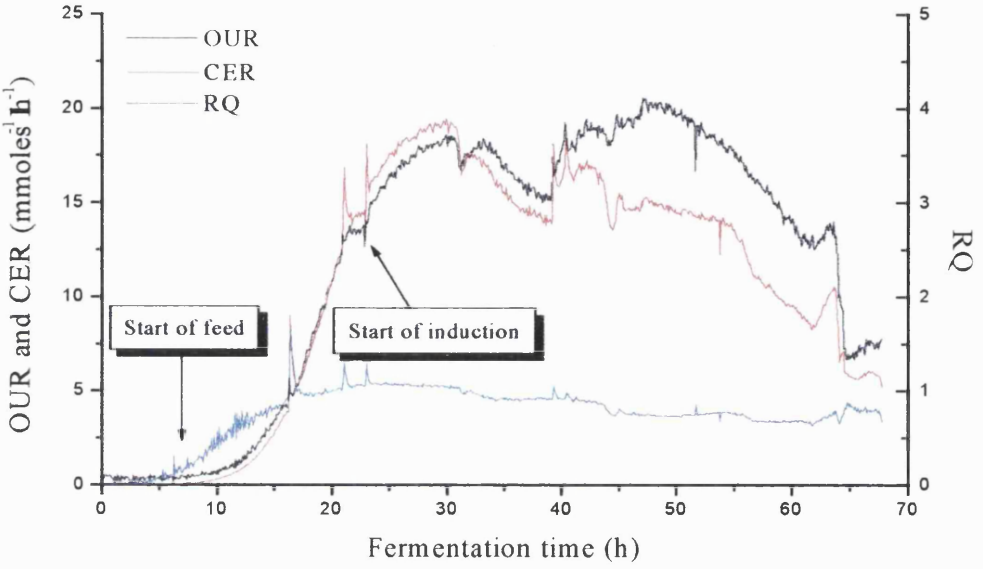


Fig 3.37 Respiration profile from pilot scale fermentation with medium D1/D2 (F12).

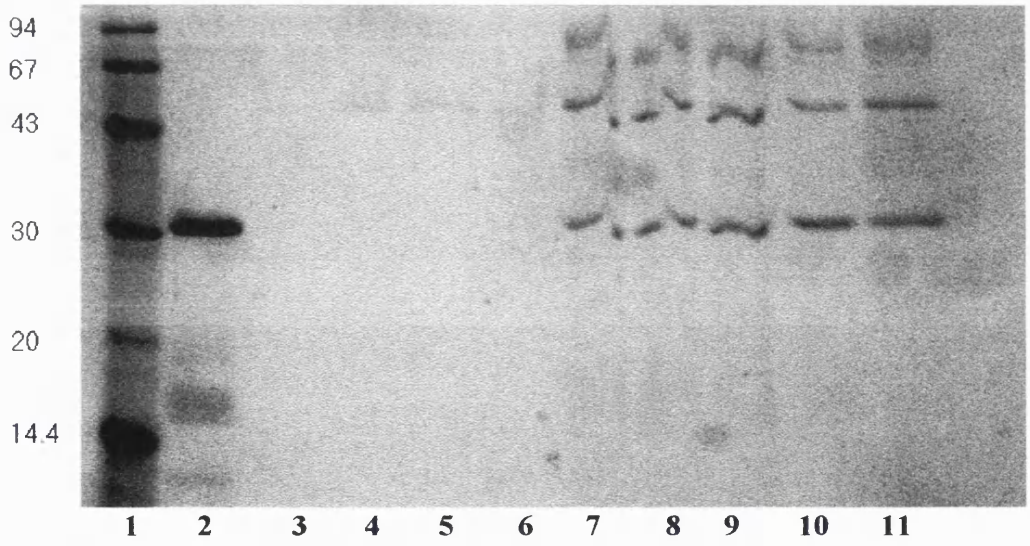


Fig. 3.38 SDS-PAGE gel of pilot scale fermentation using medium D1/D2 (F12). Bands (from left to right): Low molecular weight marker, scFv standard, 16 h, 19 h, 21 h, 24 h, 39 h, 43 h, 48 h, 64 h, 68 h. The protein evident at the top of the gel is glucoamylase. Molecular weights of the marker are in kDa, as shown on the left hand side of the gel.

The results indicated that the scale-up was successful. Although oxygen uptake rate was lower than the small scale, dry biomass was comparable and the maximum concentration of the product was increased by almost 50 % to 160 mg L^{-1} after 42 hours of fermentation (fig. 3.35). Also the SDS-PAGE gel showed that there was scFv secreted in the fermentation broth. A direct comparison of the two scales of operation with respect to product concentration and specific yields is presented on figures 3.39 and 3.40.

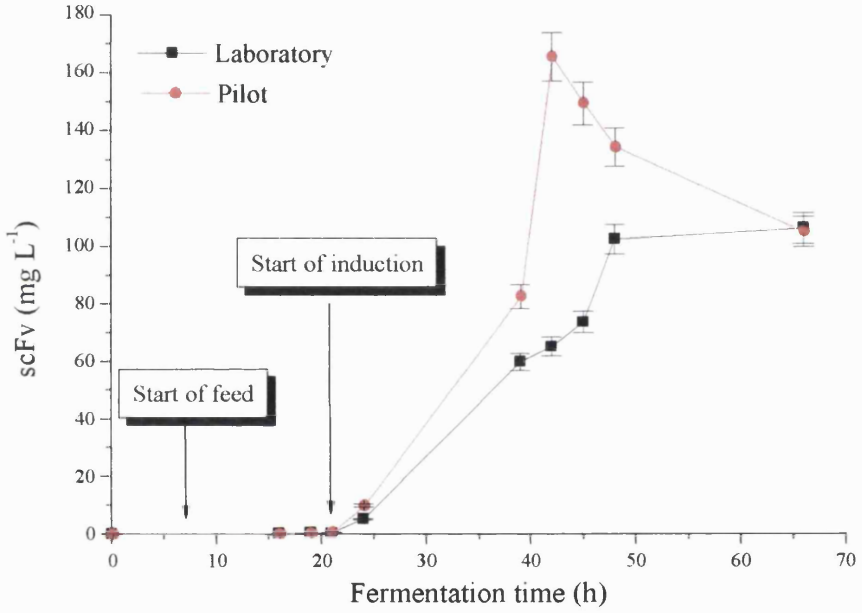


Fig. 3.39 Comparison of product concentration between laboratory and pilot scales (Runs F11 and F12).

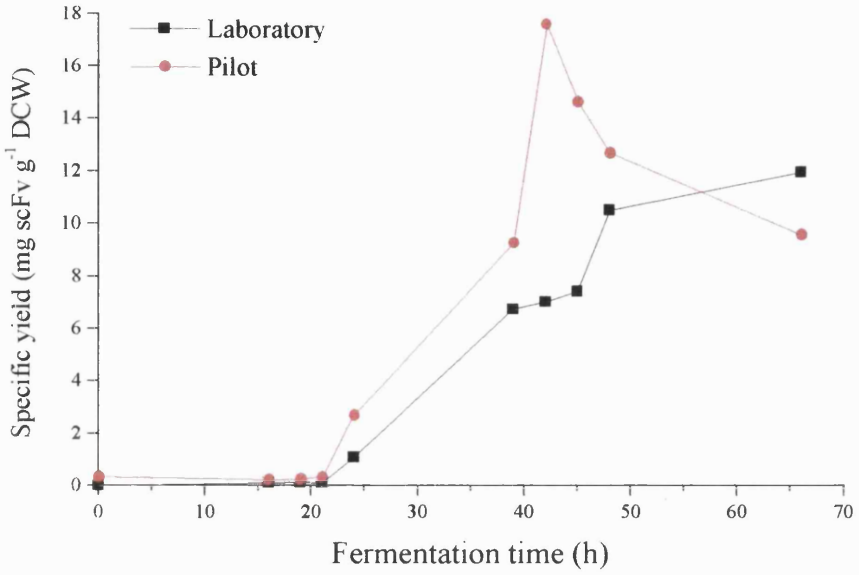


Fig. 3.40 Comparison of specific yield between laboratory and pilot scales (Runs F11 and F12).

3.7 PROTEASES.

As mentioned before, *Aspergillus* is known to produce extracellular proteases that can degrade the product of the fermentation. In order to investigate this possibility a series of experiments were carried out, first to establish the production of proteases from this particular strain of *Aspergillus*, under the adopted fermentation conditions and then to investigate the effect they have on the scFv. Figure 3.41 shows a comparison of the activities of the proteases produced during laboratory and pilot scale fermentations employing growth medium D1/D2.

