PROTEIN EXTRACTION FROM MUSTARD (*B. juncea* (L.) Czern) MEAL USING THIN STILLAGE

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ABSTRACT

Oilseeds may be processed to yield a number of potentially valuable compounds and fractions including oil, protein and small molecules. However, energy costs associated with industrial processing of oilseeds can be significant. For example, processes that use water to dissolve and separate materials are burdened with the costs associated with concentrating value-added products from dilute solutions. The ethanol industry produces large amounts of an aqueous solution called thin stillage that has little value and is used in animal feed. Thin stillage contains some of the necessary salts used in protein extraction but has a low pH. Protein extraction and protein isolate production is commonly conducted at higher pH. Waste alkali from biodiesel production has a high pH and can be used to adjust the pH of thin stillage to improve its ability to extract protein from oilseed meal. By combining the properties of the waste products of both the ethanol and the biodiesel industries, a complementary process is possible that may have greater economic potential than current practices in industry.

In this study, processes for protein extraction from mustard (*Brassica juncea* (L.) Czern.) meal using thin stillage from ethanol production and glycerol from biodiesel production were studied. The osmotic potential of thin stillage used in this research was lower than that of water, whereas both the density and the viscosity were higher. The pH was typically 3.7-3.8, and the total Kjeldahl nitrogen was approximately 0.08–0.10 %, w/w. Organic compounds identified in thin stillage were isopropanol, ethanol, lactic acid, 1,3-propanediol, acetic acid, succinic acid, glycerophosphorylcholine, betaine, glycerol and phenethyl alcohol. In addition, yeasts, bacteria and fungi were also found. Moreover, the salt types and their concentrations in thin stillage were predictable. The salt types present in thin stillage were $CaCl_2$, NaCl, K_2SO_4, $NaNO_3$, $Mg(OH)_2$, Na_2SO_4 and KOH. A model thin stillage synthesized for the purposes of this research had components and chemical and physical properties comparable to those of thin stillage from ethanol production. Protein was extracted from ground, defatted meal using thin stillage at different pHs and salt concentrations. The results showed that pH and salt content

affected protein extraction efficiency. However, no differences were found in the efficiency of extraction, SDS-PAGE profile, digestibility, lysine availability or amino acid composition of protein extracted with thin stillage, model thin stillage or sodium chloride solution. Moreover, extracted protein did not display significant hydrolysis. The results from peptide sequencing showed that napin and cruciferin were the most prevalent proteins in the extracted fractions. When increasing the scale of the extraction, the efficiency of protein extraction and the percentage of protein in the extracted protein were decreased. Protein recovery achieved with the complementary protocol was higher than that reported for a published protocol. Allyl isothiocyanate was found in protein extracts.

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ABBREVIATIONS

ACN	Acetonitrile
AITC	Allyl isothiocyanate
AOAC	Association of official analytical chemists
ASTA	American Spice Trade Association
DMF	Dimethylformamide
DS	Distiller soluble
DR-FTIR	Diffuse reflectance fourier transform infrared
FAO	Food and Agriculture Organization of the United Nations
g	Gravity
GPC	Glycerophosphorylcholine
HPLC	High performance liquid chromatography
ICP-AES	Inductively Coupled Plasma Atomic Emission Spectroscopy
MALDI	Matrix-assisted laser desorption ionization
MASS	Magic angle sample spinning
MWCO	Molecular weight cut-off
MPa	Megapascal
NMR	Nuclear magnetic resonance
NNED	N-1-naphthylethylene diamine dihydrochloride
OPA	o*rtho*-phthaldialdehyde
PAG	Phenylacetylglycine
PLD	Phospholipidosis
ppm	Parts per million
PSE	pre-press solvent extracted
SDS	Sodium dodecyl sulphate
SHMP	Sodium hexametaphosphate
TFA	Trifluoroacetic acid

1 INTRODUCTION

Brassica spp. oilseeds are grown throughout the world as a source of vegetable oil and protein-rich animal feed (Kimber and McGregor, 1995). According to statistical data from the Canada Grains Council (2008), the average annual production of Canadian mustard and Canadian canola from 1998-2007 was 196.2 and 7,489.2 thousand tonnes, respectively. From 1997-2006, the Canadian oilseed crushing industry produced an average of 1,270.9 thousand tonnes of canola oil and 1,864.6 thousand tonnes of canola meal annually (Canada Grains Council, 2008). *Brassica* spp. seed is utilized in both food and non-food applications. Heath and Earle (1995) stated that non-food applications were increasing. Examples of these included industrial lubricants, biodiesel fuel (Körbitz, 1995), biopesticides (Bones and Rossiter, 1996; Brown and Morra, 2005), soil amendments/fertilizers, adhesives and surfactants (Bonnardeaux, 2007).

Industrially, oilseeds can be expeller-pressed to produce oil and meal, and the residual oil in the presscake can be removed using a solvent such as hexane (Carr, 1995). Both oil and meal from oilseed processing have potential industrial uses. Vegetable oils can be utilized for food and non-food purposes. For food use in the USA and Canada, canola (low erucic acid rapeseed) oil contains less than 2% of erucic acid (Booth and Gunstone, 2004). Oil meeting the low erucic acid level specification is used in food products such as shortenings, salad and cooking oils, and margarines. In addition to food applications, the oils can be used for industrial purposes, such as in the production of emulsifers, lubricants, plasticizers, surfactants, plastics, solvents and resins. Industrial products that contain vegetable oils in place of petroleum-based oils may be deemed to be more environmentally benign than the equivalent petroleum based product (Erhan, 2005). However, oil composition may not be ideal for all applications. For example, polyunsaturated fatty acids (e.g., linoleic and linolenic acids; McKeon, 2005) are less stable than monounsaturated fatty acids and saturated fatty acids (Johnson *et al*., 2002). Stable fatty acids are preferred in industrial applications where shelf life and high

temperature stability are important (e.g., motor oil), whereas polyunsaturated fatty acids are preferred in applications where lower oxidative stability is preferred (e.g., paints and coatings).

Canola meal remaining after vegetable oil extraction is widely used as a protein source in poultry, swine, beef and dairy cattle feeds (Hickling, 2001) because of its excellent amino acid profile (Canola Council, 2008). Bonnardeaux (2007) stated that "canola meal is sold as a protein source for a variety of animal species with markedly different digestive capacities and nutrient requirements". For fish, according to Higgs *et al*. (1995), high dietary protein is needed to feed farmed salmon and as much as 67% of the feed cost originates from protein sources. Much of this protein is currently sourced from small ocean fish such as herring and menhaden. The supply of fish meal from ocean fish is unreliable and both price and quality fluctuations occur. Therefore, *Brassica* oilseed meal may be an alternative to fish meal as its supply is more stable and its amino acid composition is suitable. In addition, canola meal is used for swine feed (Hickling, 2001), as the protein content of the swine diets is very important. The swine diet can be supplemented with up to 15 percent of the mass as canola meal (Bonnardeaux, 2007). Davey and Morgan (1969) found that the carcasses of swine fed a high protein diet had more lean than those fed a low protein diet. Furthermore, canola meal can be used as a valuable supplement for sheep feed because of its high methionine and cysteine contents (Bonnardeaux, 2007).

However, even though defatted canola meal has high protein content, there are anti-nutritional factors such as glucosinolates, tannins (Higgs *et al*., 1995), phytates, other phenolic compounds and crude fibre (Bonnardeaux, 2007) which would have to be removed before using canola meal products in some non ruminant animal and fish rations. Higgs *et al*. (1995) stated that while the protein of canola meal is a desirable component for inclusion in most animal rations, it is necessary to eliminate anti-nutritional factors to make the meal suitable for animals that do not tolerate these materials well. According to Sakorn *et al.* (1999 and 2002), enriched protein fractions with a balanced amino acid composition prepared from *Brassica* oilseed meal may be used in animal feed and human food (Hiron *et al.*, 2006).

The manufacture of enriched protein fractions from oilseed meal produces large volumes of waste products. Glucosinolates are a by-product in the aqueous solution from protein extraction from rapeseed meal and have to be removed from the protein (Tzeng, *et al.*, 1990). Glucosinolate breakdown products are biologically active (Vaughn and Berhow, 2005). Some of the reported activity may include health enhancing properties (Berhow *et al.*, 2006). Certain glucosinolates and/or their metabolic products have anti-cancer effects and may be used in the diet to reduce the risk of cancer, whereas other glucosinolate breakdown products, such as allyl isothiocyanate, possess herbicidal, fungicidal and bactericidal activity (Brown and Morra, 2005). In addition, these products have potential use in organic synthesis. The production of protein-enriched fractions from *Brassica* oilseeds may lead to co-products (glucosinolates) that will supply new markets for glucosinolate-rich products and breakdown products of glucosinolates.

Thin stillage is a dilute stream of organic and inorganic compounds produced as a co-product from the ethanol industry. Usually, it is processed by drying to generate solids called distillers dired grains with solubles (DDGS) that can be used in animal feeds. To make DDGS, thin stillage has to be concentrated into syrup before mixing with wet cake [Van der Vorst, J. (personal communication) from Terra Grain Fuels, Inc., Belle Plaine, Regina, Saskatchewan; Wilkins *et al*., 2006]. The concentration of thin stillage is not energy efficient as it consumes about 40-45% of the thermal energy required to evaporate and dry thin stillage and 30-40% of the electrical energy utilized in a dry-grind facility (Wilkins *et al*., 2006). According to Meredith (2003), "a large mechanical vapour recompression unit might evaporate 100,000 lb/hr of vapour with a power requirement of 1000 kW". The energy required to evaporate the large amount of water entrained in thin stillage is a major cost in the ethanol industry.

If the ethanol, oil and protein processing plants are physically close together, the thin stillage from the ethanol production plant could be used after ethanol distillation (the temperature of thin stillage would be around 80-85°C). After transport and heat exchange, the temperature of thin stillage could still be 40-60°C, which would be suitable for protein extraction according to Rhee *et al*. (1972) who stated that 40-44°C was satisfactory for protein extraction from raw peanut. The ethanol industry would save energy (from evaporating thin stillage to make the syrup) as they would not have to treat

thin stillage before discarding it. The protein industry would not have to purchase water for the process or energy to heat the water for protein extraction. In addition, the oil industry would provide defatted meal. In the case of an oilseed plant that produces biodiesel, alkaline glycerin, a by-product from biodiesel plants, could be used for pH adjustment in protein extraction. Thus, the ethanol, biodiesel and protein industries would all benefit. Furthermore, thin stillage contains minerals (Ojowi *et al.*, 1996; Mustafa *et al.*, 1999) which may enhance protein extraction. It also contains yeast cells, soluble nutrients and grain protein molecules (Wheals *et al*., 1999) as protein sources. Therefore, the protein content of the extracted protein may increase. Ethanol residue remains in the thin stillage after the distillation process (Wilkie *et al.*, 2000), along with other organic compounds (Dowd *et al.*, 1994; Wilkie *et al*., 2000), which might have an inhibitory effect on myrosinase activity (Botti *et al*., 1995) in defatted meal. This would allow more efficient isolation of intact glucosinolates.

The use of thin stillage for protein extraction from canola or mustard meal has not been examined. However, as described above, the use of thin stillage might have advantages for protein extraction. Therefore, this research was conducted with the following three objectives:

1) To study the composition of thin stillage from the ethanol industry;
2) To prepare a model thin stillage to study the effect of the compounds in thin stillage on protein extraction; and
3) To use thin stillage to extract protein from defatted meal of *Brassica juncea* and study the qualities of the extracted protein.

2 LITERATURE REVIEW

2.1 The composition of *Brassica* seeds

The production of *Brassica* spp. oilseeds (canola, rapeseed, mustard, etc.) has increased and it has become one of the most important crops for vegetable oil production (Kimber and McGregor, 1995). In addition to using *Brassica* spp. crops as a source of edible oil, the seed is also used to produce lubricants, other industrial oils, biodiesel (Körbitz, 1995) and high protein meal that can be used for feeding animals (Kimber and McGregor, 1995).

Brassica seeds typically contain 23-50% oil (w/w) (Pouzet, 1995) of which most is triglyceride (more than 90%). The oil also contains mono- and diacyl glycerols (partial glycerides), free fatty acid and phytosterol. Depending on extraction conditions, the oil will contain more polar lipids including phospholipids and glycolipids (4-5 %), sterols (0.27-0.36 %), esterified sterols (0.4-1.2 %). Furthermore, *B. napus*, *B. juncea* and *S. alba* oils have tocopherol contents between 50 mg/100g and 70 mg/100g (Uppström, 1995).

After oil extraction, the meal by-product of the oil extraction contains 36-44% protein and is generally used as an animal feed, although some work has explored the preparation of protein isolates for human consumption (Uppström, 1995).

Carbohydrates, including soluble and insoluble carbohydrate are the second largest component of the meal. Soluble carbohydrate varies from 10.5 to 16.0 % in oil-extracted rapeseed meal. The major components of soluble carbohydrate, in the order of concentration, are sucrose, raffinose and stachyose. Insoluble carbohydrate is composed of polysaccharides and represents 29% of oil- extracted, dehulled, rapeseed meal which include amyloids, arabinans, arabinogalactans, pectin and starch (Uppström, 1995). In addition, Bell (1995) stated that low glucosinolate rapeseed meal contains crude fibre (12-13%), acid detergent fibre (17-24%) and neutral detergent fibre (21-31%).

Uppström (1995) stated that several glucosinolates are present in cruciferous plants and the glucosinolate concentration varies between plant type and plant part.

DeBonte and Fan (1999) reported that canola meal contains allyl-glucosinolate (0-1.16μm/g), 3-butenyl glucosinolate (1.65-3.44 μm/g), 2-hydroxy 3-butenyl glucosinolate (2.40-7.32 μm/g), 2-hydroxy 4-pentyl glucosinolate (0-0.43 μm/g), 4-hydroxyl 3-indolylmethyl glucosinolates (2.60-4.40 μm/g), 4-pentenyl glucosinolate (0-1.14 μm/g) and 3-indolylmethyl glucosinolate (0-4.18 μm/g).

2.2 Myrosinases

2.2.1 Occurrence of myrosinase

Myrosinase (thioglucoside glucohydrolase; EC 3.2.1.147) is a glucosinolate-degrading enzyme (Van Eylen *et al.*, 2006) which is normally found in myrosin cells, a form of idioblast cell, in Brassicaceae (Andréasson *et al*., 2001). Myrosinase can be found in all glucosinolate containing plants such as *Brassicaceae* and some bacteria and fungi (Rakariyatham *et al.*, 2006). Björkman and Janson (1972) separated and enriched three myrosinase *isoenzymes* from *Sinapis alba* seed using DEAE-cellulose chromatography on Whatman DE-52, followed by gel chromatography on Sephadex G-200 and isoelectric focusing on an LKB-column (Sphensorb ODS-2). They found three myrosinase subgroup enzymes which they called MA, MB and MC. Similarly, Li and Kushad (2005) demonstrated the presence of three myrosinase subgroup enzymes in *Brassica napus* and *Sinapsis alba*. MB was located in most *Brassica* tissue, whereas MA and MC were found only in seeds. Furthermore, myrosinase activity was reported in the fungi *Aspergillus sydowi* and *Aspergillus niger*, the intestinal bacteria *Enterobacter cloacae* and *Paracolobacterium aerogenoides*, in mammalian tissue and in the cruciferous aphids *Brevicoryne brassicae* and *Lipaphis erisimi* (Bones and Rossiter, 1996). Sakorn *et al*. (1999) reported that *Aspergillus* sp., strain NR-4201 isolated from a rotting mustard seed meal sample collected from Lumphun, Thailand, produced myrosinase enzyme. Subsequently, Sakorn *et al*. (2002) developed a technique based on opaque barium sulphate zone formation to detect myrosinase-producing fungi. In addition, Rakariyatham *et al*. (2006) found that *Aspergillus* sp., strain NR-4617 isolated from the soil sample obtained from Thailand also produced myrosinase.

2.2.2 Characteristics of myrosinase

Myrosinase enzymes hydrolyse a wide range of glucosinolates to form the aglycone and D-glucose, thus myrosinase is typically not substrate specific (Björkman and Lönnerdal, 1973; Bones and Rossiter, 1996; Van Eylen *et al.*, 2006). In addition, Botti *et al*. (1995) stated that the activity and stability of myrosinase decreased when a variety of organic solvents, including simple alcohols, were present. However, the alcohol did not prevent the reaction from taking place.

2.3 Glucosinolates

2.3.1 Occurrence of glucosinolates

Glucosinolates are thioglucosides (Rauth, 2002) found among family members of the order Capparales: Tovariaceae, Resedaceae, Capparaceae, Moringaceae, and Brassicaceae (Brown and Morra, 2005). Glucosinolates were found in various parts of the plant and the concentration varied among plant parts (Verhoeven *et al.*, 1997). Glucosinolate concentrations vary within plants of a single species depending on developmental stage, environmental factors (space, moisture regime and nutrient availability) and tissue type (Brown and Morra, 2005). In *Arabidopsis thaliana*, seeds had the highest concentration of glucosinolates (over 60 μmol per gram of dry weight in mature seed). When compared with the other organs, the glucosinolate concentratation in the seed was at least twice as high (Brown *et al.*, 2003).

2.3.2 Characteristics of glucosinolates

Glucosinolates are defined as thioglucosides linked to the carbon of a sulphonated oxime (Fig. 2.1). In addition, the central carbon is linked to a moiety or side group. The side group and the sulphate group have an anti-stereochemical configuration (Witczak, 1999). The structure of the side group is determined by its biochemical origin. Many side groups are derived from amino acids and thus may be aliphatic, cyclic or heterocyclic (Uppström, 1995). In addition, Mithen (2001) stated that glucosinolates with more than one hundred different side chain structures have been discovered. Nevertheless, only seven amino acids (alanine, valine, leucine, isoleucine, phenylalanine, tyrosine and

tryptophan) are reported to contribute to the variety of side groups. The side groups of the remaining glucosinolates arise from: 1) chain elongation of amino acids, particularly methionone and phenylalanine; 2) modification of the structure of the side chain after amino acid elongation and glucosinolate biosynthesis, for example by the oxidation of the methionine sulphur to sulphinyl and sulphonyl, and by the subsequent loss of the ω-methylsulphinyl group to produce a terminal double bond. The modifications may subsequently involve the hydroxylation and methoxylation of the side group; and 3) some glucosinolates have complex side groups, for instance the o-(α-L-rhamnopyranosyloxy)-benzyl and sinopoyl moieties. The sulphate group is strongly acidic and it is associated with a cation (usually potassium) (Mithen, 2001). "The sulfate group and the thioglucose moiety impart nonvolatile and hydrophilic properties to all glucosinolates. This is important when proper methods for analysis of intact glucosinolates have to be considered. The R group, although perhaps always derived from amino acids, varies in properties from lipophilic to marked hydrophilic" (Olsen and Sørensen, 1980). The general structure of a glucosinolate is shown in Fig. 2.1. The side group and trivial names are provided in Table 2.1.

2.4 The myrosinase/glucosinate system

"Myrosinase is normally segregated from glucosinolates, sugar anionic thioesters containing beta-thioglucosinolate glycoside bonds, in plant tissues" (Rakariyatham *et al*., 2006). When glucosinolate-rich seeds are crushed in the presence of water, myrosinase enzyme is brought into contact with glucosinolates. The enzyme will cleave the thioglucosidic bond and produce D-glucose and thiohydroximate-O-sulphonate (an aglycone; Uppström, 1995). The aglycone is not stable and spontaneously rearranges into nitriles, thiocyanates, isothiocyanates or indoles depending on the side group, pH and the presence of ferrous ions (Fenwick *et al.,* 1983; McGregor *et al.,* 1983; Uppström, 1995; Vaughn and Berhow, 2005; Van Eylen *et al*., 2006) and protein epithiospecifier protein, for example (Fenwick *et al*., 1983; Uppström, 1995; Van Eylen *et al*., 2006). The temperature, age and condition of the plant tissue and availability of ascorbic acid are also factors that may contribute to the production of a variety of products (McGregor *et al*., 1983; Uppström, 1995). The products of myrosinase hydrolysis are shown in Fig. 2.2.

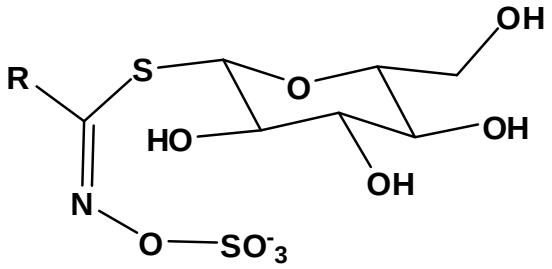

Fig. 2.1 The general structure of glucosinolates.

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Table 2.1. Systematic and trivial names of the prevalent glucosinolates found in *Brassica* and related seeds (Uppström, 1995).

Systematic name	**Trivial name**	**Oil seed**[1]
Aliphatic		
2-Propenyl (Allyl)[2]	Sinigrin	Bj
3-Butenyl	Gluconapin	Bj[3] Bn Br
4-Pentenyl	Glucobrassicanapin	Bn Br
S-2-hydroxy-3-butenyl	Progoitrin	Bn Br
R-2-hydroxy-3-butenyl	Epiprogoitrin	Ca
2-Hydroxy-4-butenyl	Napoleiferin	Bn Br
Cyclic		
2-Phenylethyl	Gluconasturtin	Bj Bn Br[4]
4-Hydroxybenzyl	Sinalbin	Sa
Heterocyclic (indole)		
3-Indolylmethyl	Glucobrassicin	Bj Bn Br Sa[5]
4-Hydroxy-3-indolylmethyl	4-Hydroxyglucobrassicin	Bj Bn Br Sa
1-Methoxy-3-indolylmethyl	Neoglucobrassicin	Bj Bn Br Sa[4]
4-Methoxy-3-indolylmethyl	4-Methoxyglucobrassicin	Bj Bn Br Sa[4]

[1]Bj = *Brassica juncea*, Bn = *Brassica napus*, Br = *Brassica rapa*, Ca = *Crambe abyssinica*, Sa = *Sinapis alba*

[2]Common systematic name.

[3]Found in substantial amounts in seeds of *B. juncea* of Indian origin.

[4]Found predominantly in roots.

[5]Found predominantly in vegetative tissues.

Isothiocyanate is the preferred product under neutral pH and alkaline conditions, whereas nitrile is favoured at low pH (Uppström, 1995; Bones and Rossiter, 1996; Brown and Morra, 2005). The addition of ferrous iron promotes reactions that produce nitrile hydrolysis products (Uppström, 1995). Epithiospecifier protein is a small protein (Bones and Rossiter, 1996) which can interact with myrosinases. In combination with ferrous iron, this protein can convert glucosinolates to epithionitriles (Uppström, 1995). Furthermore, "SCN^- production from glucosinolates is controlled by the presence of specific R-groups" (Brown and Morra, 2005). Vaughn and Berhow (2005) studied the hydrolysis products from white mustard defatted seed meal using HPLC (high performance liquid chromatography). They found that when defatted seed meal was extracted with 2M HCl, 4-hydroxybenzyl nitrile was the dominant compound. After extraction of the same material with both 0.1 N HCl and pH 7 buffer, the prevalent compound was 4-hydroxybenzyl isothiocyanate. In addition, Morra (2005) stated that the half life of 4-OH benzyl isothiocyanate decreased when the pH was increased.

2.4.1 The myrosinase/glucosinate system as a biopesticide

"It is considered that glucosinolates themselves possess limited biological activity until they are hydrolyzed" (Tsao *et al.,* 2002). Through hydrolysis, glucosinolates are converted to nitriles, thiocyanates, isothiocyanates (Van Eylen *et al*., 2006; Vaughn and Berhow, 2005), epithionitrile and oxazolidinethione (Vaughn and Berhow, 2005) which are toxic to living organisms, for example insects, fungi, animals and humans (Brown and Morra, 2005).

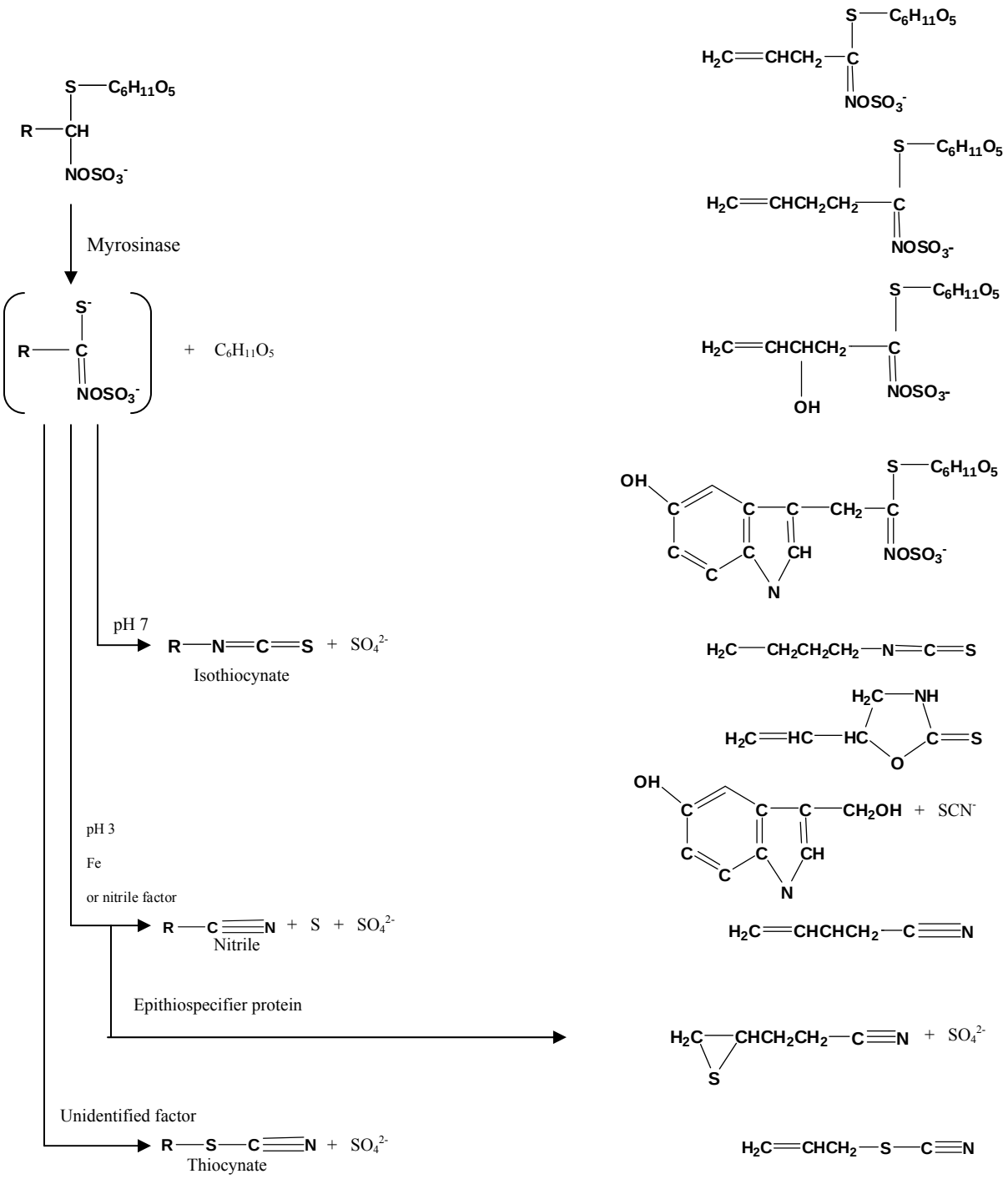


Fig. 2.2 Products of myrosinase hydrolysis (modified from Uppström, 1995).

The toxicity of these compounds in living organisms differs depending upon the type of compound. Brown and Morra (2005) stated that isothiocyanates have the highest toxicities (isothiocyanate > thiol ≈ thiocyanates > sulfides). In addition, both nitriles and isothiocyanates appear to play a role in attracting parasitic wasps that prey on plants (Kliebenstein *et al.*, 2005). "Allyl isothiocyanate is the most toxic compound formed from allyl glucosinolate hydrolysis in *B. juncea* L., and possibly the most important for biofumigation" (Noble *et al.*, 2002). Additionally, isothiocyanates can be lost by volatilization. However, for fumigants, volatilization is a significant mechanism enabling the compound to move through soil. Volatilization may also be a concern for health and environmental reasons.

Glucosinolate hydrolysis products have herbicidal, fungicidal and bactericidal activities, and have effects on insects and other invertebrates (Brown and Morra, 2005). Noble *et al*. (2002) used allyl isothiocyanate and fresh tissue of *B. juncea* as a source of allyl isothiocyanate. They found that allyl isothiocyanate was positively correlated to masked chafer beetle larval mortality. Tsao *et al*. (2002) found that "the fumigation toxicity of some of the glucosinolate products was very close to, or better than, that of the commercial insect fumigants such as chloropicrin (LC_{50}: 0.08 and 1.3 μg cm^{-3} against *Monodelphis domestica* (opossum) and *Rhyzopertha dominica*, respectively) and dichlorovous (LC_{50}: < 0.02 and 0.29 μg cm^{-3} against *M. domestica* and *R. dominica*, respectively)". Also, they concluded that the glucosinolate products might interfere with the insect respiratory system in their mode of action. Ernesto *et al.* (2006) studied the impact of isothiocyanate on fungal diseases. They found that "several isothiocyanates can control soil-borne phytopathogenic fungi, as well as fungi that produce infections in fruits. Furthermore, experiments have shown that some of the most fungitoxic isothiocyanates can provide better control of fungal infections in fruits than commercial fungicides can, even in an advanced stage of development. Fruits treated with these compounds did not have any deleterious effects on fruit physiology and quality" (Ernesto *et al.,* 2006).

However, hydrolyzed glucosinolate products are toxic compounds which interfere with thyroid function and cause liver and kidney damage in animals (Sadeghi *et al.,* 2006). This is supported by Mithen (2001) who observed that thyroid size, structure and

function in animals are altered after consuming rapeseed meal and that continued consumption can cause liver and kidney damage. Mieth *et al.* (1983) stated that hydrolyzed glucosinolate products negatively stimulate iodine metabolism and disturb the functioning of other vital organs.

2.5 Binding of allyl isothiocyanate (AITC) and protein

Allyl isothiocyanate can bind with protein. Murthy and Rao (1986) studied the interaction of allyl isothiocyanate with mustard 12S protein (cruciferin, which has six subunits arranged as a trigonal antiprism). Each subunit contains two polypeptide chains. In addition, its secondary structure has 11 percent α-helix structure and 31 percent β-structure, whereas 58 percent of the structure is aperiodic (Mieth *et al*., 1983). The precursor of cruciferin has a molecular weight of approximately 59 kDa and is processed to a 30 kDa acidic α-peptide chain and a 20 kDa basic β-peptide which are linked together by a single interchain disulfide bond (Wan *et al.,* 2007). Murthy and Rao (1986) found that AITC could bind to cruciferin and that the reaction rate increased when pH, temperature and interaction time increased. They explained that AITC was more reactive with NH_2 than with NH_3^+. In addition, temperature increases the kinetic energy of the molecules which, in turn, increases the frequency and energy of collisions and reactions of AITC and protein molecules. They also found that AITC can bind with ε-groups of lysine and phenolic groups present on tyrosine residues. Kawakishi and Kaneko (1987) studied the interaction of proteins with AITC. They discovered that AITC can react with model proteins (insulin, bovine serum albumin, ovalbumin and lysozyme) by cleaving the disulfide bond in the cystine moiety and forming polymers (Fig 2.3). In addition, AITC can react with the free amino group of lysine and arginine residues to form thiourea-like derivatives (Kawakishi and Kaneko, 1987).

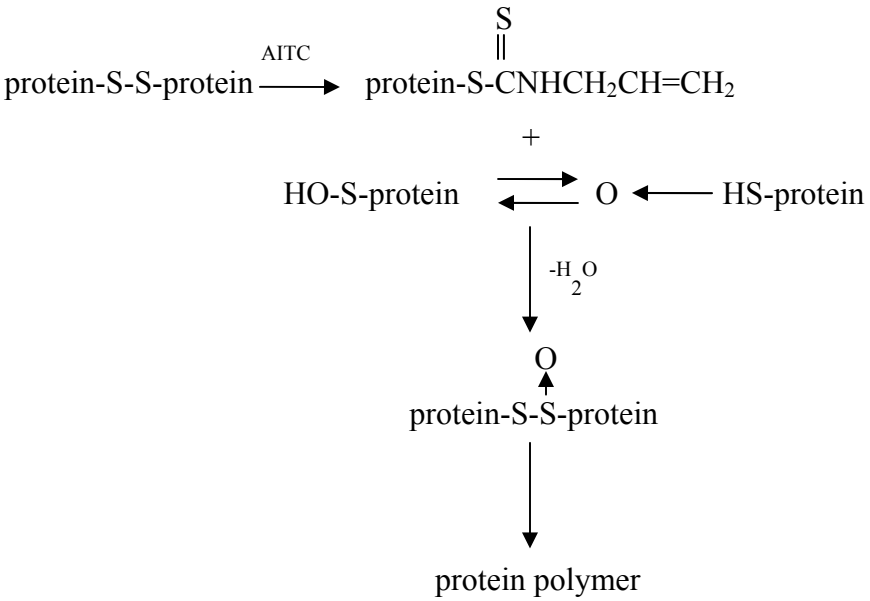


Fig 2.3 Interaction of protein with AITC (modified from Kawakishi and Kaneko, 1987).

2.6 Protein extraction and isolation from canola and other *Cruciferae*

Seeds of canola and other *Cruciferae* contain a great deal of protein, about 20-30 per cent (Uppström, 1995). Many studies have reported protein extraction from canola and *Cruciferae*.

Sims (1971) described a process whereby a protein extract was prepared from dehulled and defatted mustard flour using a 1:3 ratio of material:water and the pH was adjusted to 8.2. Subsequently, a paste of ground, cold, defatted mustard seed was added as an enzyme source. The paste was reacted for 4-5 hours at room temperature to allow for glucosinolate hydrolysis, after which steam distillation was used to remove the liberated volatile mustard oil. The pH of the slurry was lowered to 5 to precipitate protein and the solids were washed using water (1:3, w/v) to remove the bitter taste. The process produced dried material containing 48% of protein and 1.5% of fibre.

Rao *et al.* (1978) studied extraction of *B. juncea* protein. Their protocol involved extracting two grams of flour with 20 mL of solvent [water, 0.1M NaCl, 1M NaCl or 2% sodium hexametaphosphate (SHMP)]. To determine the optimum pH, samples of the slurry were adjusted to pH values that ranged from 1 to 11 using 1N HCl or 1N NaOH. Two minima and two maxima of protein extraction efficiency (solubility values) occurred at pH 4.0 and 7.8-8.0 (minima) and at pH 5.8 and 11 (maxima; 65 and 92%, respectively). The solubility profile of mustard protein extracted with 0.1M NaCl showed a similar trend to an intermediate value between water and 1M NaCl. However, for 2% SHMP, two solubility minima occurred at pH 3 and 10.

Klockeman *et al.* (1997) isolated canola protein from defatted meal using 0.1 to 0.4% (w/v) NaOH (5% meal to NaOH solution ratio) and varied extraction times from 10 to 60 minutes followed by precipitation of protein at pH 3.5. They found that when the extraction time was fixed at 60 minutes, 95.2-99.6% of the available protein could be extracted with 0.4% NaOH. When the concentration of NaOH was fixed at 0.4%, maximum protein extractability occurred after 60 minutes.

Xu *et al.* (2003) extracted protein from yellow mustard at a 1:18 ratio of meal:solvent using alkaline solutions at pH 10 and 12. In these studies, the pH was maintained at a constant value for 30 minutes. The results showed that protein

extractability increased when the pH was increased, and at pH 12, protein extractability reached >80% of available meal protein.

Pedroche *et al*. (2004) extracted protein from *Brassica carinata* defatted flour using alkaline solutions (pH 10, 11 or 12) at a 1:10 ratio of defatted flour:water. Protein was precipitated by bringing the solution to the isoelectric point (pH 3.5). They found that extracted nitrogen at pH 12, 11 and 10 was approximately 80, 78, and 55%, respectively.

Maenz *et al.* (2004) used aqueous salt solutions (<2 % (w/v) NaCl) to extract protein from oil-extracted desolventized flakes from canola or rapeseed [the ratio of meal:aqueous solvent was 10-50% (w/v)]. In addition, they described the use of phytase to digest phytate present in the meal. The resulting product was a protein-enriched dephytinized fraction that contained ≥ 45% protein (dry basis).

Prapakornwiriya and Diosady (2004) extracted protein using alkaline solutions (pH 10 to 13) from dehulled yellow mustard flour and concentrated proteins using ultrafiltration. In the described method, the extractions were achieved with a 1:18 meal:solvent ratio and an extraction time of 30 minutes, after which protein was precipitated at pH 5.5. They found that at pH 12, approximately 90% of the protein could be extracted.

Diosady *et al.* (2005) used an alkaline solution (pH 12) containing salt (NaCl, 0.02-0.5M), surfactant (sodium lauryl sulphate, 0.02-0.05%, w/w) and reducing agent (Na_2SO_4 and/or ascorbic acid, 100-5000 mg/kg). In addition, polyvinylpyrrolidone was added (1-5%,w/w) to absorb at least a portion of the phenolic compounds present. Protein was then concentrated by using isoelectric precipitation. The protein content of the precipitated protein obtained by this method was reported as over 80 percent (dry basis) with phenolic compounds of less than 0.02% (w/w).

Milanova *et al.* (2006) extracted canola protein from defatted meal using salt solutions followed by ultrafiltration and diafiltration to concentrate the protein. Subsequently, they precipitated a protein concentrate by lowering the salt concentration (salting out) using cold water to form protein micelles. The resulting product consisted of protein micelles containing at least 90% (w/w) protein.

Schweizer *et al.* (2006) extracted protein from canola meal using NaCl solution (0.15M) and then enriched specific proteins from the solution. *Brassica* and *Cruciferae* seed storage proteins are composed of 2S (napin) and 12S (cruciferin) globulin proteins (Mandal and Mandal, 2000). However, 2S and 7S globulin can be generated from the 12S protein, as outlined in Fig. 2.4 (Mieth *et al*., 1983). The 2S protein is a basic protein composed of two polypeptide chains (molecular weights of 9 and 4 kDa) linked by disulfide bonds (Ericson *et al.,* 1986; Kohno-Murase *et al*., 1994). The 7S globulins are trimeric proteins and have molecular weights of approximately 150-190 kDa (Shewry, 1995). To achieve the separation, protein was isolated using selective membrane techniques to increase the proportion of 2S protein, and decrease the proportion of 7S protein. In an initial ultrafiltration with a molecular weight cut-off (MWCO) of 50-100 kDa, 12S and 7S proteins were retained. A second ultrafiltration using 5-10 kDa MWCO membranes separated the 2S protein from lower molecular weight contaminants. Final step, the 2S solution was then spray dried. The alternative novel process used heat to precipitate 7S protein. The canola protein was extracted using salt solution (0.1-0.15M). The protein solution was concentrated using ultrafiltration and diafiltration with a MWCO 5 kDa membrane. The concentrated solution was diluted using cold water (approximately 5°C, about 10-fold) to form a protein micelle (predominantly 7S). The solution was allowed to settle to precipitate and remove micelle. The supernatant (2S and 7S proteins) was ultrafiltered using 5-10 kDa MWCO membrane to get rid of lower molecular weight contaminants and concentrate the supernatant. The retentate after ultrafiltration was heated at 75-95°C for 5-15 minutes to precipitate the 7S protein. The 7S protein was separated from 2S protein using centrifugation and 2S solution was spray dried. From both processes, the dried canola protein isolate contained at least 90% (w/w) of 2S protein (Schweizer *et al*., 2006).

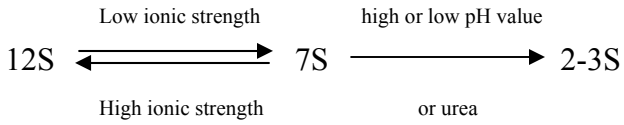


Fig 2.4 Generation of 2-3S and 7S protein from 12S protein (modified from Mieth *et al*., 1983).

Marnoch and Diosady (2006) studied production of mustard protein isolates from *B. juncea* after extracting protein with alkaline solutions (pH 7-13) and concentrating the protein using membrane-based processes. They found that the optimum extraction pH was 11. Ultrafiltration was used to concentrate the protein in the extract four-fold. Subsequently, the ultrafiltration retentate was diafiltered using three volumes of water and precipitated at pH 5. With this method, 81% of the protein was recovered. The crude protein was distributed among the fractions, which included 47.3% in the precipitated protein isolate, 3.8% in the soluble protein isolate, 29.9% in the residual meal, 6% in the permeate and 13% in the unrecovered fraction.

Maximum protein extraction efficiency is achieved at high pH and salt concentration. However, economic concerns including the value of protein and the cost of inputs must be considered in process development. In addition, isoelectric point precipitation, membrane concentration and "salting out" are well known techniques to isolate protein from the solution.

2.7 Quality of protein from canola and other *Cruciferae*

2.7.1 Protein types measured by gel electrophoresis

Two major storage protein fractions, napin (20-30%) and cruciferin (60%), were found in mature *Brassica napus* seeds (Crouch and Sussex, 1981). This reference appears to be one of the first reports on the napin and cruciferin proteins. Monsalve *et al*. (1997) stated that two types of napin (12 kDa and 15 kDa) were found in rapeseed. Aluko and McIntosh (2001) conducted SDS-PAGE analysis of proteins from canola meal under reducing and non-reducing conditions. Under non-reducing conditions (without 2-mercaptoethanol), four major polypeptide bands appeared at 16, 18, 30 and 53 kDa. However, under reducing conditions, the intensities of those major four bands were reduced significantly ($P<0.05$) and bands at 11 and 13 kDa, not seen under non-reducing conditions, appeared. They concluded that the band observed at 53 kDa was a component of the 12S globulin fraction. In addition, the four major polypeptides (16, 18, 30 and 53 kDa) were composed of smaller subunits linked together with disulfide bonds.

Aluko and McIntosh (2004) studied non-reducing and reducing SDS-PAGE of protein from defatted seed meal of *S. alba*, AC Vulcan (*B. juncea* L. Czern) - an oriental condiment mustard with yellow seed colour, commercial Brown (*B. juncea* L. Czern) - a condiment mustard with brown seed colour, and Dodolla (*B. carinata* Braun). The electrophoretic patterns of protein fractions prepared under non-reducing conditions varied by genus. SDS-PAGE bands at 135 and 50 kDa were apparent in *S. alba* meal, but not in *Brassica* meal. All *Brassica* protein extracts contained a polypeptide at 29 kDa not detected in *S. alba* meal. After the same fractions were exposed to 2-mercaptoethanol, the electrophoretic patterns changed. The SDS-PAGE band at 135 kDa observed in extracts of *S. alba* meal was no longer observed and the intensity of the band at 50 kDa was reduced significantly. In their place, a band at 66 kDa appeared in SDS-PAGE analysis of reduced protein, suggesting that larger polypeptide structures were held with disulfide bonds and that the polypeptide at 66 kDa was a component of the 135 kDa polypeptide. Conversely, the intensity of the major band observed at 29 kDa in non-reduced polypeptides of *Brassica* was unaffected by 2-mercaptoethanol. The authors concluded that the 29 kDa polypeptide did not contain a disulfide bond(s). Moreover, 12 kDa and 13 kDa polypeptides (subunits of 2S (or 1.7S) proteins of mustard seed), which were not detected in non-reducing SDS-PAGE, appeared in reducing SDS-PAGE.

Aluko and McIntosh (2004) also reported SDS-PAGE analysis of acid-precipitated and calcium-precipitated protein isolates from *S. alba*, AC Vulcan, commercial Brown and Dodolla. Protein was extracted using an alkaline solution (0.1M NaOH). For acid precipitation, protein was precipitated at pH 4 using 0.1M HCl. For calcium precipitation, the pH of the protein solution was adjusted to pH 6 and solid $CaCl_2$ was added until the concentration was 1M. The patterns of SDS-PAGE for these protein isolates were similar to the patterns of SDS-PAGE from defatted meal, except that the polypeptides observed at 16 and 20 kDa were resistant to acid precipitation but very susceptible to calcium precipitation. Conversely, peptides observed at 50 and 55 kDa were subject to acid precipitation and not precipitated by calcium.

Aluko *et al.* (2004) studied calcium-soluble and calcium-insoluble protein isolates prepared from *S. alba*. Electrophoretic patterns of calcium-soluble protein isolates under non-reducing conditions indicated major polypeptides with molecular masses of 15, 28,

50 and 55 kDa. However, the predominant peptide observed in calcium-precipitated protein isolates had a mass of 130 kDa. The isolates produced different patterns under reducing conditions. In the calcium-soluble protein isolate, the intensity of the bands observed under non-reducing conditions at 15, 50 and 55 kDa decreased, whereas the intensity of bands at 5, 22 and 35 increased. A 28 - kDa polypeptide was not affected by the presence of a reducing agent. These results suggest that the 15, 50 and 55 kDa polypeptides were composed of subunits linked with disulfide bonds. Additionally, these compounds were calcium-soluble. Bands at 5, 22, 28 and 35 were cut and analyzed to determine the peptide sequence. The data showed that the subunit at 5 kDa was the small subunit of the *S. alba* major allergen peptide, whereas bands at 22, 28 and 35 kDa were cruciferin subunits.

Aluko *et al.* (2005) studied calcium-soluble and acid-soluble protein extracts of defatted *S. alba* and *B. juncea* meal. The results showed that for non-reducing SDS-PAGE of defatted *S. alba* and *B. juncea* meals, four major polypeptides were found in *S. alba* (16, 50, 55 and 135 kDa) and in *B. juncea* (16, 29, 48 and 55 kDa). Notably, the intensity of the 55 kDa polypeptide in *S. alba* was higher than that in *B. juncea*. For *S. alba* and *B. juncea* meal peptide isolates exposed to reducing conditions, the electrophoretic patterns changed substantially. Polypeptides formerly observed at 16, 50, 55 and 135 kDa disappeared, indicating that these four polypeptides contained subunits that were held together with disulfide bonds. For acid-precipitated protein concentrates of *S. alba* and *B. juncea*, the electrophoretic patterns were similar to those observed for defatted *S. alba* and *B. juncea* meal. However, 12 kDa and 13 kDa polypeptides (2S or 1.7S) appeared in acid-precipitated protein concentrates but not in the defatted meals. Additionally, the intensity of the 16 kDa polypeptide was lower, and that of the 135 kDa, higher, in acid-precipitated concentrates than was observed for defatted meal. For calcium-precipitated protein concentrates of *S. alba* and *B. juncea*, the intensities of the 50 and 55 kDa polypeptides were lower than they were for the defatted meal and acid-precipitated protein concentrates under non-reducing SDS-PAGE. The 135 kDa polypeptide was absent completely in the presence of 2-mercaptoethanol.

2.7.2 Protein digestibility and bioavailability

Protein digestibility is a useful parameter when studying protein nutrition (Sarwar, 1987) and is the key determinant of the amino acid availability of protein (Hsu *et al.,* 1977). Because of both the high protein content of rapeseed meal (34-40%) and its excellent amino acid composition (Tzeng *et al.*, 1990), the digestibility and bioavailability of rapeseed/canola protein have been studied extensively.

2.7.2.1 *In vivo* digestibility

Barley *et al.* (1969) studied swine fed both a corn-soy diet and a corn-soy-rapeseed diet (substitution of 11% rapeseed meal (36% protein) by mass in a basal diet of meal and pellets). They found that feed consumption was not affected by the treatments and that 11% rapeseed meal in a diet could be substituted without any effect on growth rate. A basal diet using rapeseed meal (60% of basal diet and 40% of rapeseed meal or reground, pelleted rapeseed meal) was used to study digestibility. The results showed that 40% substitution by rapeseed decreased the digestibility of the basal diet (the per cent dry matter digestibilities of the basal, rapeseed meal and reground, pelleted rapeseed meal diets were 90.5, 80.7 and 82.9, respectively).

Sauer *et al.* (1982) studied the amino acid availability and protein quality of canola and rapeseed meal for pigs and rats using a corn-starch-based diet formulated to contain 14% protein. They found that true ileal or true fecal digestibility of canola and rapeseed meals were not different for pigs and rats, but were lower than for soybean meal. Bell (1984) stated that the protein digestibility of high and low glucosinolate rapeseed meal was about 81%. Newkirk *et al.* (1997) studied the nutritional evaluation of low glucosinolate *B. juncea* meals in broiler diets using oil-extracted meals. Nutritional value for broiler chickens was judged by nutrient retention (apparent metabolizable energy and ileal protein digestibility) and growth performance. They found the following ileal protein digestibilities: *B. napus*, 75.34%; *B. rapa*, 76.72%; *B. juncea* 78.02, 82.99, 76.65 and 76.39%; and soybean meal, 83.22%. Harazim *et al.* (2002) studied the intestinal digestibility of crude protein and amino acids of extracted rapeseed meal using the mobile bag method in Black Piels cows. They found the intestinal digestibility of crude protein to be 65.9%.

Bos *et al*. (2007) studied the digestibility of rapeseed protein in humans. The protein was extracted from defatted ^{15}N-labeled rapeseed flour at pH 11, then ultrafiltered at pH 7. The test meal used included 30 grams of ^{15}N-labeled rapeseed protein isolate, 96 grams of carbohydrate (75% of maltodextrin and 25% of sucrose), 23 grams of canola oil, and water to reach a final volume of 500 mL. The total energy content of the meal was 700 kcal, of which 15% was protein, 30% was fat and 55% was carbohydrate. They discovered that real ileal digestibility was 84.0 ± 8.8%; the postprandial biological value was 83.8 ± 4.6%.

2.7.2.2 *In vitro* digestibility

Hsu *et al*. (1977) developed a multienzyme technique for estimating protein digestibility. They used 5 mL of multienzyme (pH 8.0) [1.6 mg/mL of trypsin (14190 BAEE units/mg of protein), 3.1 mg/mL of chymotrypsin (60 units/mg of powder) and 1.3 mg/mL of peptidase (40 units/g of powder)] with 50 mL of aqueous protein suspension (6.25 mg of protein/mL, pH 8.0) and pH was measured at 10 minutes. They concluded that apparent digestibility = 210.46 -18.10 X where X= pH at 10 minutes, which gave a correlation coefficient of 0.9 with a 1.72 standard error of estimate with *in vivo* digestibility of rat models. Brulé and Savoie (1988) investigated *in vitro* digestibility of amino acids in protein mixtures based on two-step proteolysis with pepsin and pancreatin at 37°C. They found that rapeseed had a nitrogen digestibility of 40.1% in 6 hours. Amino acid digestibility after 6 hours was determined to be: arginine, 73.4%; histidine, 46.1%; isoleucine, 44.9%; leucine, 46.1%; lysine, 64.2%; methionine, 39.7%; cysteine, 22.1%; phenylalanine, 50.1%; tyrosine, 62.2%; threonine, 34.1%; valine, 46.5%; alanine, 41.3%; asparagine, 31.7%; glutamine, 32.1%; glycine, 32.9%; proline, 18.8%; and serine, 35.7%. Sadeghi *et al*. (2006) examined *in vitro* digestibility of defatted *B. juncea* meal and a protein isolate using pepsin and pancreatin enzymes. They found the digestibility of defatted *B. juncea* meal and protein isolate were 80.6 ± 0.5% and 92.4 ± 0.6%, respectively.

2.7.2.3 Lysine availability

"Lysine is a strictly essential amino acid and as it is sensitive to thermal processing, its availability in poultry feeds needs to be known" (Larbier *et al.,* 1991). Sauer *et al.* (1982) studied the amino acid availability of canola meal and rapeseed meal for pigs and rats using corn-starch-based diets formulated to contain 14% protein from canola meal or rapeseed meal. They found that the true ileal availabilities of lysine, threonine and methionine in canola meal and rapeseed meal for pigs were 77.7, 72.7 and 84.5%, respectively. Larbier *et al*. (1991) studied the availability of lysine in rapeseed and soybean meals via a digestibility trial in cockerels and a chick growth assay, using a basal diet supplemented with whole rapeseed meal, dehulled rapeseed meal and soybean meal as the source of lysine. They found that the true lysine digestibilities for whole rapeseed meal, dehulled rapeseed meal and soybean meal were 76.9, 81.4 and 87.5%, respectively. Lysine availability values were 72.8, 78.3 and 85.5%, respectively. Rozan *et al*. (1996) examined the lysine availability of rapeseed meal using the Carpenter method. The results showed that the availability of lysine in rapeseed meal was 2.3 g/16 g of nitrogen from a total of 5.2 g lysine /16 g of nitrogen.

However, lysine availability might be decreased due to the interactions of lysine and glucosinolates, polyphenols or phytates (Pedroche *et al*., 2004). In addition, there are two forms of phenolic acids in all plant-derived products: free phenolic acids (such as sinapine) and bound phenolic acids (tannin). The enzymatic oxidation of free phenolic acids produces *o*-quinones, which can bind with lysine and methionine. After such reactions, lysine and methionine become unavailable. Moreover, a high concentration of tannin can suppress the absorption of protein in the small intestine. Binding of tannin to essential amino acids and digestive enzymes might play an important role in modifying nutritional quality (Rozan *et al*., 1996). Murthy and Rao (1986) discovered lysine availability decreased when: 1) the ratio of AITC to mustard 12S protein was increased; 2) the reaction time of mustard 12S and AITC was increased (at pH 10; ratio of AITC to mustard 12S protein, 100; and temperature, 30°C); and 3) when the pH of the system was increased (reaction time, 2 hours; ratio of AITC to mustard 12S protein, 100; and temperature, 30°C).

2.8 Components of thin stillage

Mustafa *et al*. (1999) stated that "Whole thin stillage is the by-product remaining from cereal grain-based ethanol production. The fermentation process utilizes starch while other nutrients such as protein and fibre are concentrated. Stillage can be fractionated into wet distillers grains and thin stillage, which can be fed separately in wet form or dried and marketed as dried distillers grains or dried distillers grains plus solubles".

Wu (1986) reported the yield and composition of fermentation products from barley on a dry basis. He found that it was composed of distiller grains (72%), centrifuged solid (3%) and stillage solubles (25%). Stillage solubles contained protein and ash, 16.7 and 13.1%, respectively, on a dry weight basis.

Dowd *et al.* (1993) stated that the low molecular weight constituents of ethanol stillage from corn were lactic acid, glycerol, alanine, a small amount of ethanol, various non-nitrogenous and nitrogenous acids, polyhydroxyl alcohols, sugars and glucosides. Dowd *et al*. (1994) used GC/mass spectroscopy and HPLC to analyze the components of filtered stillage from sugarcane molasses, citrus waste and sweet whey. They found that the major components in cane stillage were lactic acid, glycerol, ethanol and acetic acid in decreasing order of concentration; in citrus stillage, they were lactic acid, glycerol, *myo*-inositol, acetic acid, *chiro*-inositol and proline. Whey stillage was composed of lactose, lactic acid, glycerol, acetic acid, glucose, arabinitol and ribitol.

Ojowi *et al*. (1996) found that samples of thin stillage from wheat-based ethanol contained dry matter, crude protein, ether extract, neutral detergent fibre, acid detergent fibre, crude fibre, ash, calcium, phosphorus and magnesium (8.4 ± 0.35, 48.5 ± 1.83, 9.6 ± 1.63, 34.5 ± 4.55, 3.4 ± 0.44, 1.5 ± 0.33, 8.0 ± 0.95, 0.37 ± 0.08, 1.09 ± 0.09 and 0.66 ± 0.04% on a dry matter basis, respectively) and cobalt, zinc, manganese and iron (11.9 ± 1.28, 69.4 ± 7.64, 105.1 ± 8.4 and 430.7 ± 105.8 mg/kg on a dry matter basis, respectively). On a dry matter basis, corn thin stillage was found to contain starch, crude protein, neutral detergent fibre and fat (220, 160, 117 and 81 g/kg of thin stillage, respectively; Larson *et al.,* 1993). Barley- and wheat-based thin stillage were reported to contain minerals (calcium, phosphorus, magnesium, copper, iron, manganese, sodium, potassium and zinc; Mustafa *et al*., 1999).

Benke *et al.* (1998) used carbon-13CP (cross polarization)/MASS (magic angle sample spinning) NMR and DR-FTIR (Diffuse reflectance fourier transform infrared) spectroscopy and GC to study sugarcane distillery waste. They found that mono-, oligo- and/or polysaccharides, lactic acid, oxalic acid, malonic acid, succinic acid, fumaric acid, cellulose, lipids and amino acids were present in vinasse (a waste liquor generated in the process of making sugar).

Pandiyan *et al.* (1999) stated that production of major intermediate products such as acetic, propionic and butyric acids occurred during anaerobic conversion of organic waste.

Mustafa *et al.* (2000) reported the chemical composition of thin stillage derived from different cereal grains (Table 2.2). Davis *et al.* (2005) found that wheat stillage contained glucose, 18.0 ± 2.1%, w/w; xylose, 21.5 ± 1.8%, w/w; arabinose, 10.6 ± 0.8%, w/w; galactose (trace); mannose (trace); protein, 31.5 ± 3.4%, w/w; lipid, 19.3 ± 1.5%, w/w; ash, 1.3 ± 0.4%, w/w; acid-soluble lignin, ≤ 1.0%, w/w; and acid-insoluble lignin, ≤1.0%, w/w, on a dry basis.

Kim *et al.* (2008) analyzed the composition of thin stillage from corn dry-grind ethanol products. The results are shown in Table 2.3.

According to Wheals *et al.* (1999), thin stillage contained yeast cells, soluble nutrients and small maize molecules that were high in protein.

The constituents of thin stillage can be categorized into three groups.

1) Yeast metabolites
   - Glycerol (Russell, 2003)
   - Ethanol (Wilkie *et al*., 2000)
   - Succinic acid (Russell, 2003)
   - Glycerophosphorylcholine (Almaguer *et al.,* 2006)
   - Phenylethyl alcohol (Schrader *et al.,* 2004)
2) Bacterial metabolites
   - Isopropanol (Lovitt *et al.,* 1988)
   - Acetic acid (Chin and Ingledew, 1993)
   - Lactic acid (Chin and Ingledew, 1993)
   - 1,3-propanediol (Cheng *et al*., 2006)

Table 2.2. Chemical composition of thin stillage derived from different cereal grains (Mustafa *et al*., 2000).