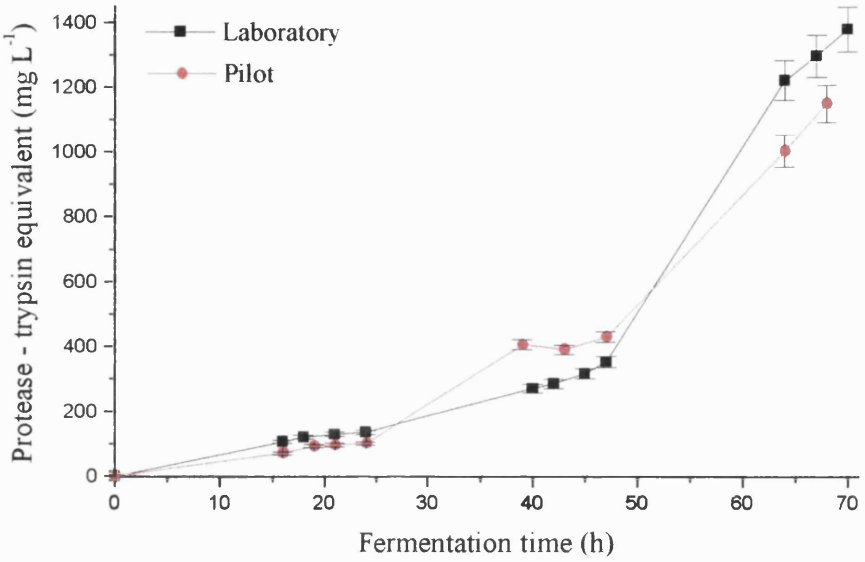


Fig. 3.41 Protease concentrations (equivalent to trypsin) from laboratory and pilot scale fermentations induced at 21 hours and using the growth medium D1/D2.

The protease test performed on the fermentation samples confirmed the presence of proteases in the broth. To investigate the effect of proteases on the product, a storage experiment was performed, as described in the "Materials and Methods" section (2.11, p. 48). The results of this experiment are summarized in figures 3.42 and 3.43.

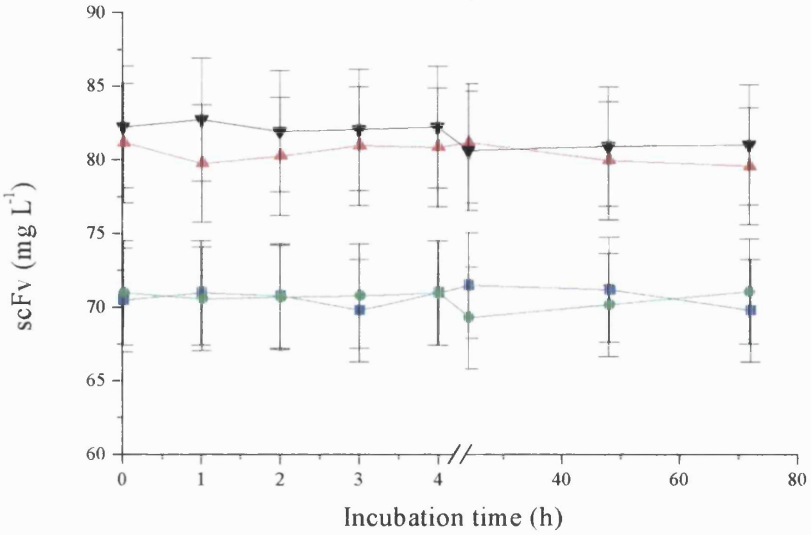


Fig. 3.42: ScFv concentration after incubation at 4 °C. ●: scFv in buffer, ■: scFv in sterile fermentation medium; ▲: scFv in fermentation broth containing proteases; ▼:scFv in fermentation broth containing proteases and 1 mg L^{-1} PMSF.

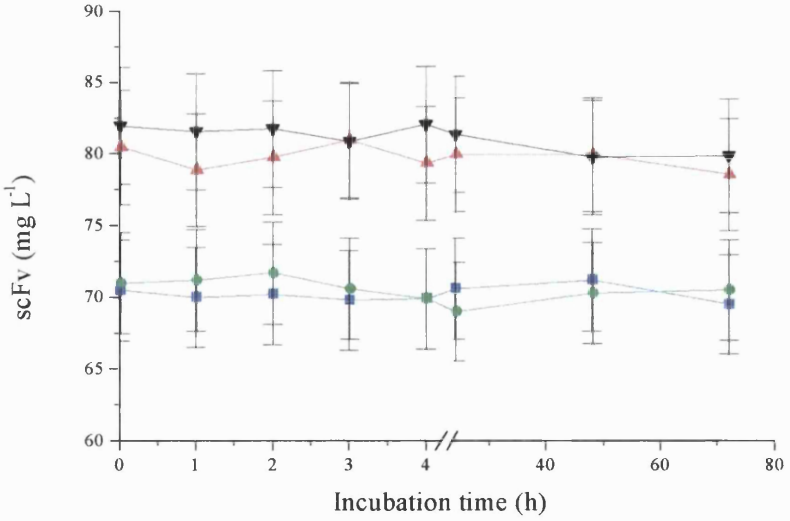


Fig. 3.43: ScFv concentration after incubation at 30 °C. ●: scFv in buffer; ■: scFv in sterile fermentation medium; ▲: scFv in fermentation broth containing proteases; ▼:scFv in fermentation broth containing proteases and 1 mg L^{-1} PMSF.

From these graphs it is evident that the scFv concentration does not decrease during the incubation period at 4 °C and 30 °C and it can therefore be concluded that proteases do not act against the antibody fragment.

3.8 IMAGE ANALYSIS.

Image analysis was used to trace changes in the morphology of the microorganism during the course of the fermentation. Table 3.2 summarizes the results from studies performed with fermentation samples using different growth media and varying times of induction.

Medium	Scale	Induct. Time (h)	Fermentation time (h)					
			24		48		68	
			PD (mm)	PA (mm^2)	PD (mm)	PA (mm^2)	PD (mm)	PA (mm^2)
A1/A2	7 L	7	0.667	1.056	0.749	1.581	0.715	1.382
B1/B2	7 L	0	0.436	0.398	0.511	0.409	0.452	0.433
B1/B2	7 L	7	0.507	0.396	0.467	0.413	0.469	0.452
B1/B2	7 L	21	0.379	0.299	0.391	0.303	0.385	0.321
B1/B2	7 L	42	0.351	0.275	0.385	0.300	0.355	0.316
C1/C2	SF	21	0.580	0.721	0.499	0.661	0.694	0.955
C1/C2	7 L	21	0.375	0.282	0.386	0.301	0.349	0.291
D1/D2	75 L	21	0.357	0.256	0.369	0.285	0.343	0.246

Table 3.2 Morphological characteristics of *A. awamori* pellets, during different fermentation runs. SF: Shaken flasks, PD: pellet diameter, PA: pellet area.

From the table several conclusions can be drawn. Firstly, the fermentations using the initial medium (A1/A2) produced significantly larger pellets than later fermentations (more than 70 % larger pellet diameter). This was either because of the different growth medium, or because the LH fermenter was used which has a different geometry than Applikon, or because of the presence of the contaminant in the culture. Subsequent fermentations produced smaller pellets. This can be attributed to the fact that, by growing faster, the oxygen demand was higher and therefore the stirrer speed had to be increased, resulting in smaller pellets. Pellet size in the laboratory and the pilot scale fermenters did not differ considerably, something explained by the similarity in geometry of the two vessels. Finally, pellets in shake flasks were almost 50 % bigger than in the fermenter, due to differences in vessel geometry, agitation regime and oxygen levels. Below photos of representative pellets from different fermentations are displayed.

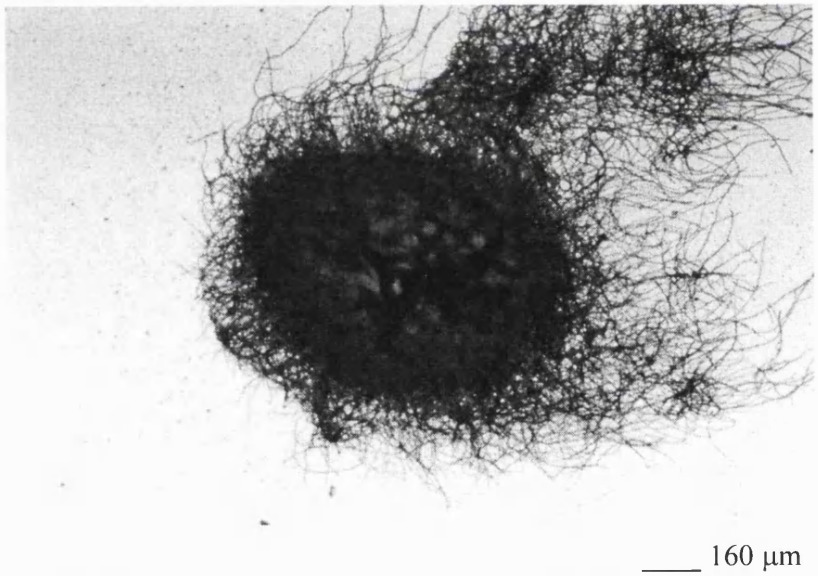
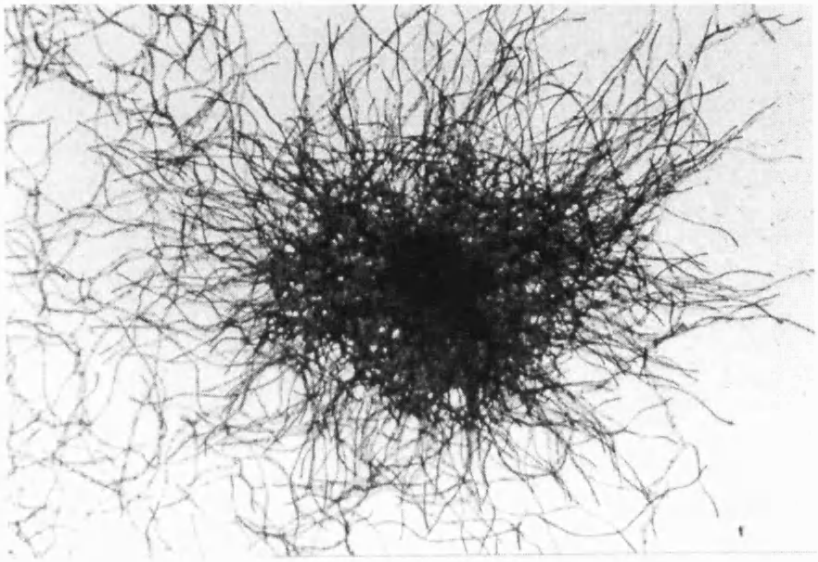
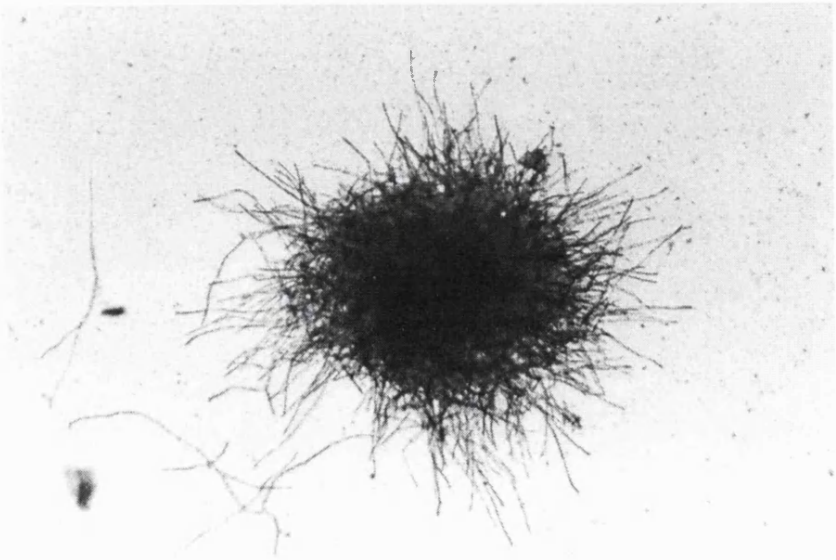