	Thin stillage			
	Wheat	**Rye**	**Triticale**	**Barley**
Ash (g/kg of DM)	64	69	87	77
Ether extract (g/kg of DM)	59	22	61	60
Carbohydrate composition				
Total carbohydrate (g/kg of DM)	522	634	455	555
Non-structural carbohydrate (g/kg of DM)	341	520	312	394
Neutral detergent fibre (g/kg of DM)	352	232	316	367
Acid detergent fibre (g/kg of DM)	85	81	72	87
Acid detergent lignin (g/kg of DM)	16	12	8	28
Starch (g/kg of DM)	30	20	13	13
Protein composition				
Crude protein (CP)(g/kg of DM)	366	275	397	308
Soluble protein (g/kg of CP)	237	358	321	174
Non-protein nitrogen (g/kg of CP)	200	341	277	160
Neutral detergent insoluble (g/kg of CP)	483	428	437	669
Acid detergent insoluble (g/kg of CP)	61	55	62	143

DM means dry matter

Table 2.3. Composition of thin stillage by (A) cellulosic biomass compositional analysis average of two batches); (B) forage/feed nutritional analysis (Kim *et al.,* 2008).

(A) Cellulosic biomass compositional analysis	
Dry matter	7.7
Glucose (g/L)	0.9
Glucan (oligosaccharide, g/L)	12.4
Xylose (g/L)	0.7
Xylan (g/L)	3.7
Arabinose (g/L)	0.4
Arabinan (g/L)	0.5
Lactic acid (g/L)	16.8
Glycerol (g/L)	14.4
Acetic acid (g/L)	0.3
Butanediol (g/L)	1.9
Ethanol (g/L)	0.6
(B) Forage/feed nutrition composition analysis **Compositional analysis**	
Dry matter	6.2
Crude protein	1.3
Crude fat	1.3
Carbohydrates	2.8
Ash	0.8
Total	100
Forage analysis	
Gross calories (kcal/kg)	28
ADF (Acid detergent fiber)	0.1
Cellulose	0.1
Starch	0.5

Table 2.3 (continued)

Mineral analysis	
Calcium (ppm)	31.0
Phosphorus	0.1
Potassium	0.2
Magnesium	0.1
Sulfur	0.1
Sodium	0.1
Chloride	0.0
Iron (ppm)	8.0
Manganese (ppm)	2.0
Amino acid analysis	
Arginine	0.1
Histidine	0.0
Isoleucine	0.1
Leucine	0.1
Lysine	0.1
Methionine	0.0
Cystine	0.0
Phenylalanine	0.1
Threonine	0.1
Tryptophan	0.0
Valine	0.1
Hydroxyproline	0.0
Aspartic acid	0.1
Serine	0.1
Glutamic acid	0.1
Proline	0.1
Lanthionine	0.0
Glycine	0.1

Table 2.3 (continued)

Alanine	0.1
Tyrosine	0.0
Ornithine	0.0
Total	1.1

All values are % dry basis except where otherwise noted.

3) Wheat metabolites

- Betaine (Kampen, 1993)

Since thin stillage contains protein, its use in protein extraction might increase the protein content of the protein product. In addition, it contains monovalent cations which may enhance protein extraction efficiency. It also contains organic molecules, such as simple alcohols, which might suppress myrosinase activity. Therefore, it may be advantageous to use thin stillage for protein extraction.

2.9 Compounds found in biodiesel glycerol

Biodiesel is a fuel produced from triglyceride oils that can be used to fuel diesel engines. Biodiesel is commonly defined as the monoester of a lower aliphatic alcohol and a fatty acid. It is typically produced by transesterification (alcoholysis) of triglyceride molecules using a catalyst and a monohydric alcohol (methanol, ethanol, etc.) to form monoesters and glycerol (Ma and Hanna, 1999; Van Gerpen, 2007). The transesterification reaction utilizes three moles of alcohol to react with one mole of triglyceride. The reaction yields three moles of fatty acid ester and one mole of glycerol. Typical transesterification reactions are conducted at temperatures between 25 and 100°C, 1.10-1.17 alcohol equivalents and 0.1-0.5% catalyst by weight of oil (Ma and Hanna, 1999). Biodiesel production commonly utilizes either hydroxide or methoxide as the catalyst. Sodium or potassium hydroxide is dissolved in methanol and forms methoxide ions, the actual catalytic agents (Van Gerpen, 2007). The transesterification reaction is presented in Fig. 2.5.

An acid such as citric acid, hydrochloric acid (Van Gerpen, 2007) or sulphuric acid (Ma and Hanna, 1999) is added to the initial wash water to neutralize the catalyst and split the soap into free acids and salts (Van Gerpen, 2007) and to acidify the system after the reaction is completed (Ma and Hanna, 1999). Thereafter, glycerol and the mono-esters are readily separated into two layers based on density. Demirbaş (2003) stated that glycerol is heavier than the esters. Therefore, the upper layer contains mostly esters and the lower layer is mostly glycerol. The flow chart of biodiesel production is shown in Fig. 2.6.

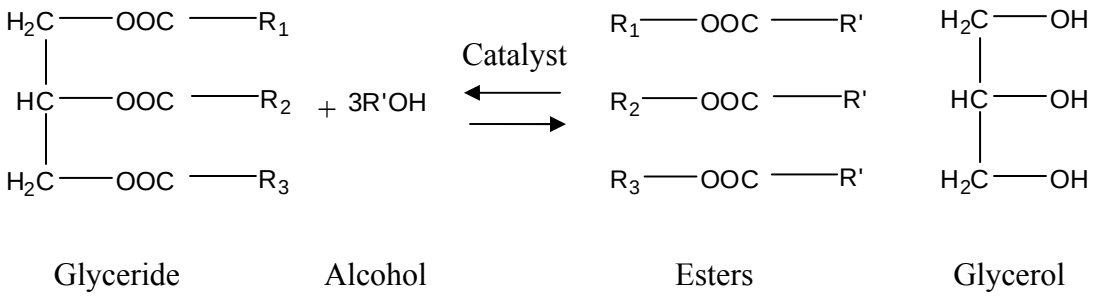


Fig. 2.5 Transesterification of triglycerides with ethanol (modified from Ma and Hanna, 1999).

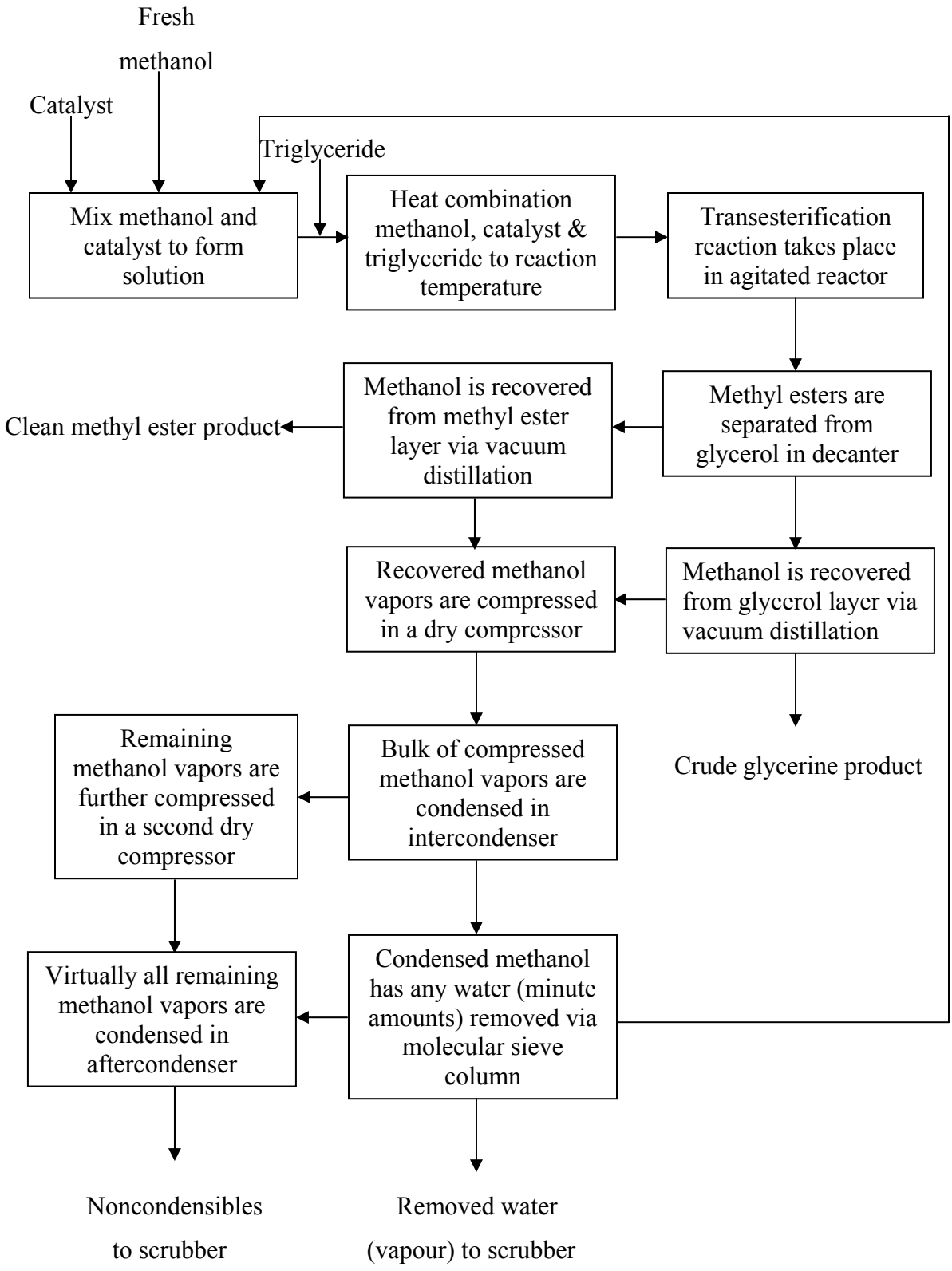


Fig. 2.6 Flow chart of biodiesel production (modified from McDonald, 2001).

A mixture of esters, glycerol, alcohol, catalyst, and tri-, di- and monoglycerides are the products of transesterification of triglycerides (Ma and Hanna, 1999). Thompson and He (2006) reported that 0.3 kg of crude glycerol is produced from 3.79 L of biodiesel. The glycerol layer is composed of glycerol, methanol and the residual catalyst. Crude glycerol (Thompson and He, 2006) contained 23.4-37.5% methanol. Van Gerpen (2007) indicated that a decanter centrifuge or coalescence may be used for glycerol separation. Furthermore, evaporation can be used to recover methanol from glycerin. Free fatty acids produced by soap splitting can be removed because they are not soluble in glycerol. From the information above, it can be concluded that after evaporating methanol and removing fatty acids produced by soap splitting, biodiesel glycerol may still have base-catalyst remaining after neutralization of the base-catalyst. Glycerol from a base-catalyzed, biodiesel process might be used to adjust the pH of other process streams. For example, protein extraction processes often require a high pH to achieve maximum protein extraction efficiency. Therefore, glycerol from the biodiesel industry could be used to adjust the pH of the protein extraction system.

2.10 Use of nuclear magnetic resonance for measurement of compounds in complex biological solutions

Nuclear magnetic resonance **(**NMR) is a spectroscopy tool that has many applications in the analysis of complex materials (Nicholson *et al.,* 1984; Rutar, 1989; Govindaraju *et al.*, 2000; Espina *et al.*, 2001; Holmes and Antti, 2002; Chen *et al.*, 2004; Crockford *et al.,* 2005) and is non-selective as it allows the measurement of a wide range of compounds. Although NMR equipment is costly, the cost for each analysis is typically inexpensive as sample preparation may take only a few minutes (Espina *et al.*, 2001; Holmes and Antti, 2002). Often, little or no sample pre-treatment is required (Nicholson *et al*., 1984). "Due to its relatively high sensitivity and ability to detect numerous tissue metabolites, proton NMR spectroscopy has become well established as a non-invasive technique for studies of biological systems *in vivo* and *in vitro*. Quantitation of the NMR-observable metabolites can provide considerable biochemical information" (Govindaraju *et al*., 2000).

Rutar (1989) used MASS NMR spectroscopy to study plant seed oils. NMR signals of oils were used to determine fatty acid composition in sunflower oil [linoleic acid (76%), oleic acid (11%), saturated fatty acids (13%)]. In addition, linolenic acid, linoleic acid and oleic acid were determined in corn and peanut oil. MASS can improve the resolution of oil spectra of plant seeds and has benefits in improving the spectra from other living organisms. For example, the spectra of small animals and bacteria are improved by MASS as the spins of ^{1}H, ^{13}C and ^{31}P in their natural abundance can be observed.

Govindaraju *et al*. (2000) used proton NMR chemical shifts and coupling constants to identify compounds from brain metabolites. They discovered that the chemical shift and *J*-coupling value of thirty-five metabolites could be detected by *in vivo* and *in vitro* NMR studies of mammalian brain and the results were confirmed with the rat brain extract spectrum.

Espina *et al*. (2001) used NMR-based metabonomic approaches to detect *in vivo* biomarkers of phospholipidosis (PLD), a lipid storage disorder in which excess phospholipids accumulate within cells. The rats were fed with the cationic, amphiphilic drugs chloroquin, aminodarone and DMP777 (a neutrophil elastase inhibitor). These drugs can induce PLD, characterized by lysosomal lamellar body and drug accumulation. Urine of rats was collected and ^{1}H NMR was employed to detect the biomarkers of PLD from urine where proton resonances arising from water, urea and drug-related compounds were eliminated from the analysis. They found that the phenylacetylglycine (PAG) concentration in urine increased when rats were treated with chloroquin and aminodarone. Therefore, they concluded that "using NMR-based metabonomic approaches, PAG was identified as a biological marker for PLD in rats dosed with chloroquin and aminodarone. Furthermore, PLD was predicted to occur in rats dosed at high levels with the pre-development compound DMP777 using this biological marker". Crockford *et al.* 2005 concluded that automated quantitation of compounds can be done using NMR technique in huge NMR data sets with minimal operator interferences.

Unfortunately, if large amounts of water are present in a sample, water suppression is required for NMR analysis to improve the dynamic range of the detector and to improve the spectra of metabolites which overlap with the broad baseline of the

strong water resonance (Chen *et al*., 2004). These authors studied signals of cells and tissues using the water suppression technique in high resolution MASS ^{1}H NMR. They concluded that a water suppression technique based on a combination of selective excitation pulses and field gradient pulses was effective for the acquisition of high resolution MASS ^{1}H NMR spectra of tissue specimens and cell samples. The pulse sequence method allowed the efficient water suppression of spectra derived from intact cells and tissue samples and eliminated signal loss from cellular metabolites. Danielsen and Henriksen (1994) utilized water suppression pulses in ^{1}H NMR spectroscopy to study compounds present in brain water and cerebral metabolites. They also concluded that the quantification method (water suppression pulses in ^{1}H NMR spectroscopy) was easily applied *in vivo* and was generally comparable with the results obtained from other studies.

3 MATERIALS AND METHODS

3.1 Raw Materials

Mustard seed (*Brassica juncea* (L.) Czern) was obtained from Agriculture and Agri-Food Canada, Saskatoon Research Centre, Saskatoon, SK. All seed was from the 2006 harvest and was grown on plots near Saskatoon. Thin stillage (wheat basis) was provided by Pound-Maker Agventures Ltd., Lanigan, SK. Samples of thin stillage were taken on four collection dates (May 18, May 27, May 28 and June 1, 2007) and were stored at 4°C (for up to 4 months) until used. However, micro-organisms can possibly grow over the 4 month storage period. Prior to all analysis of physical properties, chemical properties, chemical constituents, micro-organism content and ion content, samples were centrifuged at 1,053 x *g* for 20 minutes at 4°C (Model Avanti® J-E, Beckman Coulter Canada Inc., Mississauga, ON). Glycerol containing approximately 10 per cent KOH was provided from an industrial biodiesel processor (Milligan Biotechnology Inc., Foam Lake, SK).

3.2 Methods

3.2.1 Defatted meal preparation

Mustard seed was extracted mechanically using a continuous screw expeller (Komet, Type CA59 C; IBG Monforts Oekotec GmbH & Co., Mönchengladbach, Germany) operated at a speed of 6.5 (approximately 93 rpm) using a 6-mm choke. Oil remaining in the presscake was removed using hexane as a solvent (Milanova *et al*., 2006; Oomah *et al*., 2006), and the residual hexane in the defatted meal was removed in a fume hood overnight. Defatted meal was analyzed for protein and oil content (sections 3.2.2 and 3.2.3 respectively).

3.2.2 Protein content

Protein content was determined on 0.5 g samples using the Kjeldahl method [modified from method 981.10 of the A.O.A.C (1990)]. Samples were digested by heating with concentrated H_2SO_4 in a heating/digestion block using a package of Kjeldahl digestion mixture #200 as a catalyst. After digestion, samples were distilled using a steam distillation unit (Model 320, Büchi Analytical Inc., New Castle, DE) with 30% (w/v) NaOH. Boric acid (4%) was used to trap ammonia from the distillation. The distillate was titrated with 0.2N HCl using N-Point indicator as an indicator. The nitrogen content was calculated using equation 3.1.

Equation 3.1: Determination of nitrogen content.

$$\% \text{ N} = (\text{mL of 0.2N HCl sample} - \text{mL HCl blank}) \times \text{normality of HCl} \times 0.014 \times \frac{1}{\text{sample weight}} \times 100 \quad (3.1)$$

The % N of the sample was converted to % protein content by multiplying %N by 6.25.

3.2.3 Oil content

Oil content was determined using a Goldfisch Extractor (Model 22166B, LabConCo Corporation, Kansas City, MO) [modified from method 960.39(a) of the A.O.A.C (1990)]. Approximately 20 g of sample was ground using a coffee grinder to pass through a 1.0-mm screen (approximately 30 seconds). Three grams of ground sample was weighed on a filter paper (Whatman No. 4) and folded. The samples were placed in cellulose thimbles (25 X 80 mm, Ahlstrom AT, Holly Spring, PA). Samples were extracted for 6 hours using 50 mL of hexane as solvent. The hexane was distilled from the oil extraction beakers, after which the beakers were heated at low temperature (30-40°C) using a hot plate placed in a fume hood. The beakers were then transferred to an oven (105°C) for 30 minutes and then allowed to cool to room temperature (approximately 25°C) in a desiccator. The oil content of the samples was calculated according to Equation 3.2.

Equation 3.2: Calculation of oil content by Goldfisch extraction

$$\% \text{ oil} = \frac{(\text{weight of beaker} + \text{oil}) - \text{weight of beaker}}{\text{weight of sample}} \times 100 \quad (3.2)$$

3.2.4 Moisture content

Moisture content was determined by heating a weighed sample [1 g of ground sample using a coffee grinder to pass through 1.0-mm screen (approximately 30 seconds)] at 100-102°C for 16-18 hours or until the weight of the sample was constant [modified from method 950.46 B.a, of the A.O.A.C (1990)]. The samples were allowed to cool to room temperature in a desiccator for at least 1 hour before weighing. The moisture content was calculated using Equation 3.3.

Equation 3.3: Moisture content determination.

$$\% \text{ moisture} = \frac{\text{weight of sample} - \text{weight of dried sample}}{\text{weight of sample}} \times 100 \quad (3.3)$$

3.3 Experimental procedures

3.3.1 Determination of the physical and chemical characteristics, chemical composition, microbial populations and ion content of thin stillage

3.3.1.1 Physical and chemical properties

- *Physical properties*

Osmotic potential

The osmotic potential of centrifuged thin stillage samples was determined using the freezing point depression method of Saupe (2007). The samples were frozen using an ice bath (NaCl was added to the ice bath). The freezing point and supercooling point of the samples were recorded using a digital thermometer. The osmotic potentials of the samples were calculated from the freezing point and the supercooling point.

Viscosity

The viscosity of centrifuged thin stillage samples was measured at 25°C using a Shell cup No.1 (NORCROSS Corporation, Newton, MA). The Shell cup method utilizes a cup with a precision drilled hole in the base. The cup is first immersed in the fluid of interest and the time required for the cup to drain after it is raised from the liquid is measured. Sample viscosity was determined using the conversion chart for Shell cup No.1 that relates viscosity and time.

Density

The density of centrifuged thin stillage was determined at 25°C using a hydrometer (range 1.000 – 1.250 g/cm^3).

- *Chemical properties*

Protein content

The protein content of samples was estimated using Kjeldahl method as described in section 3.2.2.

Moisture content

The moisture content of samples was determined using the oven drying method as described in section 3.2.4.

pH

The pH of samples was measured using a pH meter (SympHony, Model SB90M5, VWR, Mississauga, ON) and a pH electrode [Model glass DJ combination, (SympHony, VWR)].

3.3.1.2 Chemical constituents

The components of centrifuged thin stillage were determined using proton NMR analysis. Prior to analysis, samples were passed through a 0.45-µm membrane filter (PTFE membrane filter, Model TF-450, Pall Corporation, Ann Anbor, MI). Deuterium oxide and dimethylformamide (DMF) were used as solvent and internal standard,

respectively. Since the water peak at around 4.7 ppm was large, NMR proton saturation methods (water suppression) were used to eliminate the water peak (see Figs. 4.2a and 4.2b). Isopropanol, ethanol, glycerol, lactic acid, 1,3-propanediol, acetic acid, succinic acid, glycerophosphorylcholine (GPC), betaine and phenylethanol were used as standards. ^{1}H-NMR (1D) spectra were recorded at 500 MHz (Model AMX 500-MHz, NMR Bruker, Missisauga, ON) and the COrrelation SpectroscopY (COSY) technique (2D NMR spectra) was used to identify and confirm the identification of the compounds present in thin stillage. The NMR results were compared with HPLC analysis (column, detector, and pump described below). Samples were passed through a 0.20-μm membrane filter before injection on LC.

- Column: Nova-Pack-C18 60A°, 4 μm, 3.9 x 150 mm, No. WAT086344, Waters, Dublin, Ireland ; flow rate, 1 mL/minute; external temperature, 90°C; internal temperature, 50°C.
- Refractive index detector: Waters, Model 2414, Serial number 712-385G
- Pump: Waters, Model 510, serial number 512-140795; operating pressure of 1,000 psi.

3.3.1.3 Microbial populations

The total plate count of thin stillage was determined using tryptic soy broth (Massa *et al.*, 1998). Yeast and mould content were determined using Rose Bengal Agar with the inclusion of an antibacterial (rehydrated Rose Begal antimicrobic supplement) (adapted from Tournas *et al.*, 2001). Total plate count and yeast and mold content were tested at 37°C.

3.3.1.4 Ion contents

Centrifuged and filtered thin stillage samples (centrifuged at 7,696 x *g* for 20 minutes at 4°C and filtered through a 0.2-μm filter membrane) were sent to Saskatchewan Research Council Analytical Services for measurement of cation (calcium, magnesium, sodium and potassium) and anion (chloride, sulfate, nitrate, hydroxide, bicarbonate and carbonate) content. Calcium, magnesium, sodium, potassium and sulfate were assayed using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES; Model

IRIS, Thermo Jarrell-Ash, Franklin, MA). Sample aerosols were injected into plasma, a stream of Argon gas ionized by an applied radio frequency field, which subjected atoms to temperatures of 6,000 to 10,000K. A computer controlled spectrometer was used to measure the emission spectra of the ionized atoms optically and a charge injection device detector was utilized to monitor a configured wavelength in a computer-controlled environment (according to the method of Eaton *et al.*, 2005). Chloride ion was assayed by the mercuric thiocyanate colorimetric method on a Discrete colorimetric analyzer (Model 200, Aquakem, Labmedics Limited, Manchester, U.K). Chloride ion replaced the thiocyanate ions in mercuric thiocyanate. The released thiocyanate ions were then reacted with ferric ions to form ferric thiocyanate, a colored complex, with absorbance measured at 480 nm using the Discrete colorimetric analyzer (Model 200, Aquakem, Labmedics limited, Manchester, U.K). The absorbance of the samples was compared to the absorbance of chloride solutions of known concentration (as the standard curve; according to standard methods of Eaton *et al.*, 2005). Nitrate content was determined by reducing nitrate to nitrite in a hydrazine sulfate solution. The nitrite ion was then diazotized with sulfanilamide and coupled with NNED (N-1-naphthylethylene diamine dihydrochloride) to form an azodye. This solution was analyzed colorimetrically using the Discrete colorimetric analyzer (Model 200, Aquakem, Labmedics limited, Manchester, U.K). Hydroxide, bicarbonate and carbonate concentrations were determined by the titration method using sulfuric acid to a fixed end point (according to the method of Eaton *et al.*, 2005).

3.3.1.5 Prediction of types and amounts of salts

The types and amounts of salts present in thin stillage were predicted using a Levenberg-Marquardt algorithm in the Solver Add-in program (a least square model for multiple linear regression to minimize the sum of the squares of deviations between parameters) of Excel® program implementation (Microsoft Corporation-Redmond, WA) [adapted from Levenberg (1944); Marquardt (1963)].

3.3.2 Design of a model thin stillage of defined composition

The impact of thin stillage components on protein extraction was studied by preparing thin stillage with a defined composition. Thin stillage composition was approximated by developing a solution that contained most of the thin stillage components observed in section 3.3.1. The model solution was developed by choosing a series of salts and organic compounds that, when mixed with water, produced a solution that had a composition similar to that found in thin stillage samples. Table 4.1 (in the results section) was derived from a spreadsheet of all salts and organic compounds found in thin stillage samples from Pound-Maker Agventures Ltd, Lanigan, SK. Using a least squares model that minimized the differences between the experimental data obtained from thin stillage and a synthetic mixture of known compounds, a recipe for thin stillage was developed. The model thin stillage was prepared and its physical and chemical properties, components, and ion contents were studied as determined in the section 3.3.1.

The cost of glycerophosphocholine (GPC) was excessively high and thus it was necessary to isolate it from thin stillage samples so that it could be included in the model thin stillage. The process for recovering GPC and betaine was developed by Jia, Y. and Reaney, M.J.T. (personal communication). The GPC and betaine used in this research were prepared from 3 L of thin stillage (batch from October 5, 2007). The stillage was placed in three 2000-mL round bottom flasks (1L/flask) and the water was evaporated under vacuum at 70°C using a rotary evaporator (Model R-200, BÜCHI Rotavapor, Brinkmann Instruments Inc., Westbury, NY). The concentrated thin stillage obtained was then mixed and stirred continuously at room temperature (25°C) with ~200 mL of acetone/flask to precipitate the GPC. After mixing, the brown, viscous syrup was collected using a Buchner funnel under vacuum and rinsed with fresh acetone several times. The solid fraction was then mixed with acetone and separated three more times. The solid matter obtained from the acetone extraction was mixed with ~150 mL of methanol three times. The brown liquid obtained from the methanol extraction was rich in GPC, betaine and lactic acid. These were obtained from the methanol solution using a rotary evaporator. The brown, viscous liquid was then diluted with water so that the final concentrations of GPC, betaine and lactic acid, as analyzed by NMR, were 0.0343, 0.0362 and 0.0513 g/mL, respectively.

3.3.3 Protein extraction using thin stillage

3.3.3.1 Comparison of the extraction efficiency of two methods for extracting protein from defatted *B. juncea* meal

Method 1 Ten grams of ground defatted meal was mixed and stirred continuously for 1 hour using a stirrer with 300 mL of centrifuged thin stillage (batch from May 27, 2007) at room temperature (approximately 25°C; Diosady *et al*., 2005). After mixing, the solution was centrifuged at 5,000 x *g* for 10 minutes (Milanova *et al*., 2006) at 4°C. The supernatant was freeze dried (Model 77540, LabConCo Corporation, Kansas City, MO) and the protein content of the freeze-dried protein and the undissolved solids were analyzed using the Kjeldahl method (section 3.2.2). The moisture content of the undissolved solids was also determined (section 3.2.4).

Method 2 Ten grams of defatted meal was blended in a blender (Eberbach Corporation, Ann Arbor, MI) with 100 mL of centrifuged thin stillage batch from May 27, 2007 (ratio 1:10) for 2 minutes. The solid material was removed by screening using a No 20 US standard sieve (mesh size = 840 μm; VWR company, West Chester, PA). After that, the solid material was then blended with centrifuged thin stillage as described above 2 more times (2 minutes each time). The liquid from 3 times extraction that passed through the screen was collected and centrifuged at 5,000 x *g* for 10 minutes at 4°C. The supernatant was freeze-dried and the protein contents of the freeze-dried protein and the undissolved solid were analyzed using the Kjeldahl method (section 3.2.2). The moisture content of the undissolved solids also was determined (section 3.2.4).