Fig. 3.44 Pellet from fermentation F2 after 48 hours of culture.



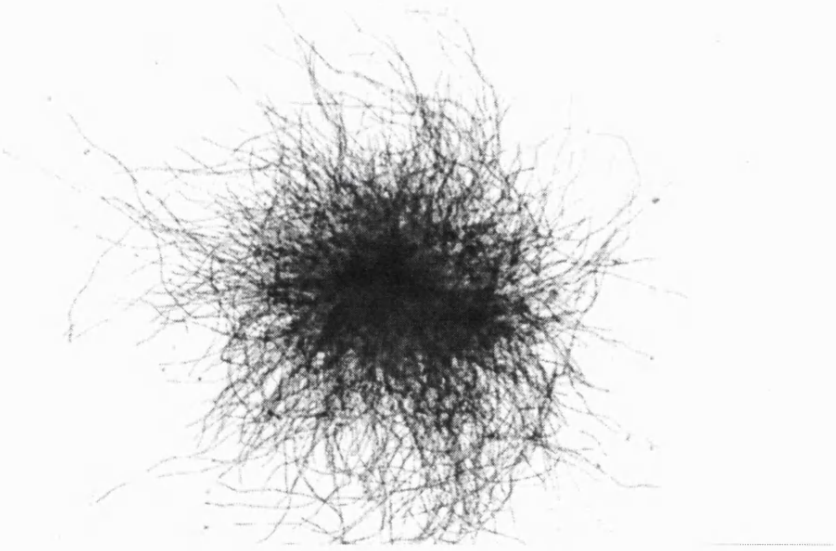
____ 100 μm

Fig. 3.45 Pellet from fermentation F4 after 48 hours of culture.



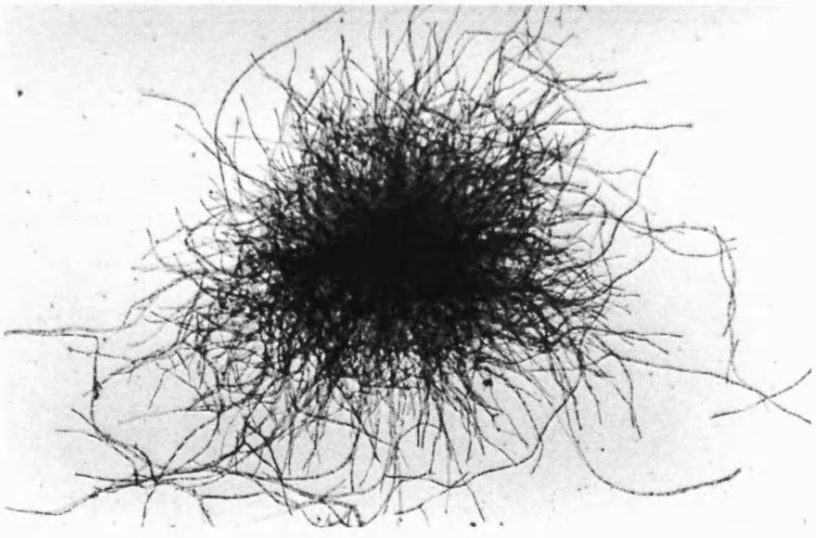
____ 100 μm

Fig. 3.46 Pellet from fermentation F7 after 48 hours of culture.



____ 100 μm

Fig. 3.47 Pellet from fermentation F9 after 48 hours of culture.



____ 100 μm

Fig. 3.48 Pellet from pilot scale fermentation (F12) after 48 hours of culture.

These photos show that there is no change in morphology and pellet size between different media and scales of operation. The exception is the pellets from runs F1 and F2 where the diameter of the pellets was almost double that of later runs. However, it should be noted that these studies were preliminary and in order to arrive

at more definitive conclusions, regarding the effect of morphology on productivity, further work should be carried out.

3.9 On-line Biomass Monitor.

The on-line biomass monitor was used in three fermentation runs (F13, F30, F31) in order to assay its suitability for such fermentation. A fact that should be noted is that this fermentation had no pH control. The reason was that the biomass monitor probe was occupying the port of the pH probe. The results obtained from that fermentation are summarized below.

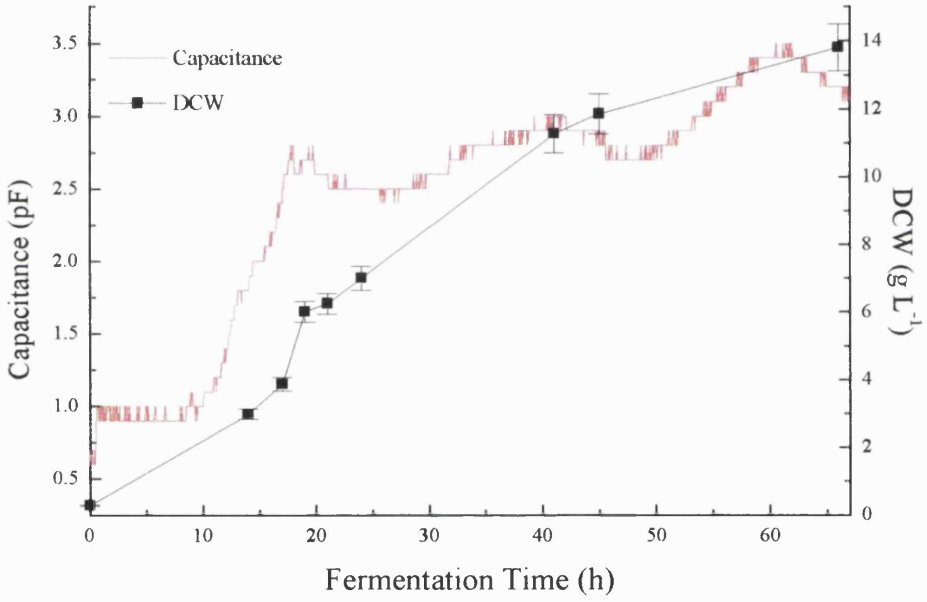


Fig. 3.49 Comparison between on-line capacitance and off-line biomass estimation by filtration.

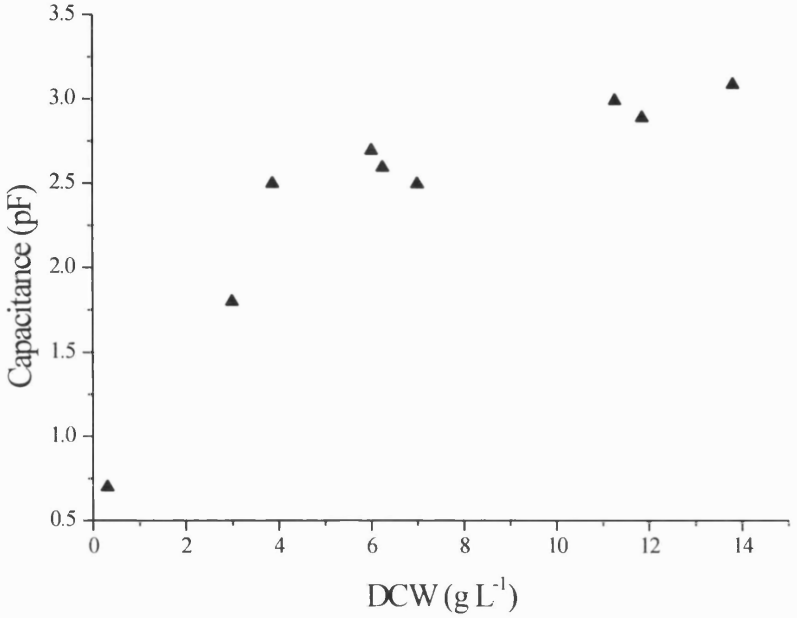


Fig. 3.50 Relationship between dry biomass and capacitance.

A non-linear correlation between capacitance and biomass at biomass levels greater than 3 g L^{-1}. However, further work has to be performed in order to establish the accuracy, reliability and suitability of this instrument for the *A. awamori* fermentation, especially due to the fact that the accuracy of the instrument is affected by parameters such as changes in stirrer speed.

3.10 CONTAMINATION.

As it was presented in table 3.1, all the runs in the LH fermenter and some runs in the Applikon were contaminated. After performing the experiments described in section 2.13, the main contaminant was identified as *Bacillus* and was traced back to the original seed stock supplied by the industrial partner. The contaminated runs were identified early by a rapid decrease in % DOT followed by an early increase in OUR. As this was happening before the end of the known lag phase of *Aspergillus*,

samples were taken and checked for sterility. This confirmed the contamination. Figure 3.51 shows typical % DOT and respiration profiles of a contaminated run.

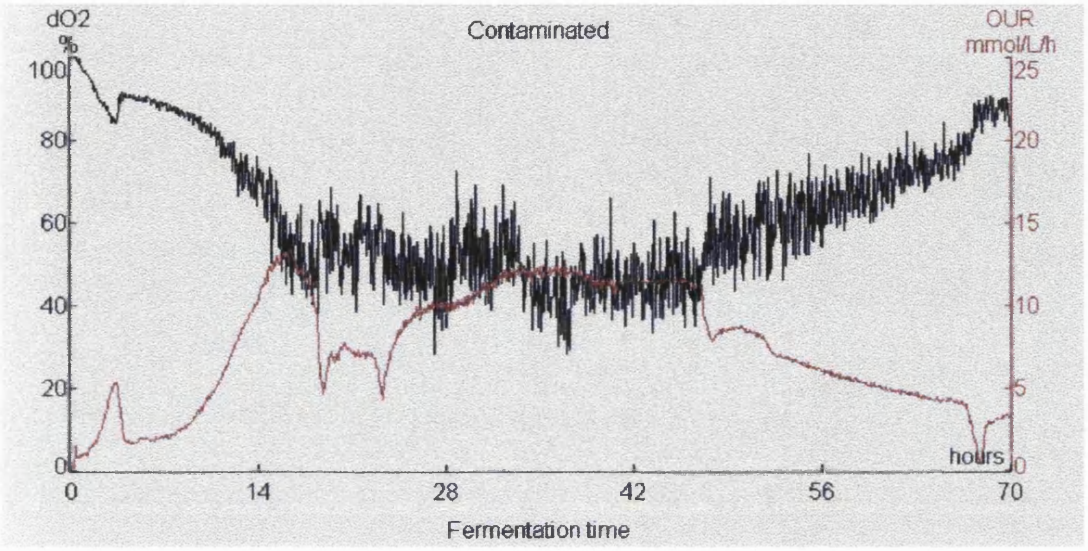


Fig. 3.51 DOT and respiration profile of a contaminated run performed in the Applikon fermenter (F14).

After the contaminant was traced and identified, several actions were taken in order to decontaminate the original seed stock. This was done successfully and further fermentations were free of contamination.

3.10.1 LH Fermentations.

All the fermentations performed in the LH vessel were contaminated. Contamination is evident from the DOT profiles (fig. 3.52, 3.54), where a sharp drop in dissolved oxygen is observed after 25-30 hours of inoculation. In order to establish the source of contamination, Nutrient Agar (NA) and PDA plates were inoculated with samples from the initial glycerol stocks, the germination shake flask and the fermenter samples. In addition, a shake flask containing fermentation medium was inoculated with the same inoculum used for the fermentation. After incubation it was found that the contaminant was present only in the fermentation samples; all the other

plates had only *Aspergillus* growing. Microscopic examination after Gram staining revealed two different contaminants, one Gram-positive (rods) and one Gram-negative (motile), which, together with the macroscopic examination lead to the conclusion that the contaminants were probably *Bacillus* and *E. coli*. Having established that the cause of the contamination was not the inoculum or the glycerol stocks, attention was focused on the fermenter. It was blank sterilised with nutrient broth and was left to run after sterilisation for 5 days at 30°C. No growth was observed and subsequently a third *Aspergillus* fermentation was performed which was also contaminated after 30 hours. At this stage the fermenter was pressure tested and it was found that the main seal was damaged, resulting in air entering the vessel and contaminating the culture. Since these runs were contaminated, no ELISAs' were performed and the biomass data cannot be considered as accurate or reliable. Nevertheless, profiles for some of these runs are included in order to provide evidence of the contamination.

As it is evident from the profiles (fig. 3.52), there is a first drop in % DOT eight hours after inoculation. This drop was expected since feed had started at seven hours after the inoculation. The second drop in % DOT levels was due to the foreign growth; *Aspergillus* was expected to grow at that point but not at such a high rate. Contamination was also evident from the sharp increase in CER and OUR values 30 hours after inoculation (fig. 3.53). Data points missing from the graphs are due to the fact that the computer malfunctioned during the first night of the fermentation.

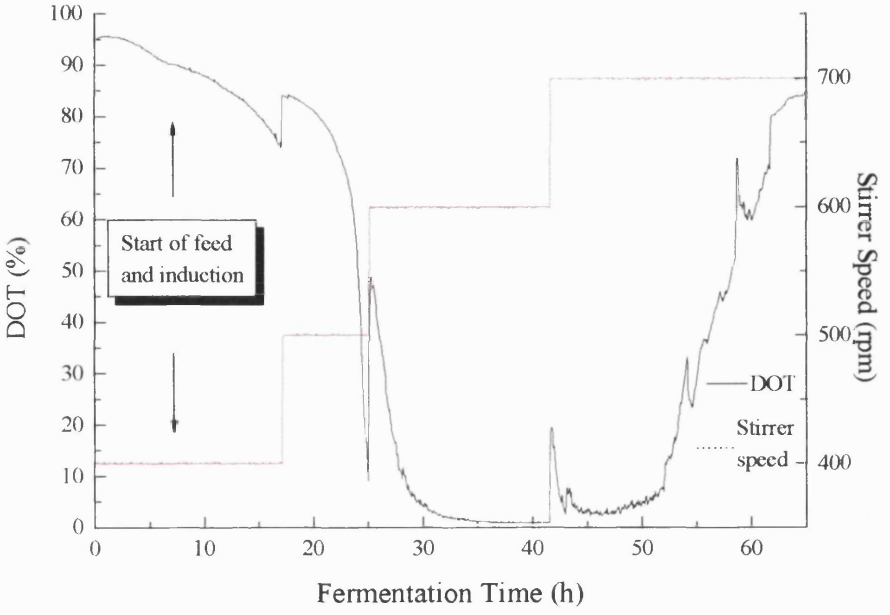


Fig. 3.52 DOT and agitation profiles for F15.

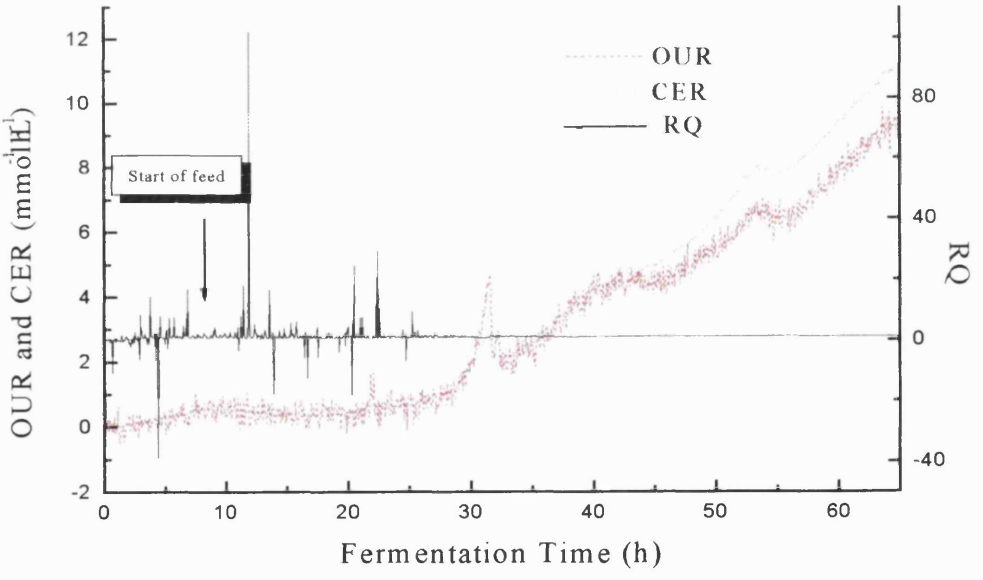


Fig. 3.53 Respiration profile for F15.