3.3.3.2 Preparation of protein titration curves (adapted from Nelson and Cox, 2005)

A salt solution was used to increase the amount of protein extracted from defatted meal. Five grams of ground defatted meal were mixed and stirred continuously for 2 hours using a stirrer with 150 mL of 0.5 M sodium chloride solution at room temperature (approximately 25°C). The solution was centrifuged at 5,000 x *g* for 10 minutes at 4°C. The small molecules (present or generated in the system) in the supernatant were removed by dialysis employing SpectraPor molecular porous membrane tubing at a ratio

of 1:1000 supernatant to distilled water (the dialysis tubing has 3,500 molecular weight cut-off (MWCO), diameter 29 mm, width volume/length 6.4 mL/cm, purchased from Spectrum Laboratories Inc., Rancho Dominguez, CA). The dialysis tubing and contents were transferred to fresh distilled water three times a day for 5 days until the conductivity of water after 8 hours of dialysis was equal to that of distilled water. The protein solution from dialysis was lyophilized and 0.05 g of freeze-dried protein was dissolved in 25 mL of water with the pH adjusted to approximately 12 using 30% (w/v) NaOH. Titration curves were prepared by titration with 0.1 mL aliquots of 0.18N HCl solution. Titration was continued until approximately pH 2. The volume of HCl solution was plotted against the pH to obtain the protein titration curve. The first derivative of the protein titration curve was plotted against the volume of HCl solution. The isoelectric point was determined from the first derivative graph.

3.3.3.3 Determining the efficiency of protein extraction

Patent literature (United States patent No. 6,992,173 B2) teaches that protein recovery from brassica family is greatly enhanced by the use of salt due to the high content of salt soluble proteins. The effect of the acidity (pH) and the ion content of centrifuged thin stillage on protein extraction were evaluated in order to determine optimum extraction conditions for recovering both albumins (water soluble) and globulins (salt soluble). In this study, NaCl was utilized for ion content adjustment of centrifuged thin stillage, with a range of final concentrations from $3.4x10^{-2}$ to 1.16 M, prior to protein extraction. Ground defatted meal (5 g) was mixed with 150 mL of centrifuged thin stillage (batch from May 27, 2007). The pH of the system was adjusted to pH 7.6, 8, 9, 10 or 10.4 using glycerol from a biodiesel plant (~ 10% KOH) and 1N HCl (details of salt concentration and pH are given in Table 3.1). The mixture was stirred continuously for 2 hours at room temperature (approximately 25°C). After stirring, the solution was centrifuged at 5,000 x *g* for 10 minutes at 4°C. The supernatant was freeze dried and the protein content of the freeze-dried protein and the undissolved solids were analyzed using the Kjeldahl method (section 3.2.2). The moisture content of the undissolved solids also was determined (section 3.2.4). The conditions that provided the maximum protein extraction efficiency from this study (ion content 1 molar and pH 10)

was used in subsequent studies of the effects of thin stillage constituents on protein extraction efficiency. A control extraction with an alkaline sodium chloride solution prepared with NaCl (1M) and made to a pH of 10 in distilled water, here after called sodium chloride solution, was conducted. The quality of extracted protein of the control and thin stillage extraction was compared.

3.3.3.4 Comparison of the relative efficiency of protein extraction using thin stillage, model thin stillage and sodium chloride solution

Efficiency of protein extraction

Ultrafiltration of thin stillage and model thin stillage were used to remove protein from thin stillage prior to using the thin stillage for protein extraction from mustard meal. Centrifuged thin stillage (batch from May 27, 2007) and model thin stillage were filtered through a 3,000 MWCO regenerated cellulose membrane (Millipore Corporation, Bedford, MA) using a stirred ultrafiltration cell (Model 8400, purchased from Millipore Corporation, Bedford, MA), running at 55 psi with a shear rate of 200 rpm. A salt concentration of 1M and a pH of 10 were selected to obtain the highest protein extraction efficiency (based on results from section 3.3.3.3). The ion content of thin stillage and model thin stillage were increased by adding 1 mole per L of NaCl prior to their use for protein extraction. Ground, defatted meal (5 g sample) was mixed with 150 mL of ultra-filtered thin stillage, model thin stillage or sodium chloride solution. Glycerol from a biodiesel plant and 1N HCl were employed for adjusting the pH of the mixture to 10. The mixture was stirred continuously for 2 hours using a stirrer at room temperature (25°C) and then was centrifuged at 5,000 x *g* for 10 minutes at 4°C. The supernatant was dialyzed using Spectra/Por molecular porous membrane tubing (3,500 MWCO, Spectrum Laboratories, Inc.) at a ratio of 1:1000 supernatant to deionized distilled water. Water exchange with fresh deionized water was repeated three times a day until the conductivity of permeate water was equal to that of deionized distilled water after 8 hours of dialysis (approximately 5 days). The protein solution obtained by dialysis was freeze dried. The freeze-dried protein and undissolved solids were analyzed for protein content (section 3.2.2) and the moisture content of the undissolved solids was determined (section 3.2.4).

Protein extracted from thin stillage, model thin stillage or sodium chloride solution (duplicate samples) was pooled together by extraction solution type and then analyzed to determine the molecular weight, peptide sequencing, amino acid composition, digestibility and lysine availability of the protein.

Molecular weight (SDS-PAGE)

Molecular weight of extracted protein was determined by the SDS-PAGE technique. SDS-PAGE was conducted using a Mini-PROTEAN 3 cell running system and Ready Gel Tris-HCl Gel**,** 4–15%, 10-well, 30 µL, 8.6 x 6.8 cm (W x L) (Bio-Rad Laboratories, Hercules, CA). Protein (20 µg) was loaded into the wells. The SDS-PAGE buffer system followed the method of Laemmli (1970). Deionized water, 0.5M Tris-HCl, pH 6.8, glycerol, 10% (w/v) SDS and 0.5% (w/v) Bromophenol Blue were used as the sample buffer. Electrode buffer (10x), pH 8.3 was utilized as the running buffer. Coomasie Blue-R250 (0.1%) in fixative (40% methanol and 10% acetic acid) was used as the staining solution and a mixture of 40% methanol and 10% acetic acid was used as a destaining solution to remove the background. SeeBlue® Plus2 Pre-Stained Standard was used as a broad range marker.

Peptide sequencing (Aluko *et al*., 2004)

Protein bands of different molecular weights obtained from SDS-PAGE were cut out and samples were sent to the Plant Biotechnology Institute, Saskatoon, SK for trypsin digestion and subsequent mass spectral analysis. Sonication in 5% trifluoroacetic acid (TFA)/50% acetonitrile (ACN) was used to recover the tryptic peptides from the gels and the solutions were dried in a SpeedVac System (Model DNA 120, Thermo Savant DNA SpeedVac System, Thermo-Savant Speed Vac, BioSurplus, San Diego, CA). The peptides were dissolved in the same TFA/ACN solution and then mixed with α-cyano-4-hydroxy-cinnamic acid in 0.1% TFA/50% ACN. The solutions were applied to a matrix-assisted laser desorption ionization (MALDI) plate, and allowed to dry. MALDI time-of-flight (TOF) mass spectrometry analysis was conducted using a Voyager-DE STR mass spectrometer (Applied Biosystems, Bedford, MA) in the reflection mode. Proteins were identified by peptide mass fingerprinting using the *m/z* ratios of trypsin peptides and the

MS-FIT program of the Protein Prospector software (publicly available software, provided by University of California, San Francisco (www.prospector.ucsf.edu)) with autocatalytic trypsin fragments as internal calibration standards.

Amino acid composition

The amino acid composition of extracted protein was determined using the method of Llames and Fontaine (1994). Performic acid and hydrocholic acid were used to oxidize and hydrolyze, respectively, the protein. Hydrolysates were analyzed for amino acids using a AMINOSep ion exchange column (Transgenomics Inc., Omaha, NE) and post column derivitization with OPA (o*rtho*-phthaldialdehyde). An Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) and fluorescence detector (RF-551, SHIMADZU, Columbia, MD) were used.

In vivo digestibility

Digestibility of extracted protein was determined using the multienzyme technique of Hsu *et al*. (1977). Lyophilized protein samples extracted from thin stillage, model thin stillage and sodium chloride solution were dissolved in water (6.25 mg protein/mL). The protein solutions (25 mL) were adjusted to pH 8 with 0.1 N HCl and/or NaOH while stirring at 37°C in a water bath. The multienzyme solution (1.6 mg/mL trypsin, 3.1 mg/mL chymotrypsin and 1.3 mg/mL peptidase) was prepared in water adjusted to pH 8. The multienzyme solution was stored in an ice bath. Digestions were conducted by adding the multienzyme solution (2.5 mL) to 25 mL of protein solution while stirring at 37°C. The pH of the protein solution was recorded over a 10-minute period. The digestibility of the protein was calculated using the equation $Y = 210.46 – 18.10 X$, where X = pH at 10 minutes. The enzyme blank was run in 0.001M phosphate buffer, pH 8.

Lysine availability

Lysine availability of extracted protein was measured using a fluorometric technique (Ferrer *et al.*, 2003). A reconstituted protein sample (50 μL) containing 0.3–1.5 mg of protein was mixed with distilled water (950 μL) and then 1 mL of sodium dodecyl sulphate (SDS) solution (120 g/L) was added. Three mL of OPA solution (80 mg OPA in 2 mL 100% ethanol, 50 mL sodium tetraborate buffer (pH 9.7–10.0), 5 mL SDS (200 g/L) and 0.2 mL β-mercaptoethanol) were added to the 100 μL of reconstituted protein solution. The mixture was incubated for 2 minutes at 25°C using a shaker table. Fluorescence was measured between 2 and 25 minutes at 455 nm (Pi-Star 180 CD spectrophometer, Applied Photophysis Ltd., Leatherhead, UK). The absorbance value of the protein sample was corrected by the absorbance of a blank and the absorbance of the interference. The blank mixture (1 mL of SDS solution, 120 g/L and 1 mL of distilled water) was incubated at 4°C for 12 hours. After that the blank mixture was sonicated (Branson 3200R-1, Sonicator, Branson Cleaning Equipment Company, Danbury, CT) for 15 minutes at 25°C. Interference in the determination comes from small peptides, free amino acids and amines. In order to determine the interference, trichloroacetic acid was added to precipitate protein in the sample solution and the sample solution was then centrifuged to remove protein. The sample preparations to determine the absorbance of the interference were prepared using 2 mL of the mixture. The mixture was prepared using 900 μL of distilled water and 1 mL of SDS solution (120 g/L) and was added to 100 μL of supernatant from the centrifuged mixture [2 mL of reconstituted protein sample mixed with 2 mL of 10% (w/v) trichloroacetic acid and centrifuged at 827 x *g* (Allegra X-22R, Centrifuge, Beckman Coulter Canada Inc., Mississauga, ON)].

A calibration curve was prepared using a mixture of casein from bovine milk at concentrations ranging from 0.1 to 3 mg/mL (lysine content varied from 8.48×10^3 to 0.169 mg lysine/mL) using 0.1M sodium tetraborate buffer, pH 9, as the solvent.

Color

Color of extracted protein was determined using HunterLab (ColorFlex, Hunter Associates Laboratory, Inc. Reston, VA). The illuminate was set at D65 (daylight), and observer was set at 10° (the angle of the light entering to the sample). Result reports in L,

a, b system. L (positive represents white and 0 represents dark), a (positive is green and negative is red) and b (positive is yellow and negative is blue).

3.3.3.5 Large-scale protein extraction

The ratio of ground defatted meal to thin stillage (1:30) in section 3.3.3.3 may be impractical for industrial scale up due to the large amount of liquid that would require evaporation after processing. The ratio of ground defatted meal to thin stillage was increased to 1:5 to simulate a more practical industrial process. One hundred and eighty grams of ground defatted meal was mixed with 900 mL of centrifuged thin stillage (from batch of December 13, 2007) with the salt concentration adjusted to 1M using NaCl. The pH of the mixture was adjusted to pH 10. The mixture was stirred continuously for 2 hours at room temperature (approximately 25°C) and was then centrifuged at 5,000 x *g* for 10 minutes. The supernatant was dialyzed using Spectra/Por molecular porous membrane tubing (Spectrum Laboratories, Inc.), 6-8,000 MWCO, at a ratio of 1:20 supernatant to deionized distilled water. The undissolved solids (extracted meal) were re-extracted two more times with 900 mL of centrifuged thin stillage for 2 hours each time (ratio 1:5 extracted meal:centrifuged stillage) The supernatant from each extraction was dialyzed as described above. Water exchange with fresh deionized water was repeated until the conductivity of the permeate water was equal to that of deionized distilled water after 8 hours of dialysis. The protein solution from the three extractions was mixed well and sub sampled (400 mL/aluminum tray for three trays). Lyophilized protein (0.1 g) and undissolved solids (0.5 g) were analyzed for protein content using the Kjeldahl method (section 3.2.2). The moisture content of the undissolved solids was also determined (section 3.2.4).

3.3.3.6 Comparison of protein extraction efficiency with that of a published protocol

Using the protocol of Milanova *et al*. (2006), ground defatted meal (20 g) was mixed with 200 mL of 0.6 M NaCl solution (the pH of the NaCl solution was adjusted to 6.2 using 0.1N HCl solution). The pH of the mixture was adjusted to 6.8 and stirred continuously for 30 minutes using a stirrer at room temperature (approximately 25°C)

and then centrifuged at 5,000 x *g* for 10 minutes. The supernatant was ultra-filtrered using a stirred cell with a 3,000 MWCO membrane until the volume of protein solution was approximately 10 mL. Subsequently, the protein solution was diafiltrated (3,000 MWCO) using 500 mL of 0.6 M NaCl solution, pH 6.0, until the volume of the solution was approximately 20 mL. The concentrated protein and salt solution (20 mL) was then diluted 15-fold (to 300 mL) with chilled water (4°C) to form a discrete protein (micelle) in the aqueous phase. The protein micelle was allowed to settle to form an amorphous, gelatinous, protein micelle mass. The protein mass was centrifuged at 5,000 x *g* for 10 minutes to separate protein particles from the liquid. Sedimented protein was lyophilized. Freeze-dried protein (0.1 g) and undissolved solids (0.5 g) were analyzed for protein content using the Kjeldahl method (section 3.2.2). The moisture content of the undissolved solids (1 g) was also determined (section 3.2.4).

3.3.3.7 Determination of allyl isothiocyanate (AITC) in defatted meal and protein extracts

AITC content in ground defatted meal

AITC content in ground defatted meal was analyzed using method 15.0 of the American Spice Trade Association (ASTA) (1997). Ground defatted meal (5 g) was mixed with 100 mL of water in a 250-mL round-bottom flask. The mixture was stirred continuously for 2 hours at 37°C in a water bath. Subsequently, 20 mL of 95% ethanol was added to the mixture to stop the reaction. The mixture was then distilled and the condensed solution was collected in a volumetric flask containing 10 mL of NH_4OH solution (1 volume of NH_4OH:2 volumes of distilled water) until the total volume in the volumetric flask was 60 mL. After distillation, the delivery tube was rinsed with distilled water and 20 mL of 1N $AgNO_3$ solution was added to the volumetric flask. The total volume of the solution in the volumetric flask was then adjusted to 100 mL. Subsequently, the volumetric flask was wrapped with aluminium foil and the solution was held at room temperature (approximately 25°C) overnight. The solution was then filtered and divided into two volumetric flasks, 50 mL in each. Five millilitres of concentrated HNO_3 was added to each flask. The contents were titrated with 0.1 N NH_4SCN solution (standardized by titrating with standard $AgNO_3$ solution) using 5 mL

of $FeNH_4(SO_4)_2.12\ H_2O$ solution (10-12% by weight) as an indicator. The amount of AITC was calculated using equation 3.4.

$$\% \text{ AITC content} = \frac{[(mL \times N)AgNO_3 - 2(mL \times N)NH_4SCN] \times 0.04958}{\text{sample weight}} \times 100 \qquad (3.4)$$

AITC content in free protein solution

Ground defatted meal (20g) was mixed with centrifuged thin stillage (100 mL) and adjusted to a salt concentration of 1M using NaCl. The pH of mixture was adjusted to 10 using glycerol:KOH. The mixture was stirred continuously for 2 hours at room temperature (approximately 25°C) and then centrifuged at 5,000 x *g* for 10 minutes. A portion of the supernatant was centrifuged at 7,696 x *g* for 10 minutes. In order to know the peak region of AITC in NMR spectra in the protein solution, the AITC was added in protein solution as the standard compound. The increases of the peak at AITC region was observed from ^1H NMR spectra. AITC content of the supernatant was determined with a Bruker NMR 500MHz using the water suppression technique and DMF as an internal standard.

The remaining supernatant was passed through a 5,000 MWCO membrane using Amicon Ultra® centrifugal filter devices (Millipore Corporation, Billerica, MA) and was used for AITC analysis by the NMR technique with DMF as an internal standard. The result was compared with the prior AITC content of supernatant of protein solution (without passing through a 5,000 MWCO membrane).

3.4 Statistical analysis

Statistical analysis was completed in two replications, and analysis of variance (ANOVA) and Duncan's multiple-range test were performed using the SPSS program (SPSS 12.0, SPSS, Inc., Chicago, IL; Hung *et al.*, 2007). A P value of 0.05 was used as the level of significance.

To study the efficiency of protein extraction, response surface methodology (RSM) was utilized. Five levels of each factor (pH and salt concentration) (Table 3.1) were chosen based on a central composite rotatable design (CCRD) (Kuehl, 2000). The

statistical analysis efficiency of protein extraction (section 3.3) was performed using the Statistical Analysis System (SAS for Windows®, Release 8.02, SAS Institute Inc., Cary, NC). For the model of CCRD and for creating response surface plots, the following second-order polynomial equation was utilized (equation 3.5).

$$Y = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} b_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} b_{ij} x_i x_j \qquad (3.5)$$

Y is the estimated response, protein content, b_0 is a constant. Linear, quadratic and interaction terms are in order, b_i, b_{ii} and b_{ij}. The independent variables, pH and salt concentration, are presented as x_i and x_j. The effect of the two independent variables studied (pH and salt concentration) was presented by surface plot.

Table 3.1 Coded value of independent variables used to study the effect of pH and salt concentration on protein extraction efficiency in section 3.3.

Independent variable	**Code level**				
	-1.414	-1	0	1	1.414
pH	7.6	8.0	9.0	10.0	10.4
Salt content (M)	$3.4x10^{-2}$	0.2	0.6	1.0	1.16

4 RESULTS

4.1 Composition of *B. juncea* (L.) Czern mustard seed and defatted meal

The protein, oil and moisture contents of mustard seed and defatted meal were determined (Table 4.1).

Table 4.1 Composition of *B. juncea* (L.) Czern seed and defatted meal.

	Protein content (%)	**Oil content (%)**	**Moisture content (%)**
Seed	25.10 ± 0.05	38.7 ± 0.2	4.80 ± 0.09
Defatted meal	36.70 ± 0.01	4.10 ± 0.01	6.30 ± 0.09

Results are means of duplicate analyses of a single sample.

4.2 Physical and chemical characteristics, chemical composition, microbial populations and ion contents of thin stillage

4.2.1 Physical and chemical characteristics

Four batches of centrifuged thin stillage were analyzed with respect to their physical and chemical properties (Table 4.2). The major component of thin stillage was water (moisture content > 95%, w/w). Thin stillage contained small amounts of protein (approximately 0.5-0.6%, w/w) and was acidic (pH 3.7-4.0). In addition, osmotic potential and viscosity of thin stillage ranged from -0.94 to -0.97 and 1.60 to 1.73 centipoises, respectively while density at 25°C was 1.01 g/cm^3.

Table 4.2 Physical and chemical characteristics of four batches of thin stillage.

Characteristics	Batch			
	May 18, 2007	**May 27, 2007**	**May 28, 2007**	**June 1, 2007**
Physical characteristics				
Osmotic potential (MPa)	- 0.96 ± 0.01	- 0.97 ± 0.00	- 0.95 ± 0.00	- 0.94 ± 0.01
Viscosity (25°C) (centipoise)	1.7 ± 0.1	1.6 ± 0.0	1.7 ± 0.1	1.6 ± 0.0
Density (25°C) (g/cm^3)	1.01 ± 0.00	1.01 ± 0.00	1.01 ± 0.00	1.01 ± 0.00
Chemical characteristics				
Protein content (%, w/w)	0.60 ± 0.00	0.60 ± 0.01	0.50 ± 0.02	0.60 ± 0.02
Protein content (corrected for glycerophosphorylcholine (GPC) and betaine phosphate) (%, w/w)	0.6	0.5	0.5	0.6
Protein content (%, w/w) (dry basis)	19.3	20.6	18.5	23.3
Moisture content (%, w/w)	96.70 ± 0.03	97.30 ± 0.01	97.10 ± 0.01	97.30 ± 0.02
pH	3.90 ± 0.01	4.00 ± 0.00	3.80 ± 0.01	3.80 ± 0.00

Results are means of duplicate analyses of a single sample of each batch.

4.2.2 Chemical constituents

The organic compounds present in thin stillage may complicate its use as a protein extraction medium. Therefore, it was important to identify the organic compounds present in thin stillage. Organic compounds that contain protons, such as ethanol, can be identified and quantified using proton (^{1}H) NMR spectrometry. The ^{1}H NMR spectrum of ethanol in D_2O was compared to the spectrum of thin stillage with added ethanol. The ^{1}H NMR spectrum of ethanol (Fig. 4.1) showed three characteristic chemical shifts (triplet at 1.1 ppm, quartet at 3.6 ppm and singlet at 4.7 ppm).

From the ^{1}H NMR spectrum of thin stillage (May 18, 2007 sample) (Fig. 4.2a), it was observed that the area of the water peak (4.7 ppm) prevented observations of peaks between approximately 3.7 and 5.7 ppm.

Since the water peak in the ^{1}H NMR spectrum was too large to allow accurate measurement of minor compounds, an NMR proton saturation method (water suppression) that irradiated the sample at the water frequency (Chen *et al*., 2004) was employed to suppress the water signal. A water suppression pulse sequence was utilized and the resulting spectrum (Fig. 4.2b) showed virtual elimination of the water peak.

The thin stillage spectrum indicated the presence of several organic components. By adding standard compounds to thin stillage and observing the impact on ^{1}H NMR signals, and using ^{1}H NMR and the COSY technique (2-D NMR spectrum), it was possible to conclusively identify each compound in thin stillage. Pure standards of isopropanol, ethanol, lactic acid, 1,3-propanediol, acetic acid, succinic acid, glycerophosphocholine (GPC), betaine, glycerol and phenethyl alcohol were added to particulate-free thin stillage. The ^{1}H NMR spectra and 2-D NMR spectra of thin stillage, before and after addition of each of the organic chemicals, were recorded. Proton spectra of thin stillage, with and without added phenethyl alcohol, are presented as an example of the NMR method of confirming the presence of a compound in thin stillage. The spectra used to confirm the presence of the remaining nine compounds are presented in Appendix A.

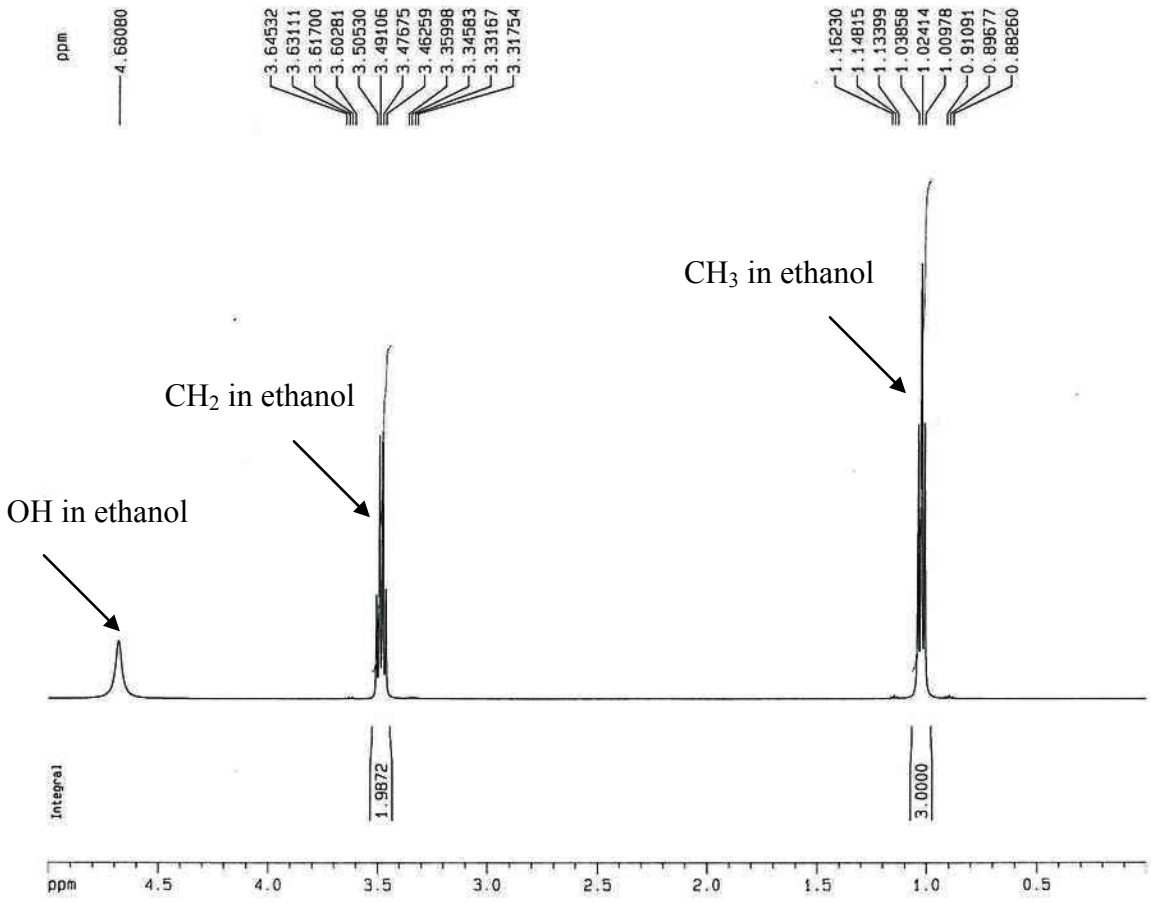


Fig. 4.1 ^{1}H NMR spectrum at 500 MHz of ethanol (CH_3CH_2OH).

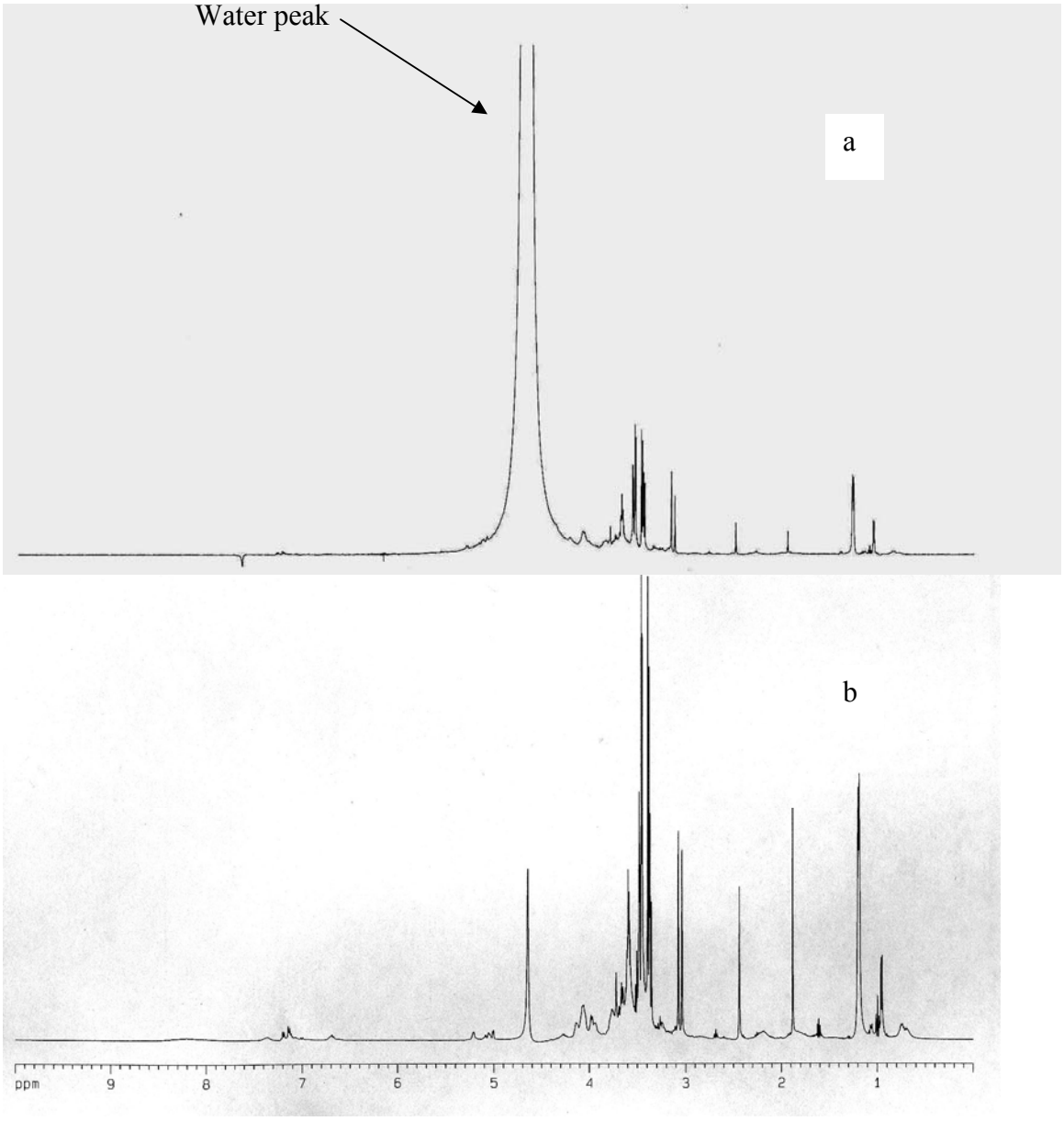


Fig. 4.2 ^{1}H NMR spectra at 500 MHz of thin stillage (May 18, 2007 sample):

a) without water suppression;

b) with water suppression.

The computer-estimated chemical shifts of phenethyl alcohol protons are presented in Fig. 4.3. As shown, phenethyl alcohol was predicted to have protons with chemical shifts at 2.77, 3.66, 4.58, 7.27, 7.29 and 7.40 ppm. When phenethyl alcohol was added to thin stillage, peaks at these chemical shifts were increased, as shown in Fig. 4.4. In addition, two dimensional NMR spectra may be used to show the interaction of protons on adjacent carbons to further confirm the identification of a compound. The COSY spectrum of thin stillage (Fig. 4.5) revealed the presence of phenethyl alcohol by the presence of correlated peaks observed at x, y coordinates of 7.29, 3.66 and 2.77 ppm.

Furthermore, ^{1}H NMR spectra of thin stillage taken after addition of isopropanol, ethanol, lactic acid, 1,3-propanediol, acetic acid, succinic acid, GPC, betaine and glycerol showed increased areas of peaks with chemical shifts of 1.02 (isopropanol), 1.1 (ethanol), 1.2 (lactic acid), 1.5-1.8 (1,3 propanediol), 2.05 to 2.09 (acetic acid), 2.47 (succinic acid), 3.03 (GPC), 3.07 (betaine), 3.3-3.6 (glycerol) and 7.06-7.36 ppm (phenethyl alcohol) when compared with spectra of particulate-free thin stillage (Appendix A). In addition, 2D NMR spectra confirmed the results from the ^{1}H NMR spectra. Therefore, the thin stillage contained isopropanol, ethanol, lactic acid, 1,3-propanediol, acetic acid, succinic acid, GPC, betaine, glycerol and phenethyl alcohol.

The ^{1}H NMR technique was also used to quantify the constituents of thin stillage. Dimethylformamide (DMF) was chosen as the internal standard. ^{1}H NMR spectra of thin stillage, with and without added DMF, are shown in Fig. 4.6. ^{1}H NMR results were compared with results from HPLC. A typical HPLC chromatogram is provided in Fig. 4.7. ^{1}H NMR and HPLC data are presented in Table 4.3. The concentrations of lactic acid and acetic acid from HPLC confirmed the ^{1}H NMR results. HPLC results also identified the residual carbohydrates present in thin stillage (dextrin, maltotriose and maltose monohydrate). HPLC measurements of glucose were not consistent within the same sample and the data were excluded from Table 4.3. NMR lacked sufficient resolution to accurately distinguish individual carbohydrates. Variances in the baseline of the HPLC chromatogram are the result of changes in Refractive index detector amplification setting made 2, 10 and 25 minute.

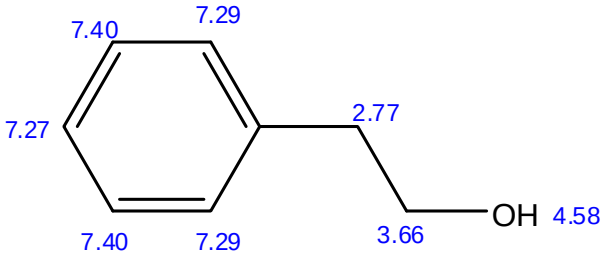


Fig. 4.3 Structure and proton chemical shifts (ppm) of phenethyl alcohol

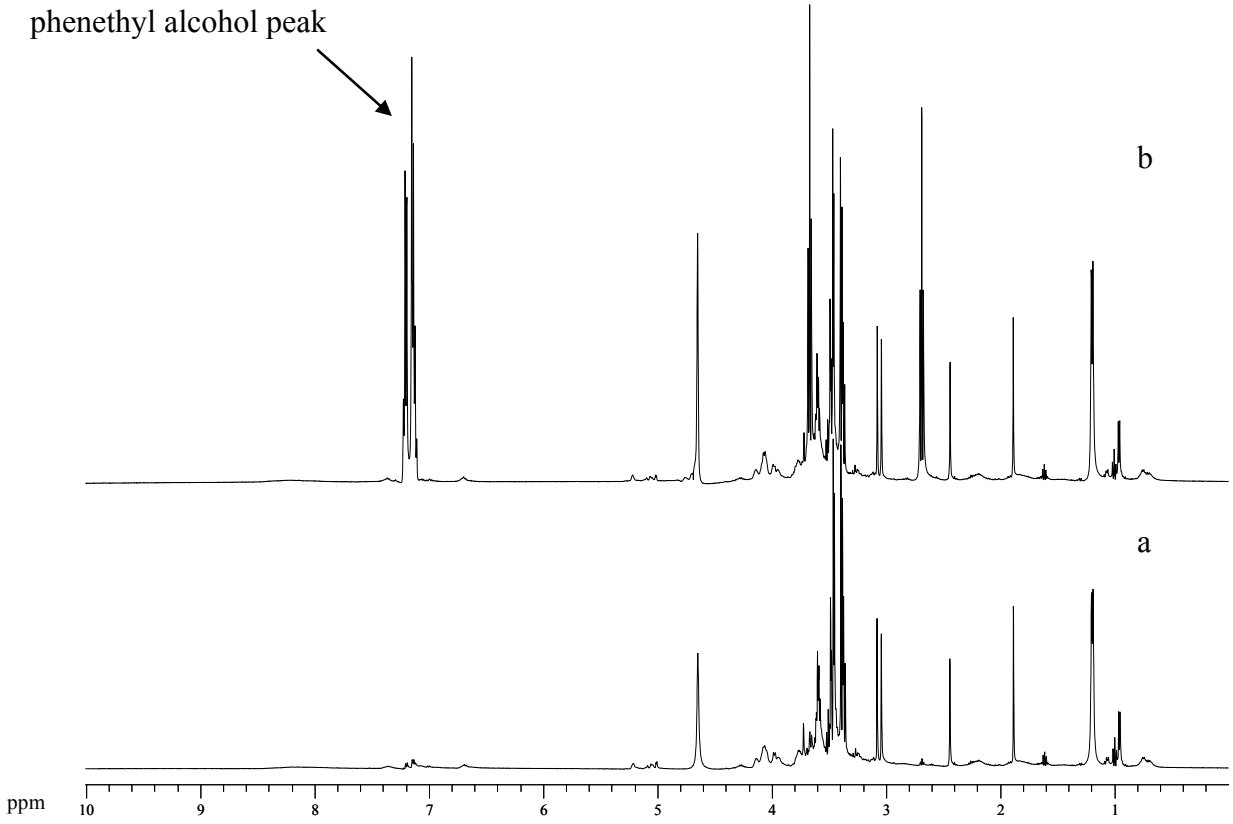


Fig. 4.4 ^{1}H NMR spectra at 500 MHz of thin stillage (May 18, 2007 sample)

a) without added phenethyl alcohol;

b) with added phenethyl alcohol.

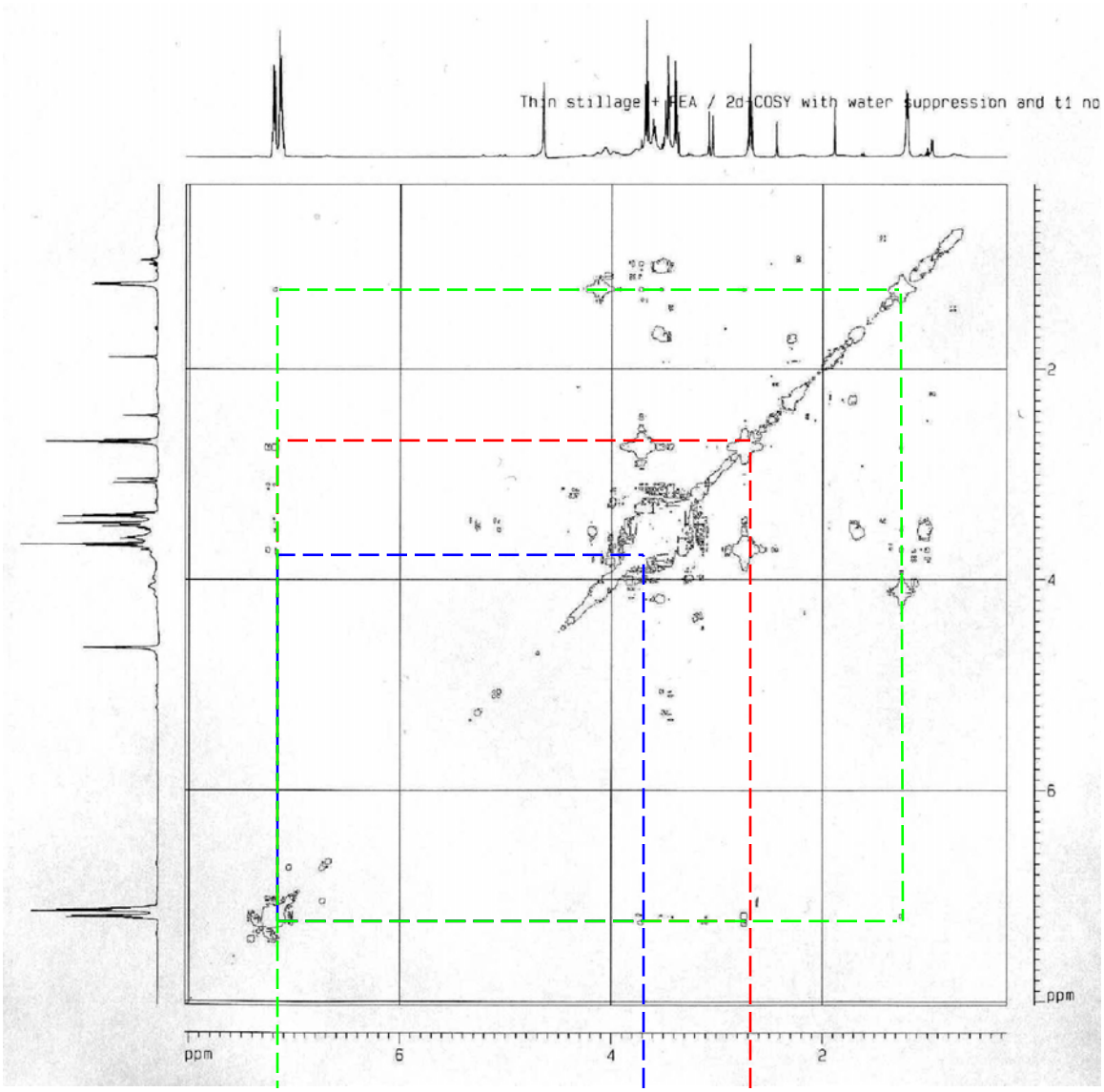


Fig. 4.5 2D ^{1}H NMR spectrum of thin stillage (May 18, 2007 sample) with added phenethyl alcohol.

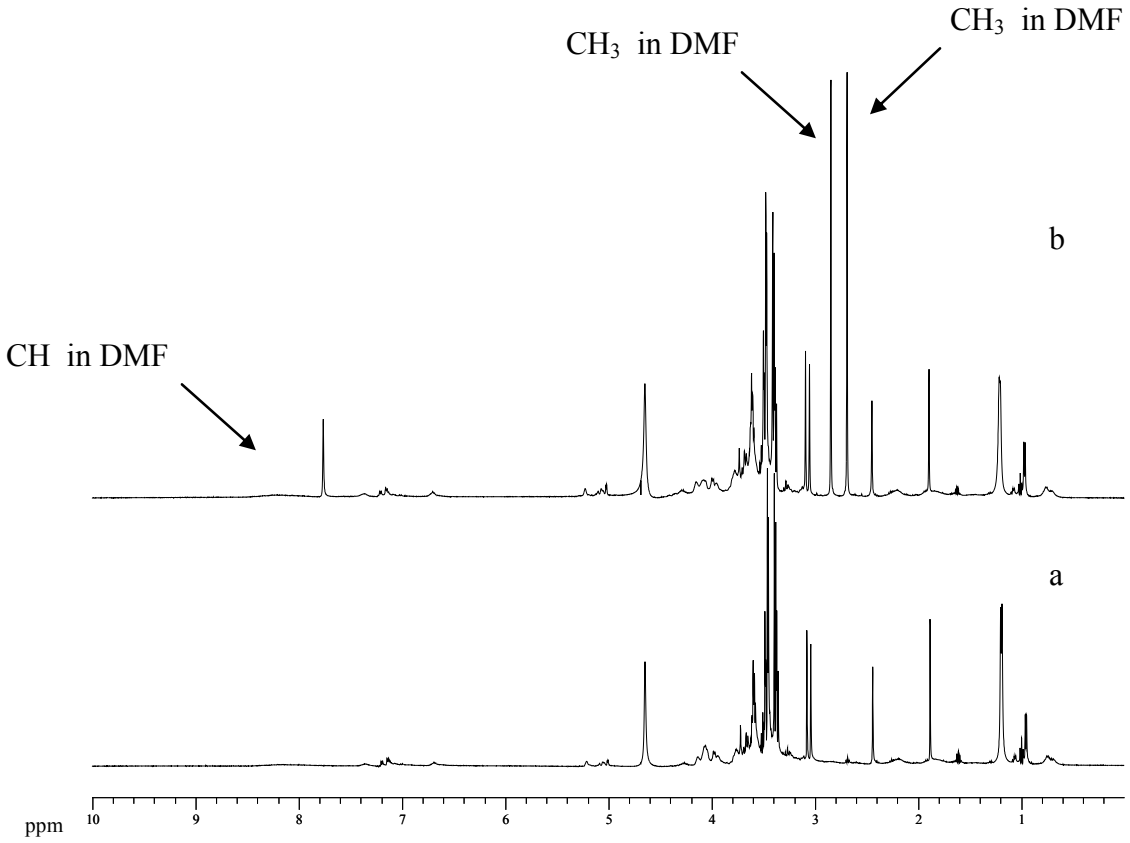


Fig. 4.6 ^{1}H NMR spectra at 500 MHz of a) thin stillage (May 18, 2007 sample)

a) without adde DMF;

b) with added DMF.

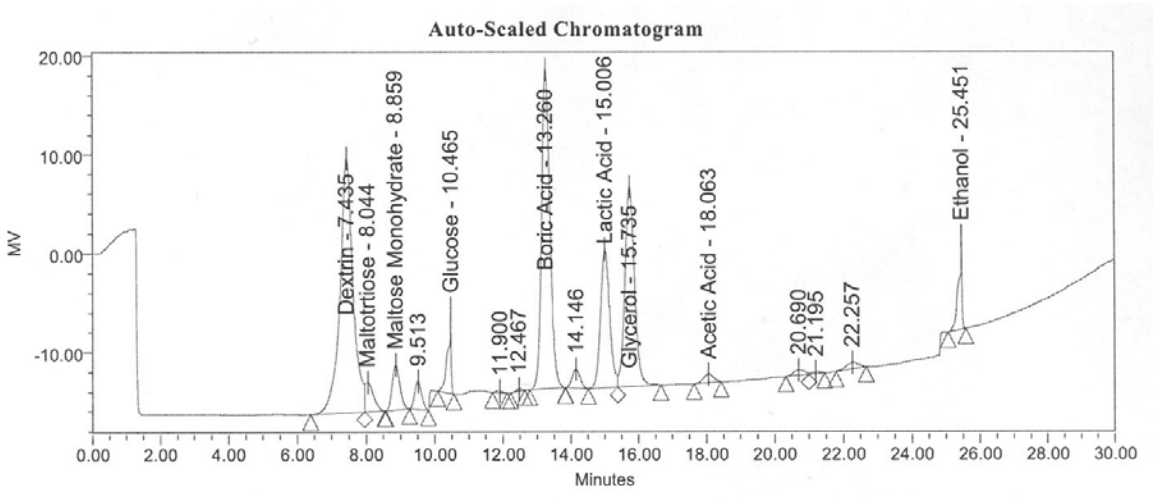


Fig. 4.7 HPLC chromatogram of thin stillage.

Table 4.3 The organic components of four batches of thin stillage analyzed by ^{1}H NMR and HPLC.

Component	Amount of the compound (g/L)							
	Batches							
	May 18, 2007		May 27, 2007		May 28, 2007		June 1, 2007	
	NMR	HPLC	NMR	HPLC	NMR	HPLC	NMR	HPLC
Dextrin	-	11.65 ± 0.05	-	9.3 ± 0.3	-	10.7 ± 0.2	-	8.47 ± 0.04
Maltotriose	-	0.73 ± 0.01	-	1.1 ± 0.8	-	0.53 ± 0.01	-	0.14 ± 0.02
Maltose monohydrate	-	1.05 ± 0.01	-	0.3 ± 0.4	-	0.97 ± 0.03	-	0.03 ± 0.00
Glycerol	-	7.87 ± 0.03	-	6.2 ± 0.2	-	6.92 ± 0.04	-	2.39 ± 0.01
Isopropanol	0.34 ± 0.00	-	0.35 ± 0.01	-	0.33 ± 0.00	-	0.31 ± 0.01	-
Ethanol	0.24 ± 0.00	1.3 ± 0.2	0.49 ± 0.00	0.59 ± 0.01	0.25 ± 0.00	0.23 ± 0.10	0.22 ± 0.01	1.2 ± 0.2
Lactic acid	5.89 ± 0.03	6.52 ± 0.06	6.3 ± 0.2	7.4 ± 0.2	4.28 ± 0.01	5.07 ± 0.04	5.7 ± 0.3	6.55 ± 0.01
1,3 Propanediol	0.41 ± 0.00	-	0.97 ± 0.03	-	0.19 ± 0.02	-	3.3 ± 0.1	-
Acetic acid	0.87 ± 0.00	0.65 ± 0.03	1.22 ± 0.01	1.14 ± 0.07	0.71 ± 0.01	0.56 ± 0.00	2.28 ± 0.08	2.7 ± 0.8
Succinic acid	0.90 ± 0.00	-	0.72 ± 0.02	-	0.63 ± 0.01	-	0.93 ± 0.05	-
Glycerophosphorylcholine	1.11 ± 0.00	-	0.95 ± 0.04	-	0.91 ± 0.02	-	0.99 ± 0.05	-
Betaine	1.03 ± 0.00	-	0.85 ± 0.03	-	0.82 ± 0.02	-	0.80 ± 0.05	-
Phenethyl alcohol	0.37 ± 0.01	-	0.26 ± 0.02	-	0.26 ± 0.02	-	0.29 ± 0.02	-

Results are means of duplicate analyses.

4.2.3 Microbial populations

The microbial population of thin stillage was determined by culturing the stillage on tryptic soy broth (for total plate count) and rose bengal agar (for yeast and fungi), and then counting the number of colonies per plate. Examples of plates are shown in Fig. 4.8. Micrographs of yeast and fungi observed in thin stillage are provided in Figs. 4.9-4.11.The results of the total plate counts and yeast and fungi plate counts for the four batches of thin stillage are shown in Table 4.4. There were significant numbers of bacteria (from total plate count) and yeast and fungi in each of the samples, and significant differences in counts among samples. For total plate count, thin stillage samples from May 18, 2007 and May 28, 2007 had the highest and lowest microbial populations, respectively. For yeast, thin stillage samples from May 18, 2007 and May 28, 2007 had the highest and lowest numbers, respectively. Thin stillage samples from May 18, 2007 and June 1, 2007 had the highest and lowest mould populations, respectively.

4.2.4 Ion contents

The anion and cation contents of thin stillage were analyzed using ion chromatography and ICP. Results are presented in Table 4.5. The four batches of thin stillage (May 18, May 27, May 28 and June 1, 2007) had similar of concentrations of the various ions. The cation present in the highest concentration was potassium, followed by magnesium, sodium and calcium, in order. The anion present in the highest concentration was sulphate, followed by chloride and nitrate, in order.

4.2.5 Prediction of types and amounts of salts

Using a Levenberg-Marquardt algorithm in the Solver Add-in program in Microsoft Excel, the types and concentrations of salts likely to be present in thin stillage were predicted. The results are presented in Table 4.6.

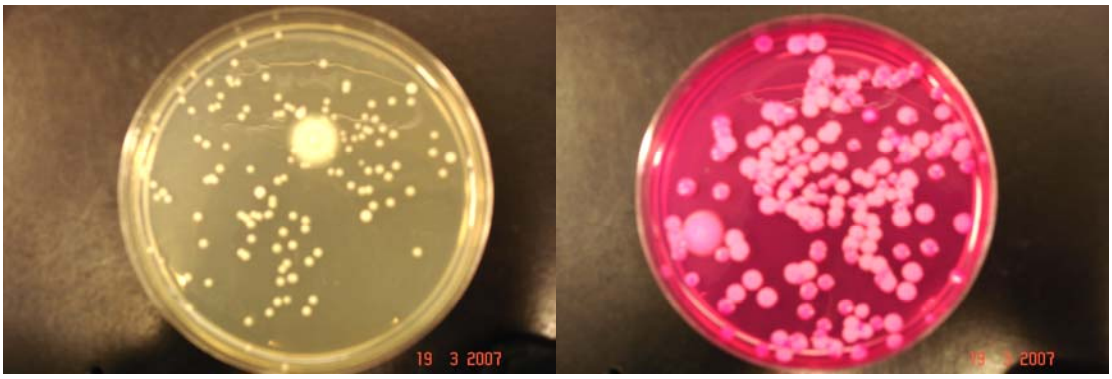


Fig. 4.8 Total plate count plate (left) and yeast and fungi plate count (right).

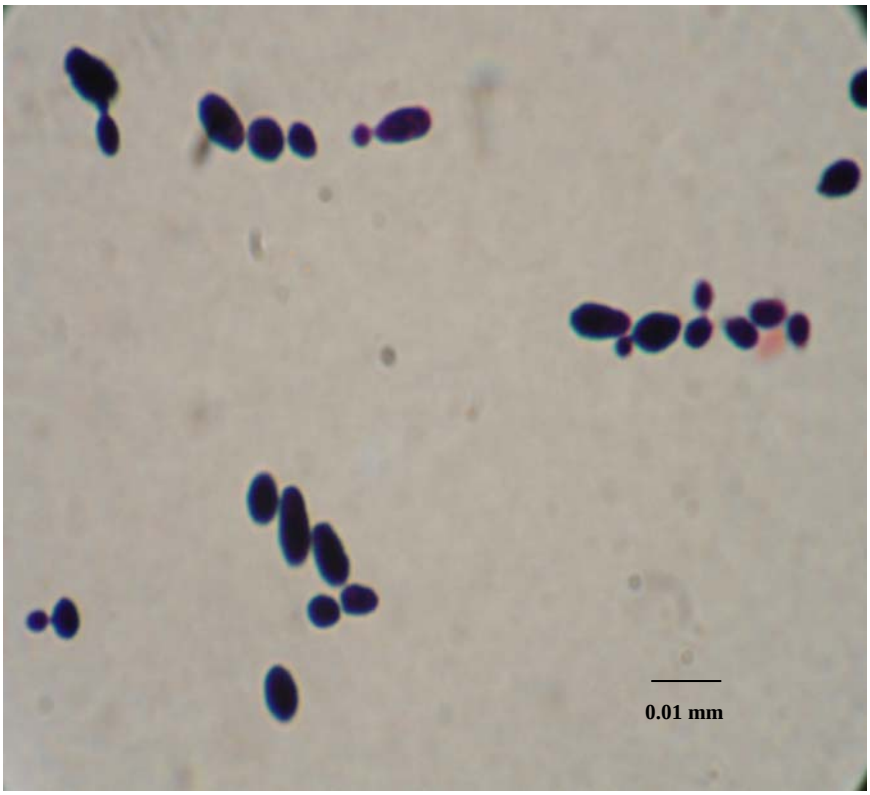


Fig. 4.9 Micrograph of yeast cells from thin stillage taken at a magnification of 400X.

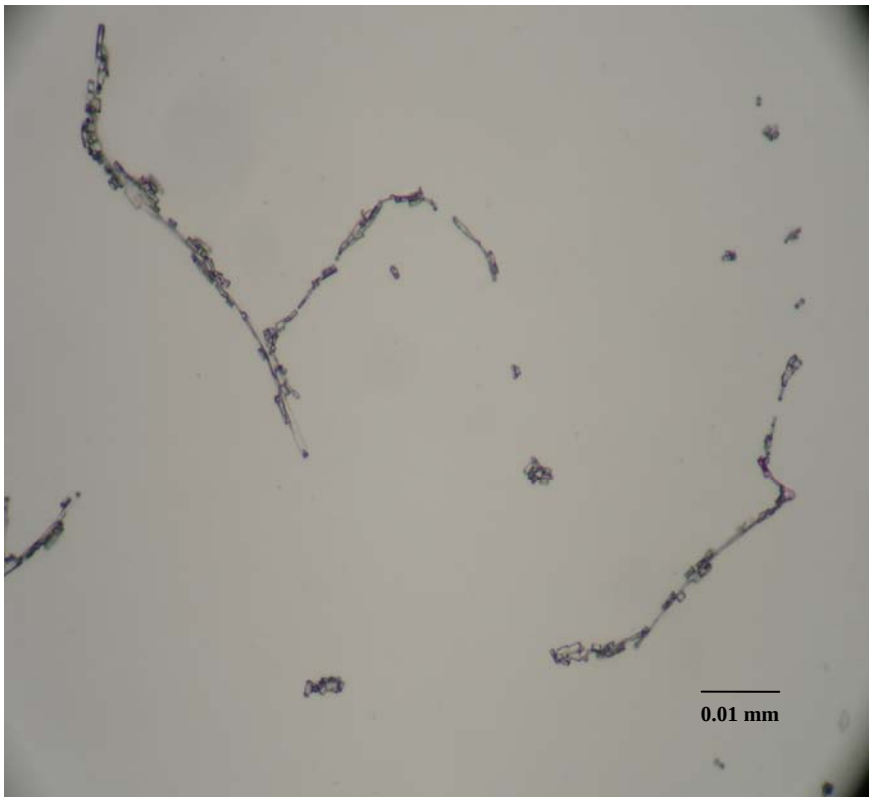


Fig. 4.10 Micrograph of fungi from thin stillage taken at a magnification of 400X.

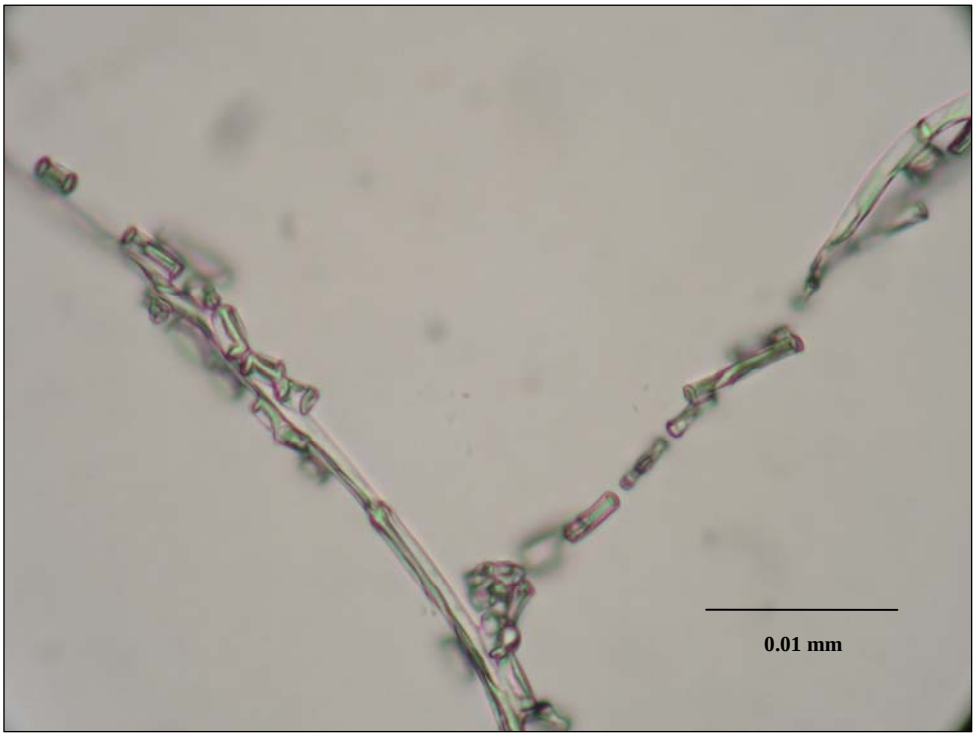


Fig. 4.11 Micrograph of fungi from thin stillage taken at a magnification of 1000X.