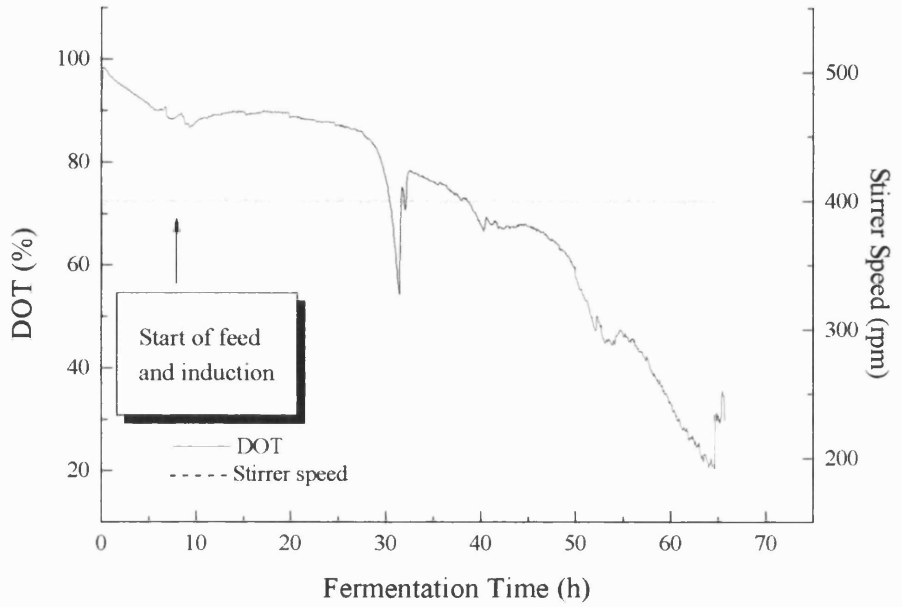


Fig. 3.54 DOT and agitation profiles for F16.

Figure 3.54 is the DOT profile for the last fermentation performed in the 7 L LH vessel. Again, contamination was observed 30 hours after inoculation and can be seen in the % DOT drop. Biomass levels reached 3.5 g L^{-1} DCW, but since they include the biomass of the contaminants as well as the *Aspergillus* biomass they are not reliable.

4. DISCUSSION

4.1 INITIAL MEDIUM.

The term "initial medium" (A1/A2) was used to describe the medium used in the initial fermentation protocol, as supplied by Unilever. The first two fermentations performed in the Applikon fermenter (F1 and F2) employed this medium and were both successful. Biomass levels reached 5 g L^{-1} DCW and scFv activity was detected in the broth. However, the results for the scFv concentration were not encouraging since only 1.5 mg L^{-1} was produced.

From the profiles for these runs it was evident that there was a delay in the initiation of the exponential growth phase of the organism, since lag phase was approximately 7 hours. The absence of a major carbon source from the "initial medium" prolonged the lag phase up to which the feeding started. It was therefore decided to include a carbon source in the "initial medium", as described below.

4.2 OLD MEDIUM.

The main difference between the improved "old medium" (B1/B2), and the "initial medium" (A1/A2) was the presence of fructose from the time of inoculation of the fermentation. The results showed that this had a positive effect on the growth of the *Aspergillus* and the scFv production (fig. 3.6). Specifically, there was an increase in biomass levels to 8 g L^{-1} DCW, from 5 g L^{-1} and an increase in scFv levels to 15 mg L^{-1} as shown in figures 3.3 and 3.6 in the "Results" section. This was the case for both fermentations performed under the same conditions (F3 and F4). The lag phase was also significantly shortened, with the exponential phase of growth beginning almost immediately after inoculation. However, maximum scFv levels were obtained at the same time in the fermentation, 42 – 45 hours after inoculation, or 35 – 38 hours after the start of induction. This time point coincides with the end of the exponential and the beginning of the stationary growth phase. Therefore it would be possible to

separate the inducer from the remainder of the feed and add it at a later stage in the fermentation. This will be discussed later in this report.

Another noticeable element from these two fermentation runs (F3 and F4) was the fact that the antibody levels reached their maximum level 40 – 50 hours after inoculation and then they either remained constant or declined. This decrease was possibly due to the presence of proteases in the broth degrading the antibody. Indeed, protease assays that were performed for some of the fermentation samples showed an increase in the protease levels after 45 – 50 hours. This also agreed with the results of Archer *et al.* (1992), who in order to overcome this problem used a protease deficient mutant strain for the expression of heterologous proteins in *A. niger*. This solution would not be the most beneficial in the case of *A. awamori* both due to resources needed for the development of such a strain and because protease levels in the broth are negligible before 40 hours. A possible solution would be the termination of the fermentation earlier, for example 45 – 50 hours after inoculation. This would also be beneficial for the whole process, as the length of the fermentation would be shortened by 20 – 25 hours.

Finally, it was evident from the respiration profiles (fig. 3.13, 3.16 and 3.19) that after induction had started the microorganism started to grow again. This would be an indication that xylose was used as a carbon source probably due to the lack of any fructose. This fact led to the experiments focusing on the medium composition.

4.3 New Medium.

The "old medium" (B1/B2) was very similar in composition to the medium used by Johansen *et al.* (1998) for *A. awamori* fermentation. The biomass values obtained were comparable. Experiments in shaken flasks, where the concentrations of nitrogen (ammonium sulphate) and carbon (fructose) sources were varied, showed a notable increase in titres and yield of scFv when the concentrations of these two compounds were doubled. It was also shown that there was a maximum concentration of fructose and ammonium sulphate, above which no enhancement of the product was detected. At that point other limiting

factors could be coming into effect, restraining any further increase in product concentration. Based on these findings, the medium with double the concentrations of fructose and ammonium sulphate was scaled-up to 7 L fermentations, in order to compare the results with the previous runs.

From the results of the fermentations with the "new medium" (C1/C2), the findings of the shaken flask runs were confirmed. Doubling the amount of these two media components resulted in 750 % increase in the maximum concentration of the product and 50 % increase in the dry biomass compared to the previous fermentations (fig. 3.23, 3.26). All fermentations using this medium were induced at the middle of the exponential phase, 21 hours after inoculation. The increase in product titre implied that the "old medium" was not optimized and lacked sufficient amounts of carbon and nitrogen, which limited the production of the antibody fragment.

Another notable result was the increase in the concentration of secreted proteases in the medium from 250 mg L^{-1} to a maximum of 1400 mg L^{-1} at the end of the fermentation. In order to investigate their effect on the product, a storage experiment was performed, the results of which are discussed in section 4.5 below.

4.4 Induction Time.

The time of induction has been shown to be a significant factor for the expression of heterologous proteins from microorganisms (Harrison *et al.*, 1997). In this study, cells were induced at four different times after inoculation in order to investigate the best time for the addition of the inducer in order to increase production of scFv fragments. Induction times varied from 0 to 42 hours after inoculation, which corresponded with early, middle exponential and stationary phases of growth. Induction at 0 hours after inoculation was chosen as the control. The results of the different induction regimes are summarized in the "Results" section 3.2. There was an increase in the titre of the antibody fragments when induction started 21 hours after inoculation. This was the result of cells being in their peak growth state, as at 21 hours, the fermentation was in the exponential

phase of growth. Induction at 21 hours had also the advantage of obtaining the maximum product concentration at an earlier stage in the fermentation. This means that the fermentation could be completed after 48 hours instead of 68 hours, making the process more efficient.

4.5 Effect of Proteases.

Citations in the literature about the detrimental effect of secreted proteases on the products of *Aspergillus* fermentations (van den Hondel *et al.*, 1992; Archer and Peberdy, 1997; van den Hombergh *et al.*,1997) and the fact that there was a drop in the concentration of the product towards the end of the fermentation led to the experiments to investigate the presence and effect of proteases. The results from these experiments performed in the samples are shown in figures 3.42 and 3.43 in the "Results" section. It was shown that there was no decrease in the scFv concentration during the period of incubation. This, together with the fact that the concentration of the scFv was constant 20 hours before the highest concentration of proteases, suggested that these enzymes did not damage the product. The presence of high concentrations of proteases could not be ascribed to cell lysis, as image analysis studies showed no difference in cell morphology during the course of the fermentation, as compared with previous runs.

4.6 Carbon Consumption.

From the experiments performed in fermentation samples to establish the rate of consumption of fructose, total carbohydrate and xylose, it was found that fructose was totally consumed after 30 hours of fermentation and also that only a small proportion of xylose was consumed (fig. 3.32). The former finding, together with the fact that only 40 % of the fructose concentration was converted to biomass, lead to the conclusion that fructose was used for the production of some other proteins, including glucoamylase. This hypothesis is also substantiated by the fact that the scFv in *A. awamori* was expressed as a glucoamylase fusion and also by the fact that this enzyme is a constitutive enzyme of *A. awamori*, being produced in concentrations up to 20 g L^{-1} (Jeenes *et al.*, 1991).

The fact that xylose was not totally taken up by the cells led to an experiment that investigated the effect of the inducer concentration on the product titre. The results of the experiments indicated that xylose concentration could be halved without a significant decrease in product concentration (fig. 3.33). This finding, together with the fact that fructose runs out after 30 hours of fermentation, led to a fermentation strategy where a step-feeding regime for addition of fructose was used with half the original concentration of xylose as inducer. Given that xylose is an expensive inducer (£120.00 kg^{-1}, 1999 prices), the resulting cost reduction is a notable factor in the fermentation process optimization.