Table 4.4 Microbial populations of four batches of thin stillage.

Stillage sample	Total plate count (CFU)	Yeast (CFU)	Mould (CFU)
May 18, 2007	$4.5x10^6 \pm 3x10^5$	$7x10^4 \pm 3x10^4$	$1.3x10^4 \pm 2x10^3$
May 27, 2007	$3.1x10^6 \pm 1x10^5$	$4.0x10^3 \pm 6x10^2$	$4x10^3 \pm 1x10^3$
May 28, 2007	$1.2x10^6 \pm 2x10^5$	$8x10^2 \pm 5x10^2$	$4x10^3 \pm 1x10^3$
June 1, 2007	$1.3x10^6 \pm 7x10^5$	$3.2x10^3 \pm 4x10^2$	$2x10^2 \pm 1x10^2$

Results are means of triplicate analyses (three plates from each sample).

Table 4.5 Concentrations (mg/L) of ions in four batches of thin stillage.

Ion	Stillage samples			
	May 18, 2007	**May 27, 2007**	**May 28, 2007**	**June 1, 2007**
Bicarbonate	< 1.00 ± 0.00	< 1.00 ± 0.00	< 1.00 ± 0.00	< 1.00 ± 0.00
Calcium	51.5 ± 0.7	51.5 ± 0.7	53.5 ± 0.7	45 ± 2
Carbonate	< 1.00 ± 0.00	< 1.00 ± 0.00	< 1.00 ± 0.00	< 1.00 ± 0.00
Chloride	224.5 ± 0.7	227 ± 7	215.5 ± 0.7	237.0 ± 0.00
Hydroxide	< 1.00 ± 0.00	< 1.00 ± 0.00	< 1.00 ± 0.00	< 1.00 ± 0.00
Magnesium	205 ± 7	185 ± 7	175 ± 7	175 ± 7
Potassium	530 ± 14	485 ± 7	475 ± 7	480 ± 7
Sodium	140.00 ± 0.00	130.00 ± 0.00	130.00 ± 0.00	120.00 ± 0.00
Sulfate	590.00 ± 0.00	460.00 ± 0.00	495 ± 7	405 ± 7
Nitrate	8 ± 2	9 ± 2	8 ± 1	9 ± 1
Total	1750 ± 14	1545 ± 7	1555 ± 7	1470 ± 28

Results are means of duplicate analyses.

Table 4.6 Types and concentrations (g/L) of salts likely to be present in thin stillage predicted with the Solver Add-in program in Microsoft Excel.

Salt concentration						
$CaCl_2$	**NaCl**	**K_2SO_4**	**$NaNO_3$**	**$Mg(OH)_2$**	**Na_2SO_4**	**KOH**
0.14	0.22	0.73	0.01	0.31	0.12	0.23

4.3 Design of model thin stillage of defined composition

Model thin stillage was formulated on the basis of the average composition of the four batches of thin stillage as presented in section 4.2 (Table 4.7). Isopropanol, lactic acid, 1,3-propanediol, acetic acid, GPC, betaine, glycerol, phenethyl alcohol, dextrin, maltotriose, maltose monohydrate, $CaCl_2$, NaCl, K_2SO_4, $NaNO_3$, $Mg(OH)_2$, Na_2SO_4 and KOH were used to prepare the model thin stillage.

The compounds (organic components, salts, carbohydrates) in model thin stillage were quantified and confirmed using the ^{1}H NMR method with DMF as the internal standard. The ^{1}H NMR results were confirmed by HPLC. The ^{1}H NMR spectrum and HPLC chromatogram of model thin stillage are shown in Figs. 4.12 and 4.13, respectively. Results are presented in Table 4.7.

The concentrations of ions in model thin stillage were determined and compared to those in industrial thin stillage (Table 4.8). The concentrations of organic components, salts, carbohydrates and ions in the model thin stillage were similar to those in industrial thin stillage, with the exception of nitrate.

The physical and chemical properties of the model thin stillage were determined (Table 4.9), and were comparable to those of industrial thin stillage (Table 4.2), although the viscosity of the model thin stillage was substantially lower (1.10 versus 1.67-1.73 centipoise). In addition, the dry matter content of model thin stillage was approximately one third less than that of thin stillage taken from a commercial source. This indicates that some components in thin stillage from this source have yet to be identified.

Table 4.7 Comparison of the average concentrations of the constituents in four batches of industrial thin stillage with those in a model thin stillage, as analyzed by ^{1}H NMR and HPLC.

Constituent	Concentration (g/L)		
	Average value in four batches of industrial thin stillage	Model thin stillage	
		^{1}H NMR	HPLC
$CaCl_2$	0.14	-	-
NaCl	0.22	-	-
K_2SO_4	0.73	-	-
$NaNO_3$	0.01	-	-
$Mg(OH)_2$	0.31	-	-
Na_2SO_4	0.12	-	-
KOH	0.23	-	-
Dextrin	10.04	-	10 ± 2
Maltotriose	0.62	-	0.91 ± 0.00
Maltose monohydrate	0.59	-	0.85 ± 0.01
Glycerol	5.85	-	6.56 ± 0.04
Isopropanol	0.32	0.25 ± 0.00	-
Ethanol	0.30	0.35 ± 0.00	0.47 ± 0.08
Lactic acid	5.55	4.42 ± 0.01	3.68 ± 0.06
1,3 Propanediol	1.22	1.36 ± 0.02	-
Acetic acid	1.27	1.46 ± 0.00	1.44 ± 0.01
Succinic acid	0.79	1.14 ± 0.01	-
Glycerophosphorylcholine	1.00	1.02 ± 0.01	-
Betaine phosphate	0.88	0.52 ± 0.02	-
Phenethyl alcohol	0.29	0.39 ± 0.03	-

Results are means of duplicate analyses.

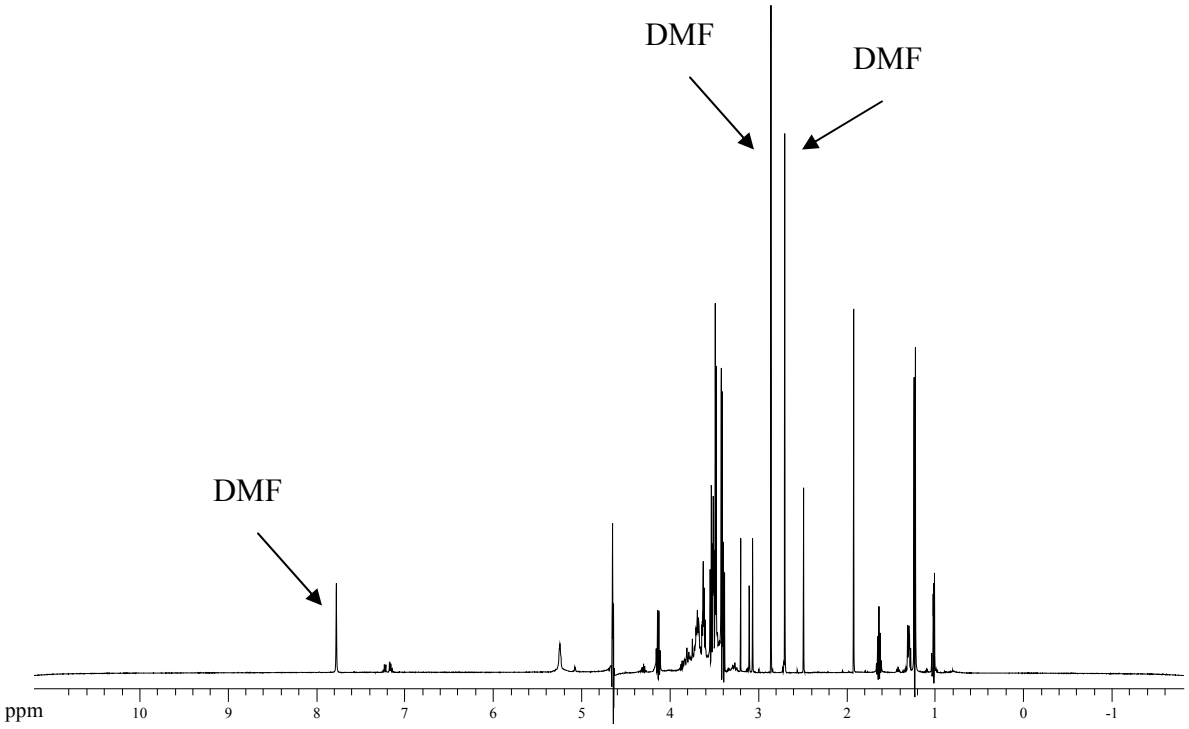


Fig. 4.12 ^{1}H NMR spectrum at 500 MHz of model thin stillage with DMF as the internal standard.

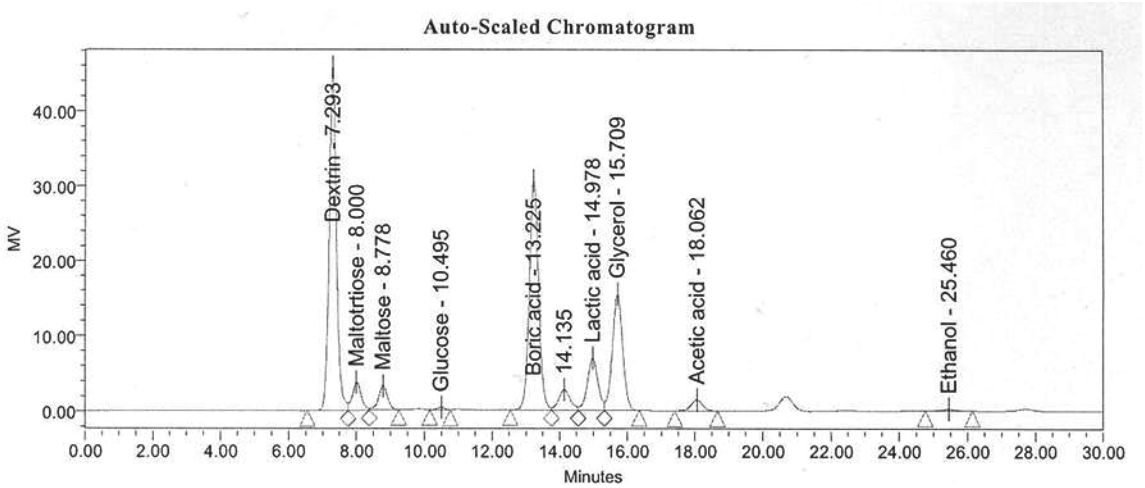


Fig. 4.13 HPLC chromatogram of model thin stillage.

Table 4.8 Comparison of the average concentrations of ions in four batches of industrial thin stillage with those in a model thin stillage.

Ion	Concentration (mg/L)	
	Average value in four batches of industrial thin stillage	**Model thin stillage**
Bicarbonate	< 1.0	< 1.0 ± 0.0
Calcium	50.3	44 ± 4
Carbonate	< 1.0	< 1.0 ± 0.0
Chloride	226.0	333 ± 2
Hydroxide	< 1.0	< 1.0 ± 0.0
Magnesium	185.0	160 ± 14
Potassium	492.5	510 ± 42
Sodium	130.0	180 ± 14
Sulfate	487.5	495 ± 7
Nitrate	8.5	< 0.04 ± 0.00
Total	1579.8	1720 ± 85

Results are means of duplicate analyses.

Table 4.9 Physical and chemical characteristics of model thin stillage.

Characteristics	
Physical characteristics	
Osmotic potential (MPa)	-0.79 ± 0.00
Viscosity (25°C) (centipoise)	1.10 ± 0.00
Density (25°C) (g/cm^3)	1.01 ± 0.00
Chemical characteristics	
Moisture content (%, w/w)	98.1 ± 0.03
pH	3.50 ± 0.00

Results are means of duplicate analyses.

4.4 Comparative efficiency of thin stillage for protein extraction

The results in section 4.2 indicated that thin stillage contained a number of components that might affect protein extraction. Therefore, the efficiency of protein extraction using thin stillage and model thin stillage were compared to that using sodium chloride solution. One of the four batches of thin stillage (batch of May 27, 2007), model thin stillage and sodium chloride solution were adjusted with NaCl and base to identify the optimum extraction conditions. The ratio of ground defatted meal to solvent and extraction time were fixed (1:30, w/v, and 2 hours, respectively) for this study.

The effect of salt concentration and pH on protein extraction was studied to identify the optimum extraction conditions.

4.4.1 Comparison of the extraction efficiency of two methods for extracting protein from defatted *B. juncea* meal

The efficiencies of protein extraction obtained by either mixing ground defatted meal with thin stillage (method 1 in section 3.3.3.1) or blending defatted meal with thin stillage (method 2) were determined. Method 1 was more efficient than method 2 (Table 4.10). It was also observed that blending defatted meal with thin stillage produced large particles that were caught on a 20-mesh screen (mesh size = 840 μm). The size of the particles remaining on the screen was larger than that of the largest particles in ground defatted meal.

Table 4.10 The efficiency of protein extraction obtained by blending defatted meal with thin stillage (using a blender) compared with that obtained by mixing ground defatted meal with thin stillage (using a stirrer).

Method	Yield of freeze-dried protein (grams)	Undissolved solids (grams)	Moisture content of freeze-dried protein, %	Moisture content of undissolved solids, %	Protein content of freeze-dried protein (%, w/w)	Protein content of undissolved solids (%, w/w)	Efficiency of protein extraction, %
Blending	14.3 ± 0.3	29 ± 3	22 ± 6	82.5 ± 0.9	19.3 ± 0.4	6.4 ± 0.1	28 ± 1[a]
Mixing	15.2 ± 0.4	34.9 ± 0.6	21 ± 5	82 ± 2	20.1 ± 0.9	6.0 ± 0.1	37 ± 2[b]

Results are means of duplicate analyses.

[a , b] Means are significantly different at $P<0.05$.

4.4.2 Generation of protein titration curves

Maximum protein precipitation occurs when the pH of a solution reaches its isoelectric point. In order to avoid protein precipitation and achieve the maximum protein extraction, it is important to know the isoelectric point of protein in the system. Protein in defatted meal was extracted using salt solution and freeze-dried. Freeze-dried protein was diluted with water and adjusted to an alkaline pH. The protein solution was then titrated with hydrochloric acid solution. The protein titration curve is presented in Figs. 4.14 a. and b. Fig 4.14 showed that the isoelectric point of freeze-dried protein was at approximately pH 7. The data from the protein titration curve was then plotted as the first derivative to determine the isoelectric point (Fig. 4.14 b.). This curve confirmed that the isoelectric point of the freeze-dried protein was at approximately pH 6.4.

4.4.3 Determining the efficiency of protein extraction

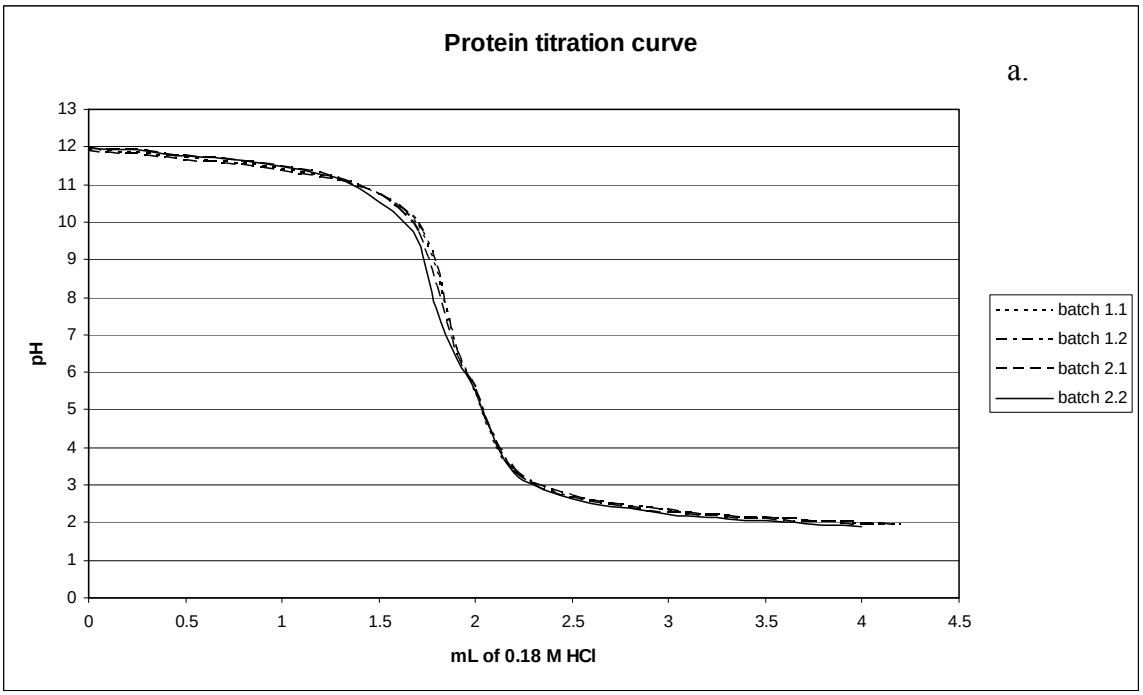
Salt concentration and pH both affect protein extraction. The effect of these two variables on the efficiency of protein extraction was studied in order to determine the optimum conditions for extraction of protein from mustard meal. The efficiency of protein extraction was calculated as described in section 4.4, and the results are presented in Table 4.11. The response surface of the efficiency of protein extraction is shown in Fig. 4.15. Using canonical analysis, the lack of fit for the model was highly significant ($P < 0.05$). In addition, there was not a good fit for a quadratic model, with the R^2 of the response surface design being 0.67. The analysis of variance for the model showed that the effects of pH, salt concentration and the interaction between pH and salt concentration on protein extraction were significant. The response surface model for protein extraction efficiency was:

$$377.25 - 64.25x_1 - 157.74x_2 + 3.14x_1^2 + 18.99x_1x_2 - 6.65x_2^2 \qquad \text{(Equation 4.1)}$$

X_1 = level of pH

X_2 = level of salt concentration

R^2 of response surface design = 0.67



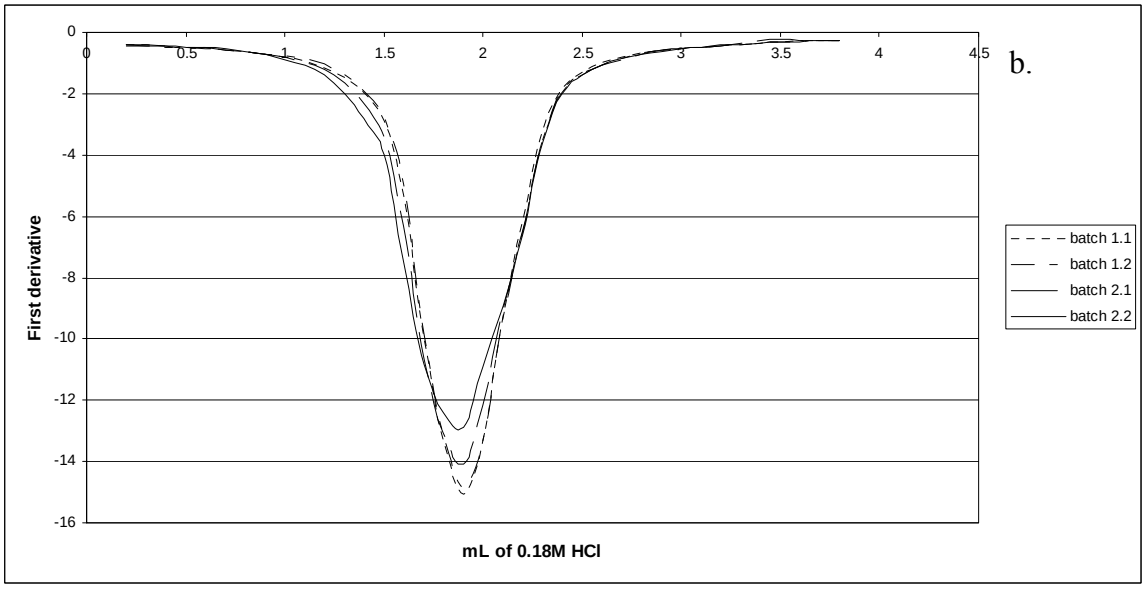


Fig. 4.14 Protein titration curve

a) Protein titration curve of protein extracted with salt solution;

b) The first derivative of the protein titration curve.

Table 4.11 Efficiency of protein extraction obtained with various combinations of pH and salt (NaCl) concentration.

pH	NaCl (M)	Grams of undissolved solids	Grams of supernatant	Grams of freeze-dried protein	Protein content (%, w/w)		Moisture content of undissolved solids, %	Efficiency of protein extraction, %
					Freeze-dried protein	Undissolved solids		
8	0.2	24.2 ± 0.4	136.9 ± 0.7	19.2 ± 0.3	10.2 ± 0.3	4.6 ± 0. 1	83.1 ± 0.3	60 ± 2
10	0.2	27 ± 1	137 ± 1	21.6 ± 0.4	8.9 ± 0.3	4.3 ± 0.1	82.9 ± 0.3	57 ± 2
8	1.0	22.5 ± 0.1	142.3 ± 0.5	23.1 ± 0.3	7.9 ± 0.5	4.4 ± 0.0	79.2 ± 0.5	52 ± 7
10	1.0	27.6 ± 0.1	139.5 ± 0.1	26.9 ± 0.6	8.7 ± 0.5	3.5 ± 0.1	80.5 ± 0.2	80 ± 10
9	0.6	24.2 ± 0.4	138.5 ± 0.1	21.0 ± 0.1	9.3 ± 0.1	4.5 ± 0.2	80.7 ± 0.6	59 ± 2
9	0.6	23.5 ± 0.9	140.8 ± 2	20.5 ± 0.2	9.1 ± 0.1	4.4 ± 0.2	80 ± 1	55 ± 0
10.4	0.6	27 ± 2	139 ± 2	25 ± 1	8.4 ± 0.1	3.8 ± 0.2	80.8 ± 0.6	65 ± 4
7.6	0.6	22.5 ± 0.5	139.9 ± 0.8	20.7 ± 0.5	9.8 ± 0.1	4.7 ± 0.1	80.9 ± 0.5	63 ± 4
9	1.16	26 ± 0	143.0 ± 0.3	25.5 ± 0.0	7.4 ± 0.1	4.4 ± 0.2	79.1 ± 0.1	56 ± 1
9	$3.4x10^{-2}$	25.9 ± 0.1	136.1 ± 0.1	18.3 ± 0.2	10.3 ± 0.1	4.7 ± 0.1	83.6 ± 0.6	55 ± 1
9	0.6	24.1 ± 0.0	139.8 ± 0.9	21.1 ± 0.1	9.4 ± 0.2	4.3 ± 0.2	81.0 ± 0.3	61 ± 2
9	0.6	24.4 ± 1	139.4 ± 0.4	21.1 ± 0.1	9.5 ± 0.1	4.2 ± 0.3	80.7 ± 1	62 ± 2

Results are means of duplicate analyses.

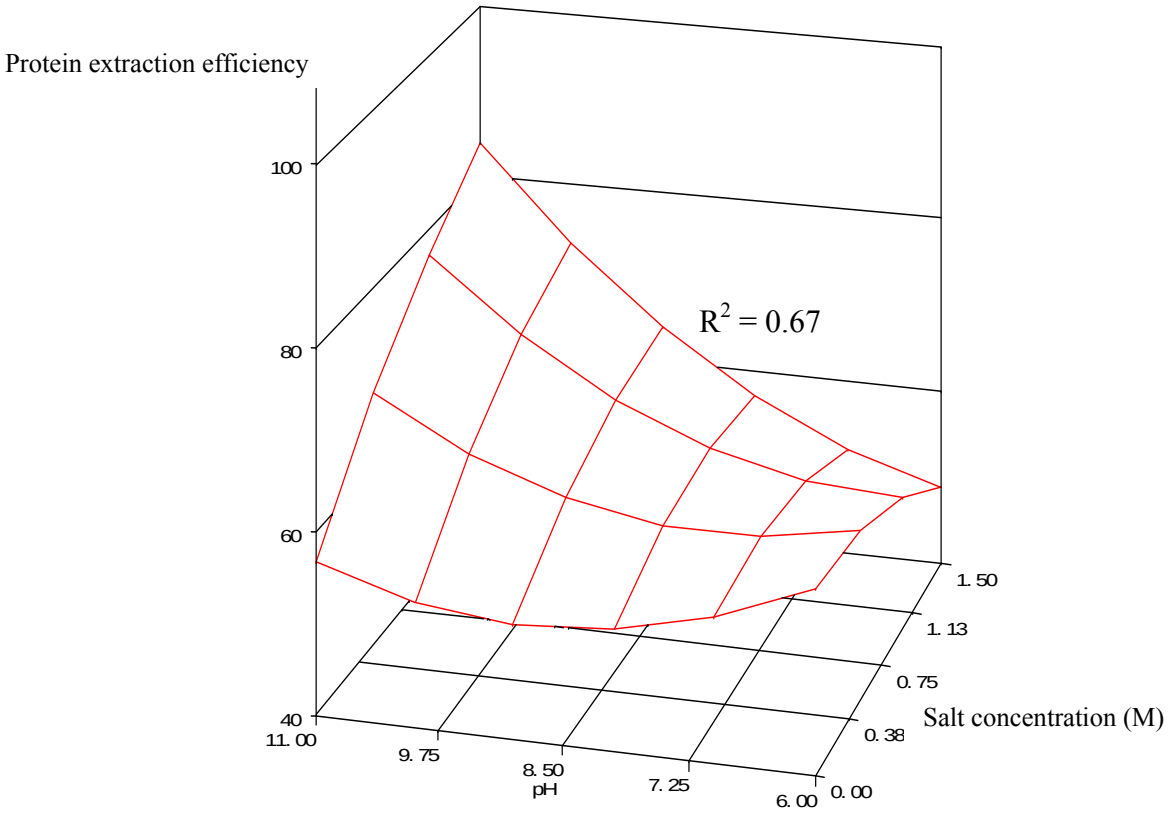


Fig. 4.15 Response surface of the efficiency of protein extraction using various combinations of pH and salt (NaCl) concentration.

In the canonical analysis, the Eigen values of the predicted response surface produced a saddle shape, and the surface did not have a unique optimum. From the response surface, it was observed that when pH and salt concentration increased, protein extraction efficiency increased. It is possible that the optimum conditions for protein extraction might be out of the range of pHs and salt concentrations used in this experiment (Table 3.1). Therefore, ridge analysis was carried out to determine the region in which further studies should be conducted. Ridge analysis indicated that further studies are needed to find an optimum centred at pH 10.2 and a salt concentration of 0.89M, with a 75% efficiency of protein extraction.

The complementary conditions [combining the optimal conditions (pH and salt concentration) of pH 10 and a salt concentration of 1M] were used in the following experiment.

4.4.4 Comparison of the relative efficiency of protein extraction using thin stillage, model thin stillage or sodium chloride solution

Efficiency of protein extraction

The efficiency of protein extraction might be affected by the presence of compounds such as divalent cations, which are found in industrial thin stillage. The relative efficiencies of protein extraction using thin stillage, model thin stillage and sodium chloride solution were used to determine the effect of these compounds. The efficiency of protein extraction, SDS-PAGE of extracted protein, amino acid sequence of tryptic peptide fragments of extracted protein, digestibility of extracted protein, and lysine availability of extracted protein were compared for protein extracted using thin stillage, model thin stillage and sodium chloride solution. Thin stillage and model thin stillage used in this study were first filtered with an ultrafiltration membrane of 3,000 MWCO. The conditions that produced the highest protein extraction efficiency (pH 10, salt concentration of 1M) in preliminary experiments (Table 4.11) were used. No significant differences in the efficiency of protein extraction obtained using thin stillage, model thin stillage or sodium chloride solution (Table 4.12) were detected.

Table 4.12 Comparison of the efficiency of protein extraction using sodium chloride solution, model thin stillage or thin stillage.

Extraction solution	Grams of undissolved solids	Grams of undissolved solids (dry basis)	Grams of freeze-dried protein	Protein content (%, w/w)		Moisture content of undissolved solids, %	Efficiency of protein extraction, %
				Undissolved solids	Freeze-dried protein		
Sodium chloride solution (1M)	18 ± 1	3 ± 1	1.1 ± 0.1	4.5 ± 0.1	105.4 ± 0.2	80.9 ± 1.0	60 ± 4[a]
Model thin stillage[1]	19.3 ± 0.6	4.0 ± 0.0	1.1 ± 0.1	4.6 ± 0.1	96 ± 2	79.1 ± 0.5	55 ± 5[a]
Thin stillage[1]	25.9 ± 0.5	4.7 ± 0.2	1.1 ± 0.1	3.9 ± 0.2	97.6 ± 0.1	81.8 ± 0.9	56 ± 4[a]

[a] Values followed by the same letter are not significantly different at $p<0.05$.

[1] 1M NaCl added adjusted pH to 10

Results are means of duplicate analyses.

SDS-PAGE

The molecular weight of protein extracted by sodium chloride solution, model thin stillage or thin stillage was determined using SDS-PAGE. The results are presented in Fig. 4.16. The results showed that the molecular weights of proteins extracted by sodium chloride solution, model thin stillage and thin stillage were approximately 14, 18-20, 20-22, 34 and 55 kDa, in all cases.