4.7 Image Analysis.

Image analysis was used in order to investigate any possible changes in morphology during the course of fermentation. The results presented in section 3.8 show that the diameter and area of the pellets did not change during the duration of the fermentation. However, there was a decrease in the diameter of the pellets when the induction time with the "old medium" (B1/B2) was varied and when the "new medium" (C1/C2) was employed. At the same time, there was an increase in product concentration. Nevertheless, these two parameters (pellet morphology and productivity) cannot be directly linked on the basis of these results. The reason being that there were parameters during the fermentation that were not controlled and could have affected the productivity. In order to perform such a study, linking productivity with morphology, a process where the size of the pellet is altered when all other parameters (medium composition, stirrer speed, induction time, aeration, etc.) are kept constant, has to be designed.

4.8 Pilot Scale.

The improved process was scaled-up 10-fold to 75 L in an effort to investigate its potential for application to production scale. The method chosen for the scale-up calculations was that of keeping constant the power consumption per unit volume. With this method shear force is kept constant and therefore damage to microorganism due to shear is prevented. Also, since power input accounts for

a large part of the total fermentation cost, keeping the power constant meant that the cost of the process per unit volume would not increase in the large scale.

A comparison of the results obtained in terms of product concentration and specific yield are shown in figures 3.39 and 3.40 and in table 4.1. It is evident from these graphs that the maximum product concentration was 50 % higher in the pilot scale. This had an effect on the specific yield of the product that was also increased by almost 50 %. Maximum volumetric and specific productivities were doubled at the pilot scale (table 4.1). Maximum product concentration in the two scales was obtained 48 hours after inoculation in the laboratory scale and 42 hours after inoculation in the pilot scale. Growth characteristics and morphology of the microorganism remained similar to those at the laboratory scale fermentations. However, after 42 hours and until the end of the fermentation, the concentration of the scFv decreased by 30 %, to 112 mg L^{-1}. Protease concentration was similar to the values observed at the laboratory scale (fig. 3.41). Circulation times were calculated for the two scales in order to see whether there was a smaller circulation time in the pilot scale, resulting in an increased exposure of the microorganism to high shear and thus decreasing the rate of scFv production. The circulation times were calculated using the equation used by Smith *et al.* (1990):

$t_c = \frac{0.76(H/D_t)^{0.6}(D_t/D_i)^{2.7}}{N}$ (t_c: circulation time; H: liquid height; D_t: fermenter diameter; D_i: impeller diameter; N: stirrer speed). It was found that circulation time for the 75 L fermenter was 2.22 sec, and for the 7 L fermenter was 0.09 sec. Image analysis performed in the samples showed that there were no significant differences between the morphology of the microorganisms between the two scales of operation and during the fermentation. Specifically, average pellet diameter was 0.386 mm in the laboratory scale and 0.369 mm in the pilot scale after 48 hours of fermentation. Similarly, pellet area was 0.301 mm^2 and 0.285 mm^2 respectively after the same period of fermentation (table 4.1). Therefore, the decrease in product concentration in the pilot scale cannot be ascribed to cell damage or proteases. Given the higher maximum specific product yields after 42 hours compared to the laboratory scale, it is possible to scale-up and harvest after 42 hours. That would have the added benefit of making the process more economical.

	LABORATORY SCALE	PILOT SCALE
Fermenter volume (L)	7	75
Fermentation duration (h)	48	42
Maximum dry weight (g L^{-1})	10.2 (±0.51)	11.2 (±0.55)
Average pellet diameter (mm)	0.386 (±0.03)	0.389 (±0.03)
Average pellet area (mm^2)	0.301 (±0.03)	0.285 (±0.03)
Circulation time (sec)	0.09	2.22
ScFv maximum volumetric productivity (mg L^{-1} h^{-1})	4.13	8.25
ScFv maximum specific productivity (mg g^{-1} h^{-1})	0.83	1.72
ScFv max. specific yield (mg g^{-1} DCW)	12.0	17.5
Maximum product titre (mg L^{-1})	108 (±5.04)	160 (±8.00)

Table 4.1 Comparison between laboratory and pilot scale processes.

4.9 ON-LINE BIOMASS MONITOR.

The on-line biomass monitor was investigated as an alternative means for measuring biomass in a fast and relatively easy manner. The results from the instrument suggested a non-linear correlation between capacitance and biomass (fig. 3.50). However, the relationship was not perfect. Also when the trace of capacitance was compared to off-line data, they did not correspond (fig. 3.49). In addition, the accuracy of the instrument is affected by changes in stirrer speed, as it was described in the instruments operation manual. Finally, the system for the particular fermenter had the added disadvantage of occupying the pH port, eliminating in this way the ability to have pH control. However, other methods (vacuum filtration and moisture analyzer) available for the estimation of the dry cell weight were simple and equally quick. Therefore, a system like the capacitance on-line biomass monitor would add unwanted and unnecessary

complexity to the fermentation process without offering any real benefits. The on-line biomass monitor system would be very useful for fermentations using complex media with insoluble particles where the estimation of the dry biomass is difficult. Also fermentations with fast growing microorganisms, where changes in biomass need to be monitored and encountered quickly, would benefit from such an on-line system. Examples where the on-line biomass monitor was useful include *Saccharomyces cerevisiae, Pichia pastoris* and *Streptomyces virginiae* fermentations (Fehrenbach, *et al.*, 1992), *Saccharomyces uvarum (carlsbergensis)* and lager yeast (Austin *et al.*, 1994) and *Saccharopolyspora erythrea* cultures (Sarra *et al.*, 1996).

4.10 CONTAMINATION.

As it was mentioned, several of the initial fermentation runs were lost due to contamination. The source of the contamination was traced back to either the equipment or, more importantly, the seed culture obtained from the industrial partner. In the case of contamination the most important action is to trace the cause. Since losing a fermentation run through contamination is very expensive and time consuming, the prevention of contamination through seed checking and equipment maintenance is a very important aspect that should be followed. Ensuring that a fermentation run is free of contamination would save valuable time and expenses.

4.11 COMPARISON WITH *E. COLI* SYSTEM.

As stated in the "Introduction", section 1.7, the aim of this project was to investigate parameters which affect the production of scFv antibody fragments in *A. awamori*, design a fed-batch strategy which can be successfully scaled-up and evaluate the process against the *E. coli* system, a well established and studied process. Table 4.2 on the next page compares key aspects of the two processes; the improved *A. awamori* system described in this report and the *E. coli* system developed by Harrison *et al.* (1997). The production in *E. coli* was aimed at maximizing the scFv antibody fragment yield in the periplasm of the microorganism. A 14 L fermenter vessel was employed, achieving high cell

density cultures by controlled exponential feeding of glucose. The effect of concentration of the yeast extract in the feed was investigated in order to achieve high product retention in the periplasmic space.

	A. awamori	*E. coli*
Fermenter volume (L)	75	14
Fermentation duration (h)	42	48
Final dry weight (g L^{-1})	11.2	52
ScFv max. volumetric productivity (mg L^{-1} h^{-1})	8.25	12.88
ScFv specific productivity (mg g^{-1} h^{-1})	1.72	2.32
ScFv specific yield (mg g^{-1} DCW)	17.5	3
Maximum product titre (mg L^{-1})	Extracellular 160	Extracellular 44
	Periplasmic 0	Periplasmic 156
Cost of growth medium (£ L^{-1})	7.9	2.2
Downstream processing	Product secreted	Product in periplasm

Table 4.2 Comparison between *A. awamori* and *E. coli* systems for scFv production.

From this comparison a number of conclusions can be drawn. The *E. coli* system produced overall 20 % more product than the *A. awamori*. However, only the portion of the product that is in the periplasm of the *E. coli* is processed. This resulted in similar usable product tires in the two systems. Volumetric and specific productivities were 50 % higher in the *E. coli* process. Dry biomass in the *E. coli* process was five times higher than in the *A. awamori* system. High cell density is not uncommon in *E. coli* and the biomass obtained by the *A. awamori* process is amongst the highest reported for submerged cultures of *Aspergillus* (Yadwad *et al.*, 1996; Favela-Torres *et al.*, 1997; Cui *et al.*, 1998; Johansen *et al.*, 1998;

Bocking *et al.*, 1999). The cost of the medium was higher for the *A. awamori* process, mainly due to the high cost of the inducer. However, the *A. awamori* system had other important advantages. The running time of the fermentation was shorter in the *A. awamori* system by 6 hours. The specific yield was almost 600 % higher in the *A. awamori* process. The product was secreted to the fermentation broth, requiring fewer steps in downstream processing than the *E. coli* process. Figure 4.1 provides a simplified flow chart showing the downstream processing requirements of the two systems prior to chromatography. The fewer unit operations in the downstream processing of *A. awamori* result in lower product loss during recovery and purification. In general, it can be said that the *E. coli* process is better in the upstream processing while the *A. awamori* process may present advantages in the downstream processing. In addition, *A. awamori* has a history of safe usage and acceptance by industry, which make the process using *A. awamori* potentially attractive. However, there is a need for the development and optimization of a downstream processing strategy for the *A. awamori* process. After such a strategy is created, a more complete study of the techno economic comparability of the *E. coli* production system may be possible.

A. awamori system *E. coli* system

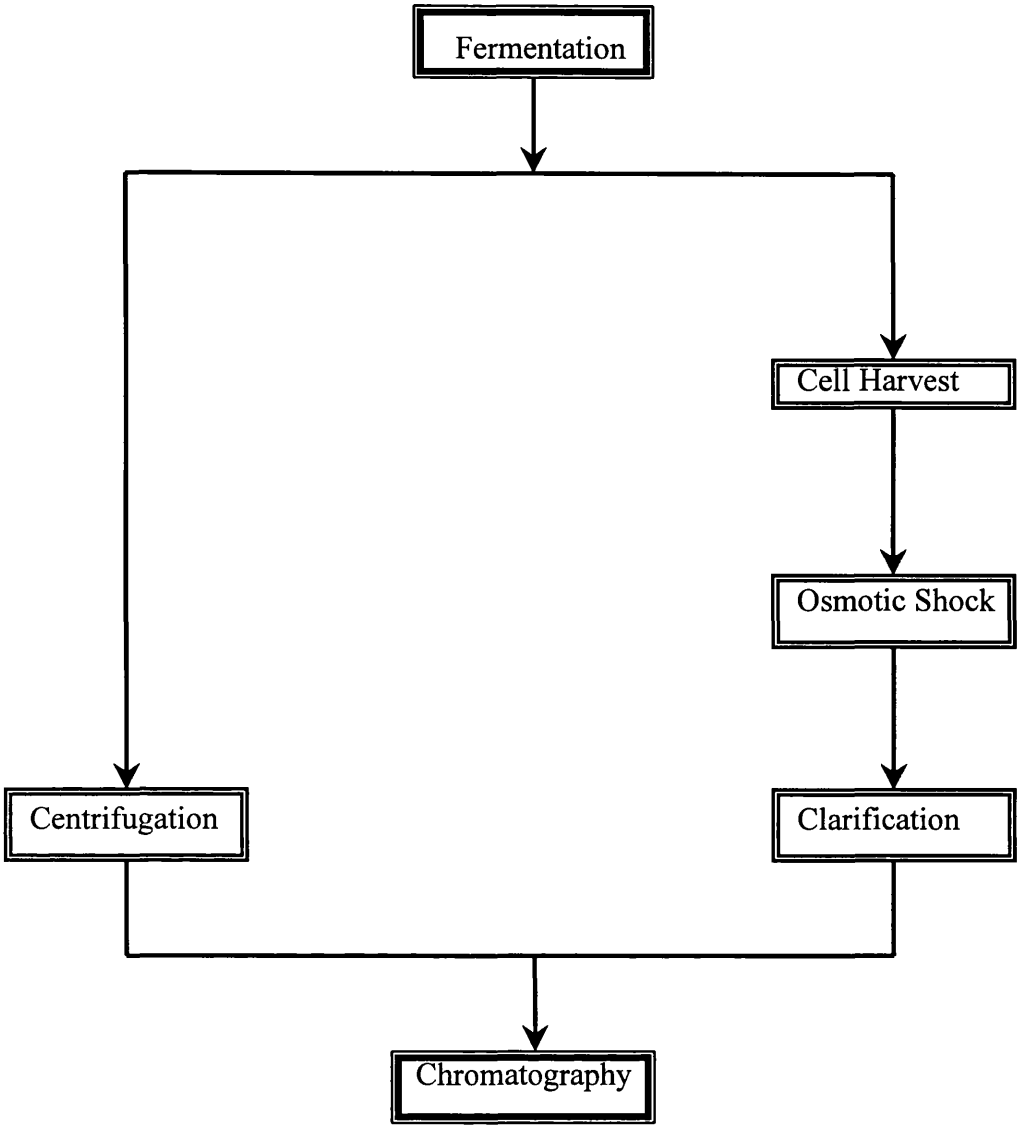


Fig. 4.1 Purification steps for the *A. awamori* and *E. coli* systems prior to chromatography.

4.12 Future Work.

The increase in the demand for monoclonal antibodies in general and antibody fragments in particular, together with the positive results obtained from this study, make the process attractive for further optimization. Specifically, factors that can be further investigated include inoculum size and condition, agitation type and intensity and fermenter design. A more radical approach would

be to investigate the possibility of using an air-lift reactor for the process. The advantages that such a system provides include high oxygen absorption efficiency, low energy requirements and absence of moving parts, resulting in reduced maintenance costs and increased reliability. Several studies have been performed, comparing the two types of reactors (stirred-tank and air-lift) (Barker and Wogan, 1981; Träger *et al.*, 1989; Okabe *et al.*, 1993; Siedenberg *et al.*, 1997a,b). In all cases results from the air-lift were comparable, if not better, to those from the stirred-tank, suggesting that utilising such a vessel can have a potential benefit.

In addition, a detailed techno-economic study of the process must be performed in order to further compare its efficiency against the *E. coli* system. This study should be combined with a suitably optimized downstream sequence for the extraction and purification of the product so that the process is assessed in its entirety.

Finally, in order to make the process even more effective with regards to product concentration, one can perform a series of media screening studies. A Plackett-Burman screening design would allow the testing of all the major ingredients of the growth medium (Strobel and Sullivan, 1999). In this way the concentrations of fructose, xylose, ammonium sulphate, sodium nitrate and yeast extract will be optimized for maximum product titre. Under the same design alternative defined and, more economical, complex carbon sources can be examined. The cost of cane or beet molasses is very competitive when compared to that of the pure carbohydrates. However, the fact that molasses contain many impurities can make the downstream processing more complicated, effluent treatment will be more expensive due to the unutilized waste materials and the process will be more difficult to validate. Also such a complex medium would not be acceptable for production of scFv for a prospective pharmaceutical use. Therefore one must take all these parameters into account when deciding which medium to use.

4.13 Conclusions.

The original strategy for the production of scFv antibody fragments from *A. awamori* was not an optimized one. The fermentation medium was carbon and

nitrogen limited. Alterations made to this medium resulted in an increase in biomass and product titre. Experiments regarding the induction strategy showed that induction at the middle of the exponential phase (21 hours) was favourable, not only because higher product concentration compared to the other induction strategies was achieved, but also because the maximum product titre was obtained at an earlier stage in the fermentation as compared with the early exponential and stationary phase induction fermentations. In addition, an investigation on the effect of the concentration of the inducer (xylose) resulted in an increase in the product yield and a subsequent decrease in the cost of the fermentation, since xylose accounts for more than 50 % of the total cost of the medium. Under the conditions stated, the fermentation can be stopped 25 hours earlier, decreasing in this way the cost of the process further. Finally, the effect of proteolytic enzymes that were secreted during the fermentation was investigated and it was found that they did not have an apparent effect on the product.

The experiments performed up to the present time have created a successful fermentation strategy for the production of scFv antibody fragments in *A. awamori*. They have improved significantly the previous process, not only with respect of productivity but also financially, by reducing the running time of the fermentation by 25 hours and by reducing the cost of the growth medium. They have also shown that the *A. awamori* system has a potential for even further improvement and optimization, especially after the encouraging results from the pilot scale fermentations. The major achievement of this project was that it has created a process that can be satisfactorily compared to the already established *E. coli* system. Specifically, scFv titres achieved after the improvements made were identical to those achieved with *E. coli*, as described by Harrison *et al.* (1997) after the same time of fermentation. Taking into account the fact that in the *A. awamori* system the product is secreted, therefore the downstream processing is simpler, and also that *Aspergillus* is in general safer than *E. coli*, this can be regarded as a successful new process.

APPENDIX I

A.1 Equations used by the computer program for the calculation of OUR, CER and RQ.

$$OUR = \frac{F_1}{V_m + V_o}\left[X_o - \frac{1 - X_o - Xc}{1 - X_{oo} - X_{oco}} * X_{oo}\right]$$

$$CER = \frac{F_1}{V_m + V_o}\left[X_{oco} * \frac{1 - X_o - X_c}{1 - X_{oo} - X_{oco}} - X_c\right]$$

$$RQ = \frac{CER}{OUR}$$

OUR: Oxygen Uptake Rate (mmol L^{-1} h^{-1}).

CER: CO_2 Evolution Rate (mmol L^{-1} h^{-1}).

RQ: Respiratory Quotient.

$\mathbf{F_1}$: Air-flow Rate (L h^{-1}).

$\mathbf{V_m}$: Molar Volume of Gas (L mol^{-1}).

$\mathbf{V_o}$: Working Volume (L).

$\mathbf{X_o}$: Molar fraction of Oxygen at Gas Inlet.

$\mathbf{X_{oo}}$: Molar fraction of Oxygen at Gas Outlet.

$\mathbf{X_{co}}$: Molar fraction of CO_2 at Gas Inlet.

$\mathbf{X_{oco}}$: Molar fraction of CO_2 at Gas Outlet.

APPENDIX II

A.2 Fermenter Dimensions and Specifications.

Dimension (mm)	Applikon 7 L	Inceltech LH 75 L
Vessel Height	350	925
Vessel Diameter	162	327
No of Impellers	3	3
Impeller Type	Rushton Turbine	Rushton Turbine
Impeller Diameter	62.5	105.5
Tip Height	12	21.4
Sparger Diameter	10	10
Baffle Height	260	612
Baffle Width	15	32

Table A2.1 Fermenter specifications.