Peptide sequencing

Peptides from protein extracted by thin stillage with masses of approximately 14, 18-20, 20-22, 34 and 55 kDa were cut from the SDS-PAGE Tris-glycine gel from the previous experiment. Trypsin was used to digest the polypeptides. Mass spectrometry (MALDI-TOF) was then used to determine accurate masses and the peptide mass fingerprint. The MS-FIT program of the Protein Prospector was used to identify proteins from the peptide fragments. The masses and peptide mass fingerprint of the peptides are presented in Table 4.13. The results showed that the peptide fragments of napin occurred at a subunit mass of 14 kDa only. Cruciferin peptide fragments were observed at subunit masses of 14, 18-20, 20-22, 34 and 55 kDa.

Amino acid composition

The amino acid composition of protein extracted from mustard meal using sodium chloride solution, model thin stillage or thin stillage was analyzed by HPLC (Table 4.14). The data showed that differences in amino acid content among proteins extracted with sodium chloride solution, model thin stillage or thin stillage were slight. The standard deviation of the valine content of protein extracted with sodium chloride solution was high because the base line of the HPLC chromatogram was not smooth. Glutamic acid and methionine were present in the highest and lowest concentrations, respectively, in protein extracted by each of the three methods. Of the essential amino acids, leucine and methionine were present in the highest and lowest concentrations, respectively.

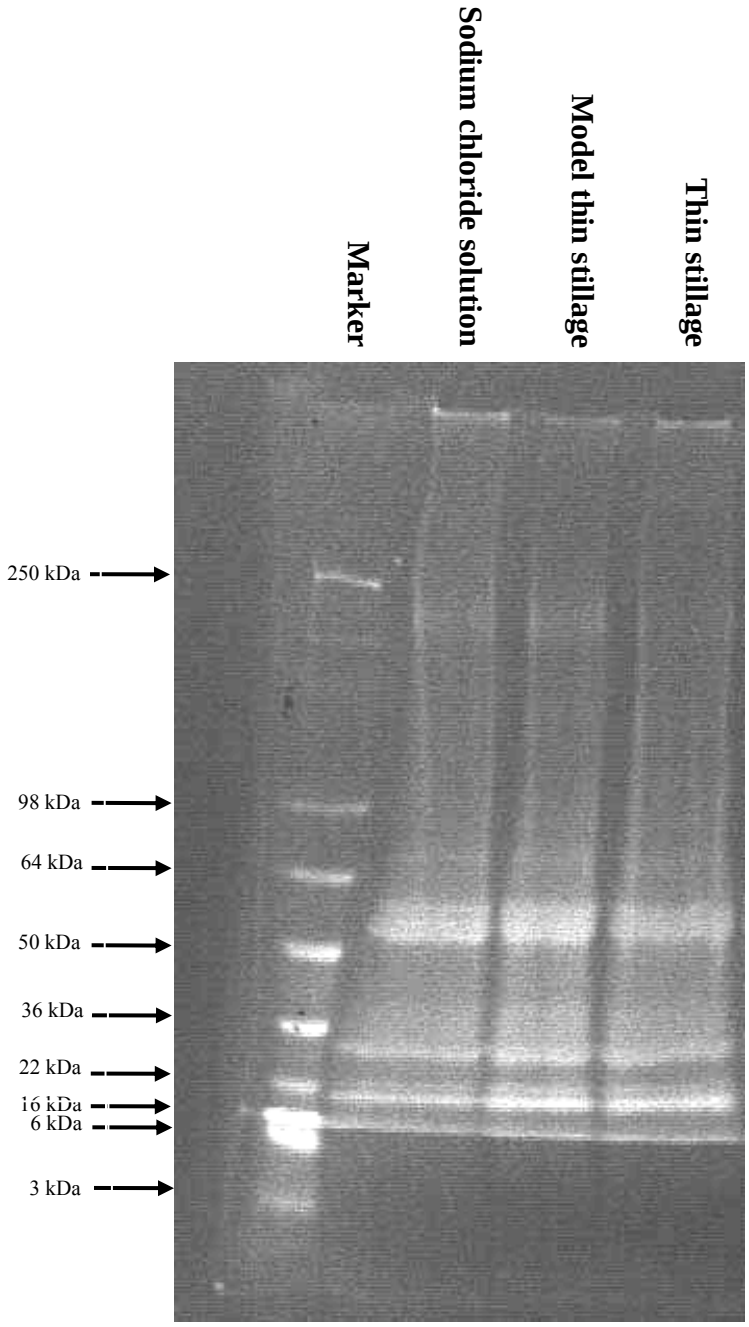


Fig. 4.16 SDS-PAGE of protein extracted from mustard meal using sodium chloride solution, model thin stillage or thin stillage.

Table 4.13 Amino acid sequences of tryptic peptide fragments of protein extracted from *B. juncea* (L.) Czern using thin stillage.

Subunit mass (kDa)	**Fragment sequence**	**Calculated mass (Da)**	**Actual mass (Da)**	**Sequence assignment**	**position**
14	EFQQAQQHLR	1155.5785	1156.6826	Allergen *B. juncea* 1-E	12-20
	IYQTATHLPR	1198.6458	1199.7471	Allergen *B. juncea* 1-E	100-109
	IEVWDHHAPQLR	1499.7633	1500.8756	Cruciferin	50-61
	GLPLEVISNGYQISPQEAR	2070.0745	2071.2039	Cruciferin	420-438
18-20	GLPLEVISNGYQISPQEAR	2070.0745	2071.2136	Cruciferin	338-386
20-22	GLPLEVISNGYQISLEEAR	2087.0898	2088.1885	Cruciferin	66-84
	GLPLEVISNGYQISPQEAR	2070.0745	2071.1917	Cruciferin	368-386
34	CSGFAFER	972.4124	973.4891	Cruciferin	62-69
	VQGQFGVIRPPLR	1465.8518	1466.9407	Cruciferin	251-263
	IEVWDHHAPQLR	1499.7633	1500.8281	Cruciferin	50-61
55	GPFQVVRPPLR	1264.7404	1265.7958	Cruciferin	288-298
	VQGQFGVIRPPLR	1465.8518	1466.9131	Cruciferin	251-263
	IEVWDHHAPQLR	1499.7633	1500.8154	Cruciferin	50-61
	GLPLEVISNGYQISPQEAR	2070.0745	2071.1272	Cruciferin	420-438

Table 4.14 Amino acid composition (g/100 g of protein) of protein extracted from *B. juncea* (L.) Czern using sodium chloride solution, model thin stillage or thin stillage.

Amino acid	Sodium chloride solution (1M)	Model thin stillage[1]	Thin stillage[1]
Cysteine	5.2 ± 0.2	5.1 ± 0.3	5.3 ± 0.0
Asparagine	6.0 ± 0.1	5.7 ± 0.3	5.4 ± 0.2
Methionine	2.3 ± 0.0	2.2 ± 0.0	2.2 ± 0.2
Threonine	3.5 ± 0.2	3.2 ± 0.0	3.1 ± 0.1
Serine	4.2 ± 0.0	3.9 ± 0.1	3.9 ± 0.1
Glutamic acid	23.0 ± 0.7	22.1 ± 0.1	22.2 ± 0.1
Glycine	4.9 ± 0.1	4.6 ± 0.2	4.6 ± 0.1
Alanine	4.3 ± 0.1	4.0 ± 0.1	3.9± 0.1
Valine	3 ± 2	4.9 ± 0.9	6.0 ± 0.2
Isoleucine	3.8 ± 0.1	3.5 ± 0.0	3.4 ± 0.0
Leucine	7.5 ± 0.1	6.8 ± 0.1	6.6 ± 0.1
Phenylalanine	4.1 ± 0.7	4.1 ± 0.6	3.6 ± 0.1
Histidine	4.5 ± 0.1	4.3 ± 0.0	4.5 ± 0.1
Lysine	5.9 ± 0.3	5.4 ± 0.1	5.2 ± 0.0
Arginine	7.6 ± 0.1	7.0 ± 0.1	7.0 ± 0.1
Tryptophan	N	N	N
Tyrosine	N	N	N
Aspartic acid	N	N	N
Proline	N	N	N

Results are means of duplicate analyses.

N means no analysis

[1] 1M NaCl added

In vitro digestibility

The digestibility of protein extracted with sodium chloride solution, model thin stillage or thin stillage was determined using a multi-enzyme technique. Trypsin, chymotrypsin and peptidase were used in this experiment. The digestibility data are presented in Table 4.15. The results showed that the digestibilities of protein extracted with sodium chloride solution, model thin stillage or thin stillage were similar.

Lysine availability

The availability of lysine was similar in protein extracted with sodium chloride solution, model thin stillage or thin stillage (Table 4.15). When the total lysine content (Table 4.14) was compared with the available lysine content, it was found that approximately 75% of the lysine in the extracted protein would be available in feed.

Color

The color of protein extracted with sodium chloride solution, model thin stillage or thin stillage was reported in table 4.16. The results showed that the color of protein extracted with sodium chloride solution was lightest comparing with protein extracted with model thin stillage or thin stillage, respectively. However, a and b value were similar in protein extracted with sodium chloride solution, model thin stillage or thin stillage.

Table 4.15 *In vitro* digestibility and lysine availability of protein extracted from mustard meal using sodium chloride solution, model thin stillage or thin stillage.

	Sodium chloride solution (1M)	**Model thin stillage[1]**	**Thin stillage[1]**
Digestibility, %	74.5 ± 0.5	73.5 ± 0.5	74.9 ± 0.8
Lysine availability (g/kg of sample)	42 ± 4	37 ± 2	43.0 ± 0.3

Results are means of duplicate analyses.

[1] 1M NaCl added

Table 4.16 Color of protein extracted from mustard meal using sodium chloride solution, model thin stillage or thin stillage.

	L	**a**	**b**
Sodium chloride solution (1M)	69.04 ± 0.04	2.34 ± 0.01	19.55 ± 0.05
Model thin stillage[1]	62.99 ± 0.01	2.75 ± 0.05	20.04 ± 0.06
Thin stillage[1]	56.36 ± 0.08	3.45 ± 0.05	19.33 ± 0.01

Results are means of duplicate analyses.

[1] 1M NaCl added

4.4.5 Large-scale protein extraction

It is known that the efficiency of protein extraction is affected by the ratio of meal to solvent. Increasing the proportion of solvent used enhances the efficiency of protein extraction **(**Esteban *et al.*, 1985). However, "higher water-to-meal ratios would be uneconomical, due to the increased cost of both water and waste water treatment" (Xu *et al*., 2003). The 1:30, w/v, ratio of ground defatted meal:solvent used in the preliminary experiments was not practical for industrial application and the use of higher ratio (1:5, w/v) was evaluated. Ground defatted meal was extracted with thin stillage. Undissolved solids (extracted meal) were re-extracted two more times and the protein solution was dialysed as described previously (section 3.3.3.5). The efficiency of protein extraction using the 1:5 ratio was approximately 60 per cent (Table 4.17), compared with approximately 80 per cent when a 1:30 ratio was used (Table 4.11).

4.4.6 Comparison of protein extraction efficiency with that of a published protocol

The complementary process developed in section 3.3.3.3 was compared with a published protocol. In the published protocol, protein was extracted from ground defatted canola seed meal using a salt solution and recovered using cold water. The cold water treatment caused the protein to salt out in micelle form. The percent recovery from the protein micelle was approximately 7.6 percent (Table 4.18). This protein recovery was significantly lower than that achieved using the complementary method (approximately 80 percent) at pH 10 and a salt concentration of 1M (Table 4.11).

4.4.7 Determination of allyl isothiocyanate (AITC) in defatted meal and protein extracts

It is important to determine the amount of AITC produced from the raw material (ground defatted meal) and the protein solution because AITC is the predominant toxic compound generated in alkaline solution. The quantity of AITC in ground defatted meal was determined using the ASTA method. It was found that ground defatted meal had an AITC content of 1.6 % (w/w; Table 4.19).

The level of AITC in the protein solution using the stillage extraction method was determined using the NMR technique with DMF as an internal standard. The chemical

shift of AITC in the protein solution was also determined. AITC was added to the protein solution and the chemical shift of AITC in the NMR spectrum was observed (Fig. 4.17). The AITC peak in the protein solution appeared at a chemical shift of approximately 4 ppm. The content of AITC was 1.0 g/1000 mL in the protein solution (Table 4.19). In this study, 20 grams of ground defatted meal was mixed with 100 mL of thin stillage. Thus, the 20 grams of ground defatted meal could generate 0.326 of AITC, whereas 0.1 g of AITC was actually generated in 100 mL of protein solution. Therefore, approximately, 32 percent of the AITC potential was generated in the protein solution.

In order to determine the level of unbound AITC in the protein solution, the protein solution developed in the complementary protocol was passed through a 5,000 MWCO Amicon ultra-centrifugal filter device (Millipore Corporation, Billerica, MA) to remove protein from the solution, and the NMR spectrum was then obtained. The results are shown in Table 4.19.

Table 4.17 Efficiency of large-scale protein extraction using a meal:solvent ratio of 1:5, w/v.

Grams of undissolved solids	Grams of freeze-dried protein[1]	Protein content (%, w/w)		Moisture content of undissolved solids, %	Efficiency of protein extraction, %
		Freeze-dried protein	Undissolved solids		
633 ± 25	62 ± 10	68 ± 3	4 ± 0	79 ± 1	59 ± 15

Results are means of duplicate analyses.

[1] Calculated from a subsample

Table 4.18 Recovery of protein following the method of Milanova *et al*. (2006).

Grams of undissolved solids	Grams of freeze-dried protein micelle	Protein content (%, w/w)		Moisture content of undissolved solids, %	Recovery of protein (calculated from protein micelle), %
		Freeze-dried protein	Undissolved solids		
48.9 ± 0.6	0.6 ± 0.0	94 ± 4	7.5 ± 0.2	76.3 ± 0.5	7.6 ± 0.3

Results are means of duplicate analyses.

Table 4.19 Allyl isothiocyanate content of defatted meal and protein extracts.

AITC content		
Defatted meal (%, w/w)	Protein solution (g/L)	Protein solution after filtering through 5000 MWCO (g/L)
1.63 ± 0.01	1.0[a] ± 0.2	1.0[a] ± 0.1

[a] Values followed by the same letter are not significantly different at $p<0.05$.

Results are means of duplicate analyses.

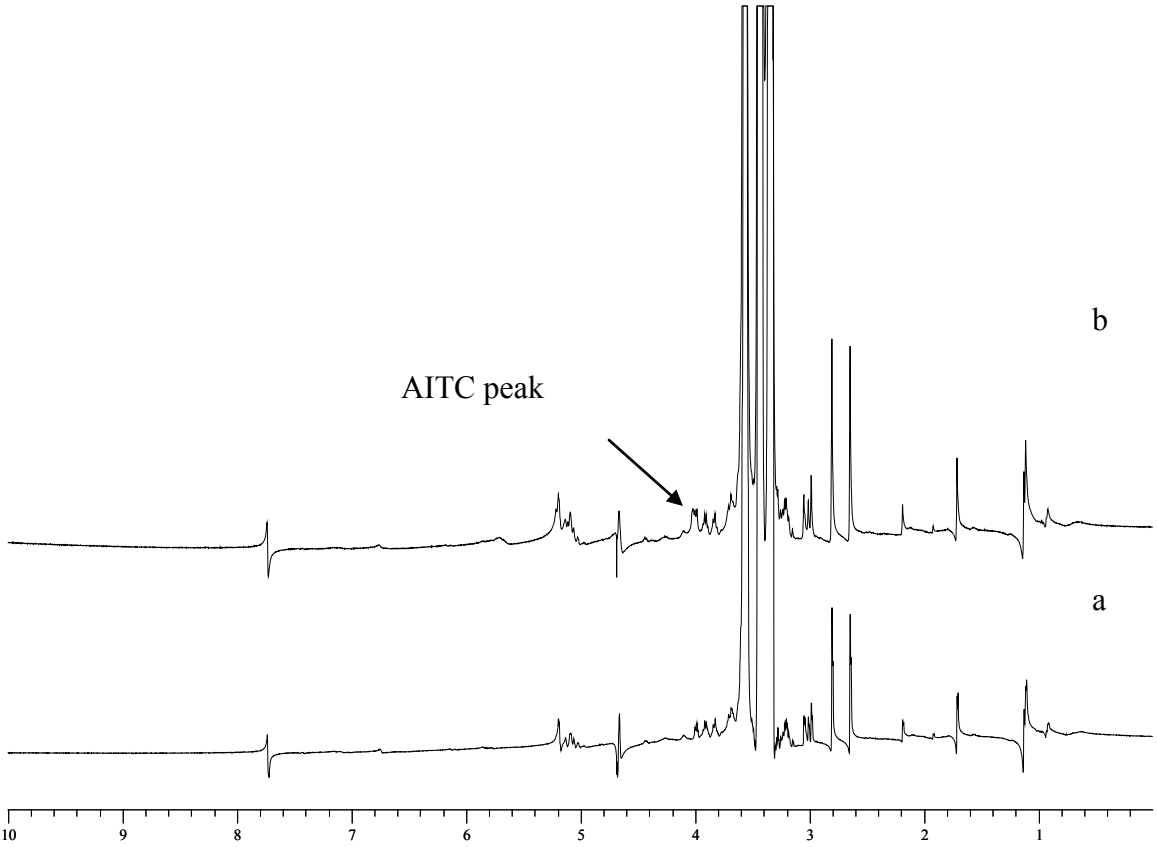


Fig. 4.17 ^{1}H NMR spectra at 500 MHz of protein solution from thin stillage extraction

a) without added AITC;

b) with added AITC.

5 DISCUSSION

5.1 Composition of *B. juncea* seed and defatted meal

Vegetable oil is a commercial product of considerable value. Vegetable oil is usually more valuable on a weight basis than the high protein meal obtained after oil extraction from most oilseeds. The oilseed processing industry in North America and much of the rest of the world utilizes a process of pressing the oilseed followed by solvent extraction with a non-polar volatile solvent such as hexane. This process yields the maximum amount of oil possible and due to the relatively high value of the oil, it maximizes the profit margin of the processor. In the present study, pre-press solvent extracted (PSE) meal was produced using small pilot-scale equipment to emulate the industrial product. When *B. juncea* seed was pressed, the majority of the oil was removed (approximately 80%), and the bulk of the remaining oil was removed by extraction with hexane. The oil content of the defatted meal was 10%, and the protein content, 150%, of that found in the unprocessed seed (Table 4.1). The decrease in oil content from 39 to 4%, on a moisture basis was associated with an increase in protein content from 25 to 37%, moisture basis. It has been reported previously that a screw press can expel 75% or more of the oil (Erickson and Bassin, 1990), which is in agreement with the results from this research. In addition, Bonnardeaux (2007) stated that canola meal in Canada contains 35% crude protein and 3.5% of oil, which is in agreement with the values for the protein and oil contents of defatted *B. juncea* meal used in this research.

5.2 Physical and chemical characteristics, chemical composition, microbial populations and ion contents of thin stillage

5.2.1 Physical and chemical characteristics

The differences in physical and chemical properties of thin stillage may have been the result of variation in the raw material used in each batch of ethanol production. The presence of organic acids, minerals and carbohydrates resulted in the osmotic potential of thin stillage being lower, and the viscosity and density higher, than that of water (data from Table 4.2). The viscosity of water at 25°C is 0.8903 (Korson *et al.,* 1969). The density of water at 25°C is 0.997048 g/cm^3 (Smakula and Sils, 1955).

Thin stillage contained protein and peptides with more than three amino acids (Ingledew, 2003). These compounds may have been produced by yeast cells present in thin stillage. Wheals *et al*. (1999) proposed that thin stillage contained yeast cells, soluble nutrients and small maize molecules. The protein content of thin stillage (dry basis; Table 4.2) is comparable to that reported by Wu (1986) - protein content of stillage solubles = 16.7% - and Mustafa *et al*. (1999) - soluble protein in barley-based thin stillage = 19%. However, the protein content in thin stillage as measured by the Kjeldahl method is not truly protein content but is based on nitrogen content, and stillage contains non-protein nitrogen. Both GPC and betaine are present in thin stillage and both contain nitrogen. Consequently, the true protein content of thin stillage should be adjusted to account for the presence of GPC and betaine and other non-protein nitrogen components. In addition, the pH of the thin stillage used in this research was relatively low (pH 3.7-4.0; Table 4.2). This could have been the result of the organic acids present in thin stillage. Thin stillage has been reported to contain organic acids such as lactic acid and acetic acid (Dowd *et al*., 1994) and in this study, lactic acid was the main organic acid found (Table 4.3). The high level of lactic acid may explain the low pH found in thin stillage. This result is supported by the work of Narendranath *et al.* (2001) who stated that lactic acid accumulation may lower pH. The pH of thin stillage reported here is similar to that of thin stillage from barley spirits (pH 3.7 – 4.1) and corn (pH 3.3 – 4.0) (Wilkie *et al*., 2000). In addition, Jones and Ingledew (1994) reported that the range of pH of thin stillage from wheat and corn was 3.6–4.7.

5.2.2 Chemical constituents

The organic acids present in thin stillage might make it difficult to utilize thin stillage as a protein extraction medium. For instance, Zhang *et al.* (2005) extracted protein from tobacco using 50 mM, pH 9 Tris buffer in the ratio of tobacco tissue: buffer of 1:10. After that, tobacco extract was precipitated at pH 3-8 using 0.2 M HCl, 0.5 M acetic acid or 0.5 M citric acid. They found that acetic acid and citric acid had less effect (pH at minimum solubility and solubility profile) on the isoelectric precipitation of native tobacco protein than did HCl. Previous studies were conducted to determine the constituents in thin stillage. Dowd *et al*. (1994) and Wilkie *et al*. (2000) found that lactic acid, glycerol, ethanol and acetic acid (in decreasing order of concentration) were constituents of thin stillage from cane molasses, whereas the major components in citrus stillage were lactic acid, glycerol, *myo*-inositol, acetic acid, *chiro*-inositol and proline. The major components of whey stillage were lactose, lactic acid, glycerol, acetic acid, glucose, arabinitol and ribitol. In addition, thin stillage contains minerals. From the research of Ojowi *et al*. (1996) and Mustafa *et al*. (1999), it was shown that barley- and wheat-based thin stillage contained minerals such as calcium, magnesium and manganese.

Dowd *et al*. (1994) reported that ethanol was found in cane thin stillage and Wilkie *et al*. (2000) reported that thin stillage from conventional and cellulosic feed stocks contained ethanol, and that the ethanol level in thin stillage should be lower than 0.1-0.2% when an efficient distillation process is used**.**

Previous research used chromatographic methods to identify stillage components, but the current study used quantitative NMR techniques. The author is not aware of a previous example of the use of NMR to quantify organic stillage components. NMR analysis of dilute aqueous solutions has become a more routine practice (Holzgrabe *et al.,* 1998). Analysis of minor constituents of aqueous solutions using NMR may require the use of extra preparative steps. When using proton NMR, for example, the strongest signal arises from the protons present in water. The Fourier transform algorithm used to interpret the spectrum divides the whole spectrum intensity according to the strongest signal in the peak, according to Jacobsen (2007), who stated that “the intensity of NMR signals is directly proportional to the concentration”. Therefore, the presence of large

amounts of solvent water will reduce the sensitivity of the spectrometer to constituents at lower concentrations. While it is possible to concentrate the dilute solution and replace the water with deuterated water, it is also possible to utilize electronic methods (suppression pulse sequences) to suppress a solvent peak. The water peak of NMR spectra of centrifuged and filtered thin stillage was too large to allow the accurate analysis of minor constituents Therefore, a water suppression pulse sequence was utilized to eliminate the water peak (Fig 4.2). This method had two important effects. First, it increased the signal:noise ratio of the whole spectrum, and second, it allowed the observation and quantitative analysis of peaks that were close to the water signal.

Proton NMR spectra of thin stillage taken after addition of pure standards showed that isopropanol, ethanol, lactic acid, 1,3-propanediol, acetic acid, succinic acid, GPC, betaine, glycerol and phenethyl alcohol were present (Table 4.3 and Fig 4.4 to 4.5 and Fig 8.1 to 8.18). According to Lovitt *et al*. (1988), isopropanol found in thin stillage might be a fermentation product of thermophilic anaerobic bacteria (*Clostridium thermohydrosulfuricum*). The ethanol residual in thin stillage may be due to an incomplete distillation process (Wilkie *et al*., 2000). Similarly, Dowd *et al*. (1993) stated that a small amount of ethanol is found in the soluble part of corn stillage. Lactic acid and acetic acid found in thin stillage (Benke *et al*., 1998; Pandiyan *et al*., 1999) were produced by lactic-acid-producing bacteria and acetic-acid-producing bacteria, which are common contaminants found in fuel alcohol plants (Chin and Ingledew, 1993). In addition, Narendranath *et al*. (2001) stated that acetic acid is also an end-product from yeast (*Saccharomyces cerevisiae*) fermentation. 1,3-Propanediol in thin stillage might be a product from the fermentation of *Klebsiella pneumoniae*, *Citrobacter freundii*, *Enterobacter agglomerans* and/or *Clostridium butyricum* using glycerol as a nutrient source (Barbirato *et al.*, 1995; Zeng, 1996; Biebl *et al.,* 1998; Cheng *et al*., 2006). Succinic acid is a second main end-product of alcohol fermentation synthesized and secreted by yeast through Kreb's cycle (Russell, 2003). GPC is synthesized from deacylation of phosphatidylcholine in *S. cerevisiae* (Dowd *et al.*, 2001; Fisher *et al.,* 2005; Almaguer *et al*., 2006). Glycerol is produced by yeast by reducing dihydroxyacetone phosphate to glycerol phosphate followed by dephosphorylation to glycerol (Russell, 2003). Betaine is a compound found in stillage as a co-product from

ethanol fermentation (Kampen, 1993). According to Paananen *et al*. (2006), betaine is found in the roots, seeds and stems of plants. Furthermore, Seibel and Walsh (2002) reported that betaine accumulated in wheat through phosphatidylcholine hydrolysis. 2-Phenylethanol is produced by yeast (*S. cerevisiae*) through the Ehrlich pathway via bioconversion of L-phenylalanine (Etschmann *et al.,* 2002; Stark *et al.*, 2002; Etschmann *et al.*, 2003; Schrader *et al*., 2004). L-phenylalanine is transaminated to phenylpyruvate. Phenylpyruvate is then decarboxylated to phenylacetaldehyde and finally reduced to 2-phenylethanol (Etschmann *et al*., 2003; Schrader *et al*., 2004). For all fermented products, 2-phenylethanol is found at concentrations of 10-35 mg/L (Brunoy *et al.*, 1999). In addition, it was a residue from distillation of thin stillage according to Brunoy *et al*. (1999) and Schrader *et al*. (2004).

The organic compounds in thin stillage can be categorized into three groups:

1) From yeast metabolites

   - glycerol (Russell, 2003), ethanol, succinic acid (Russell, 2003), GPC (Almaguer *et al.,* 2006) and phenethyl alcohol (Schrader *et al.,* 2004);

2) From bacteria metabolites

   - isopropanol (Lovitt *et al.,* 1988), acetic acid, lactic acid (Chin and Ingledew, 1993) and 1,3-propanediol (Cheng *et al*., 2006); and

3) From wheat metabolites

   - betaine (Kampen, 1993).

To quantify the concentrations of compounds in thin stillage by NMR, DMF was used as an internal standard because the chemical shift of DMF (chemical shift of CH = 7.92 and CH_3 = 3.01 and 2.85; Gottlieb *et al.*, 1997) did not interfere with the chemical shifts of thin stillage constituents (Fig 4.6). The results from the NMR technique were compared with the results of HPLC (Table 4.3) and it was found that the concentrations of lactic acid and acetic acid were comparable for the two techniques. Therefore, the NMR technique can be used to quantify the compounds present in thin stillage.

HPLC chromatography also identified the residual carbohydrates remaining in thin stillage after fermentation (dextrin, maltotriose, and maltose monohydrate) (Fig 4.7). Dextrin is the product of a liquefaction step in the ethanol industry whereby gelatinized starch is hydrolyzed to dextrin using α-amylase (Martinez-Gutierrez *et al.*, 2006). The α-

amylase enzyme hydrolyzes α-1,4 glucosidic linkages in amylose and amylopectin, resulting in dextrins. In addition, maltotriose and maltose can be generated in the long liquefaction step (1.5 hours; Kelsall and Lyons, 2003).

HPLC is currently the standard method of stillage analysis, but proton NMR could be used to quantify many of the compounds present in thin stillage. The advantages of using the NMR technique are several. Very little technical input is required to prepare the sample. The analysis is rapid, requiring less than a minute per sample. The sample is not destroyed and the proton NMR method can detect and identify compounds even when there are no standards available (Nicholson *et al*., 1984; Rutar, 1989; Espina *et al*., 2001; Holmes and Antti, 2002). The variable cost of proton NMR analysis can be low when compared with HPLC, but the capital cost of proton NMR equipment is approximately $500,000-1,000,000 which is significantly higher than the cost of HPLC equipment ($75,000-100,000). It is unlikely that proton NMR could be recommended as the analytical tool of choice for stillage analysis. However, even though the price of the NMR instrument is very expensive, the advantages of little sample preparation, less time consuming process, no sample destruction and no standard requirement should be considered.