APPENDIX III

A.3 Determination of Antibody Fragment Concentration by ELISA.

ELISA was performed as described in section 2.5 generating a standard curve every time the assay was carried out. Standards of the scFv were in the concentration range of 0 to 1,000 μg L^{-1} and the absorbance readings attained by these at the end of the ELISA were plotted against concentration to produce a standard curve, as in figure A3.1.

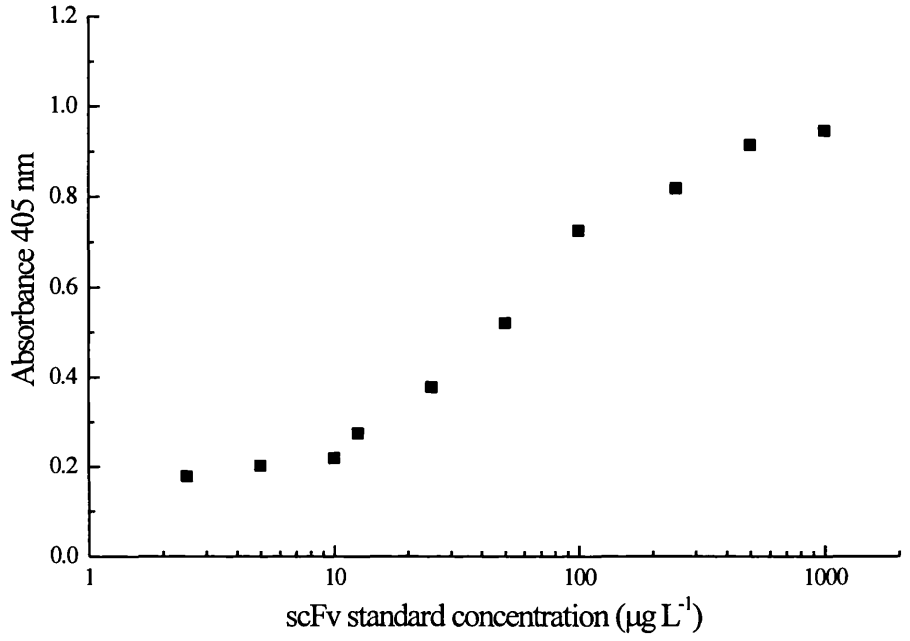


Fig. A3.1 Typical standard curve for ELISA.

Each sample dilution was assayed in triplicates. Sample concentrations were determined from the dilutions that gave absorbance readings within the linear part of the standard curve. The mean value of the three readings was used for the plots in the "Results" section. Error bars on the graphs represent the range of the readings. When the triplicate sample readings were more than 10 % different between each other, the experiment was repeated for that sample.

APPENDIX IV

A.4 Calibration Curve for DNS Total Reducing Sugars Assay.

A DNS assay was performed as described in section 2.9 in order to determine the nature of the carbon source left in the fermentation broth. Standards of xylose were in the concentration range of 0 to 4 g L^{-1} and the absorbance readings attained by these at the end of the DNS assay were plotted against concentration to produce a standard curve, as in figure A4.1 below. A linear fit (red line) was determined using Microcal Origin computer software (Microcal Software Inc., Northampton MA, USA) generating an equation which was: y=0.06+0.49x, where y is the absorbance reading and x the concentration of xylose.

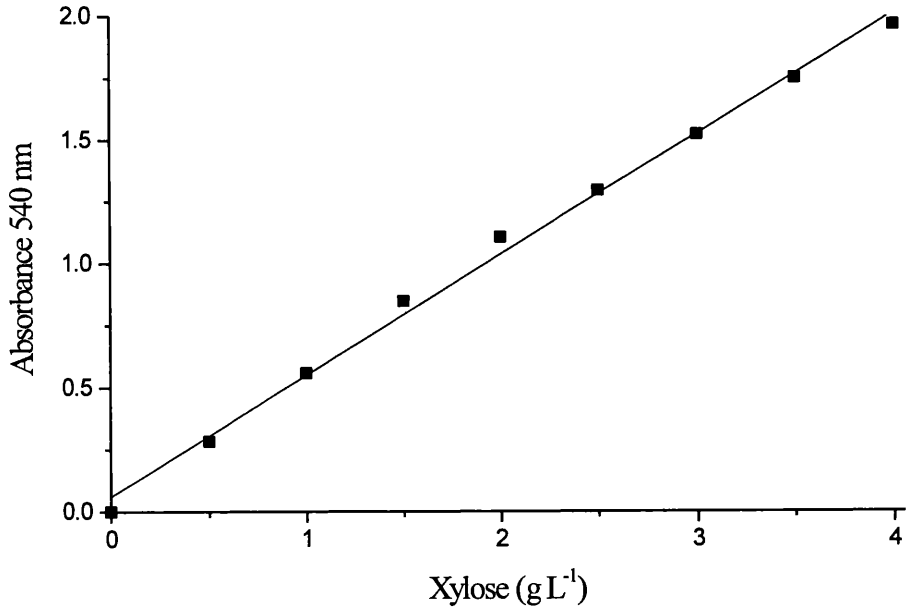


Fig. A4.1 Xylose standard curve for DNS assay.

APPENDIX V

A.5 Calibration Curve for Protease Assay.

A protease assay was performed as described in section 2.10 in order to establish the presence of proteolytic enzymes in the fermentation broth. Standards of trypsin were in the concentration range of 0 to 500 μg L^{-1} and the absorbance readings attained by these at the end of the protease assay were plotted against concentration to produce a standard curve, as in figure A5.1 below. A linear fit (red line) was determined using Microcal Origin computer software (Microcal Software Inc., Northampton MA, USA) generating an equation which was: y= 0.0002x-0.0005, where y is the absorbance reading and x the concentration of trypsin.

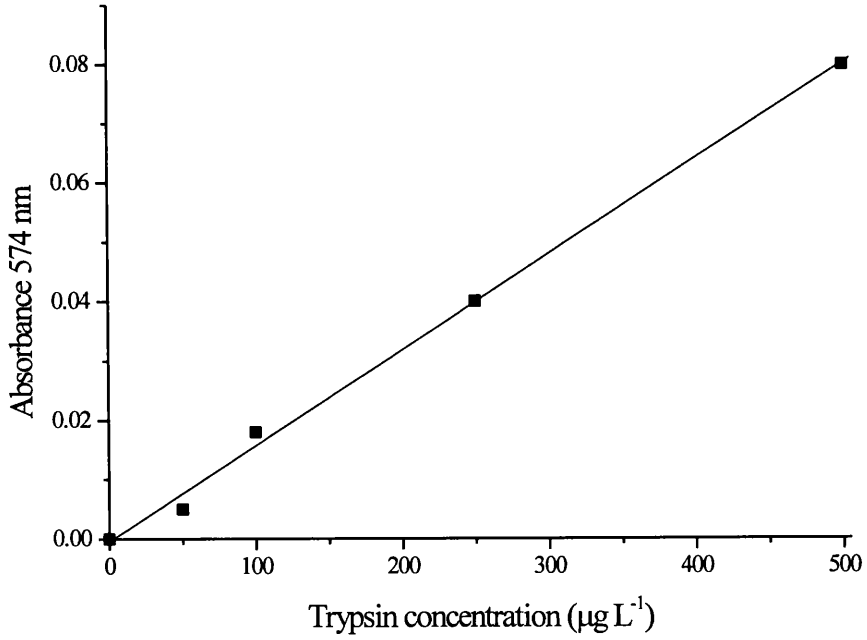


Fig. A5.1 Trypsin standard curve for protease assay.

APPENDIX VI

A.6 Fermentation Runs.

FERMENTATION CODE	MEDIUM AND FEED CODES	INDUCTION TIME (H AFTER INOC.)	FERMENTER USED	REMARKS
F15	A1/A2	7	7 L LH	Contaminated
F16	A1/A2	7	7 L LH	Contaminated
F17	A1/A2	7	7 L LH	Contaminated
F18	B1/B2	7	7 L LH	Contaminated
F19	B1/B2	21	7 L Applikon	Fermenter Failure
F20	B1/B2	0	7 L Applikon	Duplicate
F21	B1/B2	42	7 L Applikon	Duplicate
F22	B1/B2	7	7 L Applikon	Contaminated
F23	B1/B2	7	7 L Applikon	Contaminated
F24	B1/B2	7	7 L Applikon	Contaminated
F25	B1/B2	7	7 L Applikon	Contaminated
F26	C1/C2	21	7 L Applikon	Fermenter Failure
F27	C1/C2	21	7 L Applikon	Fermenter Failure
F28	C1/C2	21	7 L Applikon	Contaminated
F29	C1/C2	21	7 L Applikon	Contaminated
F30	C1/C2	21	7 L Applikon	On-line Biomass Monitor Trial
F31	C1/C2	21	7 L Applikon	On-line Biomass Monitor
F32	D1/D2	21	75 L LH	Contaminated
F33	D1/D2	21	450 L	Contaminated

Fermentation Code	Medium and Feed Codes	Induction Time (h after Inoc.)	Fermenter Used	Remarks
F34	Various	21	500 ml S.F.	Medium trial
F35	Various	21	500 ml S.F.	Medium trial
F36	Various	21	500 ml S.F.	Inducer conc. trial

Table A6.1 Description of fermentation runs.

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