5.2.3 Microbial populations

Microbial (yeast and bacteria) present in fermentation broth may generate some of the soluble and suspended protein present in the thin stillage (Weller *et al*., 1981). In addition, a portion of the protein may arise from suspended microbial. From the results of microbial population counts (total plate count and yeast and mould contents), it was concluded that yeast, bacteria and fungi were present in the thin stillage used in this research (data from Table 4.4). Furthermore, the bacteria found in thin stillage might be lactic-acid-producing bacteria, acetic-acid-producing bacteria and/or other anaerobic bacteria. The presence of yeasts in thin stillage has been reported by Wheals *et al*. (1999). The presence of lactic-acid-producing-bacteria and acetic-acid-producing-bacteria in breweries, distilleries and fuel alcohol plants has been noted previously (Chen and Ingledew, 1993). Hsieh *et al.* (2005) stated that thin stillage contains nitrogen and other nutrients, such as carbohydrate, which are used as energy and carbon sources for fungi

growth. Furthermore, bacteria found in thin stillage (lactic acid bacteria, acetic acid bacteria, other anaerobic bacteria) and yeast could produce high value compounds such as 1,3-propanediol and 2-phenethyl alcohol.

Bacteria found in thin stillage might produce valuable compounds that will accumulate in the stillage. It was discovered that stillage samples had high levels of 1,3-propanediol. This was likely due to the presence of bacteria that convert glycerol to 1,3-propanediol.

In addition, 2-phenethyl alcohol [a product of yeast fermentation (Schrader *et al.,* 2004)], which is used in perfumes and fragrances (Mitchell and Freehold, 1982), was found in thin stillage. Therefore, if the valuable compounds can be isolated or extracted from thin stillage, thin stillage might be more valuable than a simple feed product.

Conversely, if thin stillage is to be used as a protein extraction medium, microbial contamination must be monitored. If any toxic compounds are produced by microbial organisms, they need to be detected and eliminated.

5.2.4 Ion contents

Ion content can affect protein solubility. Monovalent ions such as sodium and potassium can increase protein solubility, whereas divalent cations often decrease protein solubility. Both cations (calcium, magnesium, potassium and sodium) and anions (chloride, sulfate and nitrate) were found in thin stillage and the results (Table 4.5) are in agreement with Kampen (1989) and Mustafa *et al*. (1999). According to Kampen (1989), the divalent cations calcium and magnesium are found in thin stillage. Moreover, Mustafa *et al*. (1999) stated that calcium, phosphorus, magnesium, copper, iron, manganese, sodium, potassium and zinc were detected in barley-based and wheat-based thin stillage.

Wilkie *et al*. (2000) found that beet molasses stillage contained total sodium, phosphorus, potassium and sulfur (as SO_4^{-2}) in the range of 56-7340, 91-222, 5560-14500 and 1042-5800 mg/L, respectively. Mustafa *et al*. (1999) found that barley-based thin stillage contained calcium, phosphorus and magnesium (5.3, 11.3, and 5.4 g/kg, respectively) and copper, iron, manganese, sodium, potassium and zinc (5.4, 493.2, 52.2, 0.6, 1.6, and 84.5 mg/kg, respectively). Moreover, wheat-based thin stillage contained calcium, phosphorus and magnesium (4.2, 12.1, and 5.9 g/kg, respectively) and copper,

iron, manganese, sodium, potassium and zinc (5.7, 419.1, 110.1, 0.2, 1.6, and 63.8 mg/kg, respectively). Ingledew (2003) reported that corn thin stillage had a mineral content of 3.64 g/L. The major ions found in corn thin stillage were phosphorus, sulfur, potassium, calcium, magnesium, copper, ion, manganese and zinc. Phosphorus levels ranged from 815 to 1762 mg/L, whereas potassium ranged from 705 to 2643 ppm and magnesium levels in all stillage samples examined were between 200-721 ppm. However, the levels of zinc, iron, copper and manganese were low, and sodium was not measured. From this information, it can be concluded that the amounts of ions found in thin stillage depend upon the materials and process used in ethanol fermentation. When comparing wheat-based thin stillage (data from Mustafa *et al*., 1999) with the thin stillage used in this research (Table 4.5), the calcium and magnesium contents of thin stillage used in this research were higher, and the sodium and potassium levels were lower.

5.2.5 Prediction of types and amounts of salts

As noted by Aluko *et al*. (2004), protein of *Sinapis alba* had significant solubility in calcium chloride (0.75 M; 3,000 mg/100 mL Ca). Murray *et al*. (1980) dissolved pea protein in sodium chloride (0.4 M; 920 mg/100 mL) and Murray and Mills (1998 and 1999) used sodium chloride (0.5 M; 1,150 mg/100 mL) to extract protein from canola meal. Therefore, this research was conducted, in part, to confirm the impact of sodium on the formation of protein solutions from mustard meal. Consequently, the effect of the type and amount of salt added were studied. The results of salt type predicted from the Solver Add-in program in Excel (Table 4.6) are in good agreement with the results of Chin and Ingledew (1993) who found $CaCl_2$, $(NH_4)_2SO_4$, NaCl, NH_4Cl, KH_2PO_4, $MgCl_2$, $MgSO_4$ and KCl in thin stillage.

5.3 Design of model thin stillage of defined composition

Thin stillage from the Poundmaker ethanol plant contained a number of components that might affect protein extraction. For example, divalent cations may reduce the efficiency of protein extraction. In addition, organic compounds present in thin stillage may complicate the use of thin stillage as a protein extraction medium and also may affect glucosinolate recovery. If the effects of the constituents in thin stillage on the

process are known, it maybe possible to design a process that can avoid the effects of the compounds, get rid of the particular constituents before using thin stillage or design a process that produces thin stillage which does not have the constituents that have the effects. Moreover, the qualities of industrial thin stillage vary depending on raw material and process. Therefore, the use of thin stillage to study the effects of constituents in thin stillage on protein extraction may be difficult because it is problematical to get the same quality of industrial thin stillage for duplication of experiments. Subsequently, it is important to design and formulate a model thin stillage in order to study the effect of the individual components in thin stillage. It was also necessary to study the physical and chemical properties, type and level of compounds in model thin stillage. The results from Tables 4.7, 4.8, and 4.9 and Figs. 4.12 and 4.13 showed that the physical and chemical properties of, and the types and amounts of compounds and ions in, the model thin stillage were similar or comparable to those of thin stillage from a commercial ethanol plant. Therefore, it can be concluded that it is possible to synthesize a model thin stillage that is similar to the thin stillage produced in the Poundmaker ethanol plant. However, due to the very small differences between deionized distilled water and thin stillage in protein extraction, there was no particular application of the thin stillage to identify problems associated with extraction.

5.4 Comparative efficiency of thin stillage for protein extraction

The amount of liquid used for protein extraction may determine both extraction efficiency and economics. According to Wang (1975), decreasing the meal:solvent ratio increased the amount of protein extracted from soybean using sonication. Moreover, Wang (1978) discovered that when using the sonifier at 60-480 Watts and a frequency of 20 KHz, the total amount of protein peptized in water increased when the ratio of meal:water was decreased. In addition, Esteban *et al*. (1985) discovered that a higher ratio of solvent to defatted almond meal enhanced the grams of protein extracted. Bello and Okezie (1989) reported that the lower the ratio of winged bean flour to solvent, the higher the percent protein extraction. Rosenthal *et al.* (1998) also stated that the ratio of solids to water affected protein extraction, in that when the solids to water ratio

decreased, protein extraction increased. Diosady *et al*. (2005) recommended that the ratio of meal:solvent be kept between 1:3 and 1:30.

Bello and Okezie (1989) and Xu *et al*. (2003) reported that increasing the extraction time increased the per cent extractability. In addition, Diosady *et al*. (2005) stated that extraction time could range from 30 minutes to 120 minutes.

A 1:30 ratio of defatted meal to solvent and an extraction time of 120 minutes were utilized in this study. Sodium chloride was used because it increased ionic strength and the selectivity of solubilization (Moure *et al.*, 2001).

5.4.1 Comparison of the extraction efficiency of two methods for extracting protein from defatted *B. juncea* meal

Processing time affects the economic feasibility of a processing venture. The efficiencies of protein extraction by either mixing ground defatted meal (prepared by grinding approximately 20 grams of defatted meal in a coffee grinder for 30 seconds) with thin stillage (method 1) or blending defatted meal with thin stillage (method 2) were determined. The results (Table 4.10) showed that the protein extraction efficiency of method 2 (28 $\pm$ 2%) was lower than that of method 1 (36 $\pm$ 1%). The result might be explained by the differences in particle size and processing time. The particle size of ground defatted meal was smaller than that of the meal blended with thin stillage. The effect of particle size on protein extraction has been studied by others. Pomeranz (1965) extracted protein from defatted, toasted soybean meal of different particle sizes. He found that protein was extracted more efficiently from finely powdered meal than from coarsely ground samples. Finnigan and Lewis (1985) discovered that the quantity of nitrogen extracted from a ground sample of defatted rapeseed meal was higher than from an unground sample at pH 10. Chen and Rohani (1992) stated that the time for dissolution, shape of the solubility curve and total nitrogen solubility of rapeseed flakes was lower for coarse flakes than for fine flakes. Rosenthal *et al*. (1998) studied the effect of particle size of flour on protein extraction. The result showed that the finer the particle, the greater the yield of extract. Russin *et al.* (2007) revealed that protein recovery was higher when extracting protein from fine defatted soybean flour using an alkaline solution as a solvent. Extraction time also affected efficiency as longer extraction times produced higher yields.

The extraction time when using ground defatted meal was longer than when defatted meal was blended with thin stillage. The more efficient method was used in the subsequent experiments.

5.4.2 Generation of protein titration curves

The maximum protein solubility and probable maximum extraction efficiency will occur at pH values well above and below the isoelectric point. In this study, a salt solution was used as a solvent for protein extraction instead of thin stillage because there are components in thin stillage that may buffer the system or affect protein charge. Lyophilized protein, obtained by dialysis, was used to determine the isoelectric point. Dialysis was necessary to remove components such as salts and small molecules from the protein solution. The isoelectric point of lyophilized protein was at approximately pH 6.4 (Fig 4.14), which agrees with the findings of Arntfield (2004). Chen and Rohani (1992) and McCurdy (1990) reported that the minimum solubility of nitrogen in rapeseed protein occurred betweeen pH 4 and 8. El-Nockrashy *et al.* (1977) found that protein isolates from rapeseed had isoelectric points at pH 6 and 3.6. In addition, the isoelectric precipitation of *B. juncea* protein has been found to occur in this range [pH 3.8-4.0 and 7.8-8.0, Rao *et al*., (1978); pH 6.0, Moure *et al.,* (2006)]. Therefore, a pH above the isoelectric point (above pH 7) was chosen for studies of the efficiency of protein extraction.

5.4.3 Determining the efficiency of protein extraction

The effect of using thin stillage and model thin stillage on protein extraction efficiency was studied, and the results were compared with those from sodium chloride solution extraction. The effects of salt concentration and pH of the mixture were also examined to identify the optimum extraction conditions. Protein extraction was affected by both. Unfortunately, the optimum conditions were not determined because the optimum yield occurred at a pH and salt concentration above those used in this study. In addition, the lack of fit for the model was highly significant ($P < 0.05$) and R^2 of response surface design was being 0.67. Therefore, only 67 per cent of data can be explained by equation 4.1. This might be an effect of the large variences in data collected from the

extraction at pH 10, salt concentration 1.0 and pH 8, salt concentration 1.0 (data from Table 4.11).

Since the response surface graph (Fig4.15) showed that when pH and salt concentration increased, protein extraction efficiency increased, the economic optimum could be determined knowing the value of protein and the cost of inputs. High pH and salt concentration are not necessarily practical if they are not cost effective even if they increase protein extraction efficiency. At extremely alkaline pH, proteins have high net charges resulting in strong intramolecular electrostatic repulsion. This would cause swelling and unfolding of protein molecules (Damodaran, 1996). Also, high salt concentrations are not economical. The data clearly showed that when pH and/or salt concentration increased, the protein extraction efficiency increased. When the pH is above the isoelectric pH, proteins carry negative charges and electrostatic repulsion and hydration occur, which enhances the solubility of protein. Consequently, the maximum solubility of proteins takes place at alkaline pH. Damodaran (1996) stated that when the salt concentration is at a low ionic strength (<0.5 M), the solubility of proteins that contain polar surface domains typically increases. The converse occurs for proteins that have non-polar surface domains because there is a decrease in the ion activity of the protein macro-ion (increasing solubility) and enhanced hydrophobic interaction (decreasing solubility). At high ionic strengths (>1M), some ions lower protein solubility (known as salting out) whereas other ions increase protein solubility (known as salting in). The effects of pH and salt concentration demonstrated in this experiment are in agreement with the results of other researchers. Rao *et al*. (1978) discovered that the solubility of protein from defatted *B. juncea* flour using a 1M NaOH solution was higher when the pH was increased above the isoelectric point, and at pH 11 the solubility of mustard protein was greater than 80 percent. In addition, the solubility of protein in a 1M NaCl solution was greater than in water at all pHs above pH 4, and the solubility of protein in 0.1M NaCl solution showed a similar trend and displayed an intermediate value between water and a 1M NaCl solution. Finnigen and Lewis (1985) conducted single protein extractions from rapeseed, commercial rapeseed meal, and presscake using NaCl and $CaCl_2$ solutions at 0-1M concentrations. They found that the most efficient extraction occurred at 0.2M NaCl and $CaCl_2$ and increased slightly when increasing the

salt concentration up to 1M (around 50% for NaCl and 60% for $CaCl_2$ for rapeseed and presscake, respectively, and around 35% for NaCl and 45% for $CaCl_2$ for commercial rapeseed meal). In addition, they used 1M NaCl or $CaCl_2$ and various pHs to extract protein from commercial rapeseed meal. They found that for a 1 M NaCl solution, per cent nitrogen extraction was increased over the range of pH 2.5 to 10.5 (at pH 10.5, they obtained about 60% nitrogen extraction from rapeseed and presscake and around 45% from commercial rapeseed meal). For $CaCl_2$, per cent nitrogen extraction increased over the range of pH 2.5 to approximately 8.5, and dropped over the range of pH 8.5-10.5 because of salting out at pH 11.5 (they obtained about 50% nitrogen extraction from rapeseed and presscake and around 40% from commercial rapeseed meal). Murray and Mills (1999) reported that "As the ionic strength of the salt solution increases, the degree of solubilization of protein in the source material initially increases until a maximum value is achieved". Xu *et al*. (2003) studied the effect of pH (over the range of 10-13) on extractability of yellow mustard protein and found that protein extractability increased as the pH was increased, with more than 80 percent being extracted at approximately pH 12. Marnoch and Diosady (2006) studied production of mustard protein isolates from *B. juncea* using an alkaline solution (pH 7-13) to extract protein and a membrane-based process to isolate protein. The optimum condition was at pH 11. Protein was ultrafiltered with a concentration factor 4, diafiltration volume of 3, and precipitated at pH 5. Eighty-one per cent of the protein was recovered (the distribution of crude protein in products: precipitated protein isolate, 47.3%; soluble protein isolate 3.8%; and meal residue, 13%). Lindeboom and Wanasundara (2007) used water at different pHs (3.5-10.0) to extract protein from *Sinapis alba*. They discovered that the protein content of extracts increased when the pH was above 7.5, and was as high as 25 mg/mL at pH 10.

Combining the optimal conditions of pH and salt concentration had a complementary effect on protein extraction efficiency. These conditions of pH 10 and 1M salt concentration (complementary process) were used when comparing the efficiency of protein extraction for thin stillage, model thin stillage and sodium chloride solution in the next experiment as these conditions afforded the highest protein extraction efficiency under the conditions tested.

5.4.4 Comparison of the relative efficiency of protein extraction using thin stillage, model thin stillage and sodium chloride solution

Efficiency of protein extraction

Compounds present in thin stillage, such as divalent cations, can affect the efficiency of protein extraction. Consequently, thin stillage and model thin stillage were used as protein extraction media (150 mL of solvent:5 grams of ground defatted meal) compared to water-based (sodium chloride solution) extraction to study the effect of these compounds. Thin stillage and model thin stillage used in this study were first filtered, using an ultrafiltration membrane (3,000 MWCO) to remove large molecules (such as proteins and carbohydrates). However, no significant differences were found in extraction efficiency (data from Table 4.12). Therefore, it can be concluded that protein extraction efficiency is not affected by these compounds present in thin stillage under the conditions used. However, the quality of the extracted protein may be affected by these compounds. The quality of a protein extract in animal feed applications is primarily determined by its amino acid composition and the ability of digestive enzymes to release amino acids from the protein (Willis, 2003).

SDS-PAGE

Gel electrophoresis can be used to demonstrate aspects of the quality of a protein isolate. The bands that appeared in the SDS-PAGE gel were clear (Fig. 4.16), showing that the protein extracted from *B. juncea* using thin stillage, model stillage or sodium chloride solution had not been hydrolyzed significantly. The molecular weights of the extracted proteins were approximately 14, 18-20, 20-22, 34 and 55 kDa. Appleqvist (1972) stated that defatted meal of *Brassica* species contained two major protein fractions, 2S (napin, 45-50%) and 12S (cruciferin, 25%). Monsalve and co-workers (1997) reported that two types of napin (15 kDa and 12 kDa) were present in rapeseed. Aluko and McIntosh (2001) reported that a "52 kDa polypeptide has previously been shown to be present in the purified 12S preparation from *Brassica napus* seed". Aluko *et al*. (2004) stated that in *S. alba* protein isolates, a napin band appeared at 5 kDa and cruciferin bands at 22, 28 and 35 kDa. Aluko and McIntosh (2004) demonstrated that 12 and 13 kDa polypeptides are subunits of the 2S protein of mustard seed. The research of

Aluko *et al*. (2005) showed that peptides with masses of 16 and 55 kDa were found in defatted *B*. *juncea* seed meal, but absent when 2-mercaptoethanol was added. In the presence of 2-mercaptoethanol, bands at 16 and 55 kDa disappeared, indicating that each of these proteins was composed of polypeptides held together with disulfide bonds. Bands at 12 and 13 kDa (2S), which were polypeptide subunits held together by disulfide bonds, were found in acid-precipitated protein concentrates. From the information above, it was concluded that the bands found using SDS-PAGE (Fig. 4.16) were napin and cruciferin, and they can be extracted with sodium chloride solution, model thin stillage or thin stillage. These results were confirmed by peptide sequencing.

Peptide sequencing

The results of peptide sequencing (Table 4.13) showed that the 14 kDa subunit was a peptide fragment of napin and cruciferin, and subunits 18-20, 20-22, 34 and 55 kDa were peptide fragments of cruciferin. The results are in agreement with those of Aluko and McIntosh (2001), Aluko *et al*. (2004) and Aluko and McIntosh (2004), as described above. In addition, the same peptide sequence of cruciferin appeared in different bands in gel electrophoresis using fragment sequences. This can be explained in two ways: 1) during processing the extracted protein may be degraded or broken down to smaller molecules by enzyme, pH or hydrolysis and 2) the cruciferin present in rapeseed is a member of the 11S globulins which are hexameric molecules consisting of homologous but not identical units (Tandang *et al.,* 2004). Procruciferin has a molecular weight around 50 kDa and the subunits, α and β-polypeptides, are linked by disulfide bonds. They can be produced from a precursor which has a molecular weight of ~30 and 20 kDa (Hinz *et al.,* 2007).

Interestingly, from the results of gel electrophoresis and peptide sequencing, it was noticed that no protein from yeast, bacteria, or wheat was found. Only napin and cruciferin were present in the extracted protein.

Amino acid composition

The amino acid contents of protein extracted with industrial thin stillage, model thin stillage or sodium chloride solution (Table 4.14) were comparable to that of protein

isolated from *B. juncea* and from rapeseed meal (Table 5.1). In addition, the results from Table 4.14 are also comparable to the amino acid composition of *B. juncea* meal as reported by Miller and Jones (1962). The extracted protein had a high content of essential amino acids; particularly sulphur amino acids (methionine and cysteine, more than 70 mg/g protein), which agrees with the findings of Bos *et al*. (2007). The quantity of essential amino acids extracted is sufficient to meet FAO standards for human requirements (Table 5.1).

In vitro digestibility

A multienzyme technique (Hsu *et al.*, 1977) was utilized to determine the digestibility of protein extracted with thin stillage, model thin stillage or sodium chloride solution. Trypsin, chymotrypsin and peptidase are enzymes that function in the gastro-intestinal tract to cleave the peptide bonds in proteins. Trypsin cleaves the peptide bond adjacent to basic amino acids (C terminal side of lysine and arginine except when either is followed by proline) (Manea *et al*., 2007). Chymotrypsin cleaves the peptide bond next to the aromatic amino acids (Olsen *at al*., 2004), methionine, asparagine, histidine and leucine (C terminal side) (Orten *et al*., 1970 and Ramakrishnan, 2001). Peptidase can cleave at endo-peptide bonds (non-terminal amino acid) and exo-peptide bonds (N or C terminal) (Lundy *et al*., 2000). Therefore, trypsin, chymotrypsin and peptidase were employed to digest extracted protein in a multienzyme technique. The digestibility of protein extracts prepared with thin stillage, model thin stillage or sodium chloride solution were similar (approximately 73-75%) (Table 4.15). In addition, the digestibility values were similar to those of partially-lactose-depleted whey (73.1%) and corn-milo grain (72.0%; Hsu *et al*., 1977). Additionally, Sadeghi *et al*. (2006) found that the digestibilities of defatted *B. juncea* meal and protein isolated from defatted *B. juncea* meal were 80.6 and 92.4%, respectively. When comparing the digestibility of extracted protein from this study, and those of defatted *B. juncea* meal and protein isolated from defatted *B. juncea* meal (data from Sadeghi *et al*., 2006), it is apparent that the digestibility of extracted protein was lower than the protein digestibility of defatted *B. juncea* meal or of protein isolated from defatted *B. juncea* meal.

Table 5.1. Amino acid composition of extracted protein (g/100 g of protein) from *B. juncea* and rapeseed meal and, FAO and human requirements for essential amino acids.

Amino acid	Protein isolated from *B. juncea*[1)]	Percentage in protein of rapeseed meal[2)]	FAO[3)]	Human requirement[4)]
Cysteine	2.94 ± 0.08	1.23	-	-
Asparagine	-	6.11	-	-
Methionine	2.73 ± 0.04	1.78	2.5[a]	1.7
Threonine	4.31 ± 0.09	4.50	3.4	3.3
Serine	4.49 ± 0.05	-	-	-
Glutamic acid	20.83 ± 0.15	-	-	-
Glycine	5.19 ± 0.04	-	-	-
Alanine	4.36 ± 0.10	-	-	-
Valine	5.17 ± 0.05	5.11	3.5	4.2
Isoleucine	3.65 ± 0.06	3.98	2.8	4.2
Leucine	7.75 ± 0.05	6.97	6.6	4.8
Phenylalanine	4.51 ± 0.04	4.01	-	6.0[b]
Histidine	2.86 ± 0.03	2.81	1.9	2.4
Lysine	4.90 ± 0.10	5.98	5.8	4.2
Arginine	9.97 ± 0.05	-	-	2.0
Tryptophan	1.55 ± 0.04	1.16	-	-
Tyrosine	2.26 ± 0.05	-	6.3[b]	-
Aspartic acid	6.97 ± 0.08	-	-	-
Proline	5.56 ± 0.05	-	-	-

[1)] Sadeghi *et al*., 2006. [2)] Bell, 1984. [3)] Pedroche *et al*., 2004. [4)] Zhou *et al*., 1990.
[a] value for methionine + cysteine
[b] value for tyrosine + phenylalanine

Lysine availability

Lysine is frequently the factor limiting the protein quality of mixed diets for human food and animal feed. Consequently, it is important to minimize lysine damage when processing high-protein feeds (Carpenter, 1960). The amount of available lysine in the protein extracted using thin stillage, model thin stillage or sodium chloride solution was measured using the reaction between OPA and the free ε-amino acid group of lysine (Ferrer *et al*., 2003). It was found that the lysine availabilities of protein extracted using thin stillage, model thin stillage or sodium chloride solution were similar (approximately 75% of total lysine) (calculated from data from Table 4.15 comparing to data from Table 4.14). The results agree with those of Larbier *et al*. (1991) who found that when using a basal diet supplemented with whole rapeseed meal, dehulled rapeseed meal or soybean meal as the source of lysine, the true digestibilities of lysine in whole rapeseed meal, dehulled rapeseed meal and soybean meal in cockerels were 80.1, 86.0, and 88.9%, respectively. In addition, available lysine values for chicks were 72.8, 78.3, and 85.5%, respectively. The lysine availability of the extracted protein in this research (75%) was much higher than the lysine availability of rapeseed meal using the Carpenter method (44%; Rozan *et al*., 1996).

Color

The HunterLab was utilized to measure the color of extracted protein. The results (Table 4.16) showed that the color of protein extracted with sodium chloride solution was lightest when compared with protein extracted with model thin stillage or thin stillage, respectively. The darker color protein extracted with thin stillage and model thin stillage may be due to the inclusion of color compounds with the protein or a reaction with compounds in the stillage that react with protein to produce color. In addition, protein extracted with conventional thin stillage can pick up the color from glycerin from biodiesel industry and carbohydrates and wheat pigments in thin stillage. Therefore, it can be concluded that the compound in conventional thin stillage may have the effects on the other qualities of protein which have not been tested in this research, for instance, *in vivo* digestibility. Consequently, the other qualities of extracted protein should be tested in the future studies.

5.4.5 Large-scale protein extraction

The ratio of ground defatted meal: solvent is very important for the economics of protein extraction. However, the ratio of ground defatted meal:solvent used in the current study (1:30, w/v) would not be practical for industrial processing of large amounts of meal. Also, the energy required to evaporate water from the protein solution in the final processing step would make the overall process inefficient at low meal:solvent ratios. However, the results showed that when the meal:solvent ratio used for protein extraction was reduced from 1:30 to 1:5, protein extraction efficiency decreased from 80% (Table 4.11) to 59% (Table 4.17).

5.4.6 Comparison of protein extraction efficiency with that of a published protocol

Using the protocol of Milanova *et al*. (2006), protein micelles were formed using cold water. However, the per cent recovery of protein was low (approximately 7.5%) (Table 4.18) compared to the efficiency of protein extraction in the present study which used thin stillage at pH 10 and a salt concentration of 1M (approximately 80%) (Table 4.11). The complementary process which was developed in this research was more efficient in terms of protein extraction than the published protocol, which required additional steps to maximize protein recovery, making it more costly for manufacturers.

5.4.7 Determination of allyl isothiocyanate (AITC) in defatted meal and protein extracts

Glucosinolate and myrosinase are present in different compartments of the mustard seed (Murthy and Rao, 1986). In the Brassicacea, myrosinase is generally found in myrosin cells (a form of idoblast cell; Andréasson *et al.,* 2001). When glucosinolate and myrosinase interact under neutral pH or alkaline conditions, isothiocyanate is the preferred product, whereas the formation of nitrile is favoured at low pH (Uppström, 1995; Boness and Rossiter, 1996; Brown and Morra, 2005). These products are toxic compounds that obstruct the thyroid function and cause liver and kidney damage (Sadeghi *et al*., 2006). In addition, Mieth *et al*. (1983) stated that "Antinutritional components of *Brassica* seed are, in particular, glucosinolates (thioglucosides, mustard oil glucosides) located in the parenchyma and their decomposition products, which not

only negatively influence iodine metabolism and thus the basic metabolic rate but also bring about disturbances in the functioning of other vital organs.". It is likely that AITC is a major product from the myrosinase/glucosinolate system under the alkaline conditions used for protein extraction because in most cruciferous vegetables except broccoli, sinigrin, an aliphatic glucosinolate, is the predominant glucosinolate (Kushad *et al*., 1999) which gives rise to AITC as a major product under myrosinase-dependent hydrolysis (Masuda *et al.,* 1996). Therefore, it is important to quantify the amount of AITC in the protein extract. The level of AITC in ground defatted meal used in this research was 1.63 (%, w/w) (Table 4.19), which is in good agreement with the results of Sadeghi *et al*. (2006) who found 18.75 mg of isothiocyanate per gram of defatted *B. juncea* meal.

It has been reported that AITC can interact with protein at the free amino groups of lysine and arginine (Kawakishi and Kaneko, 1987) and the phenolic groups of tyrosine residues (Murthy and Rao, 1986) to form thiourea-like compounds (Kawakishi and Kaneko, 1987). In addition, it can react slowly with cystine to cleave the disulfide bond in the cystine moiety and form polymers (Kawakishi and Kaneko, 1987). In order to examine free AITC in the protein extracts generated in the complementary protocol, the protein extract was filtered to remove protein and the NMR spectra were examined. When the amount of AITC in the protein extract and in the filtered extract were compared, no significant difference was found (Table 4.19). It was concluded that the AITC did not bind with the extracted protein. Consequently, when the protein solution is lyophilized or further processed, AITC could be evaporated easily from the protein solution as it is a volatile compound. Thus, the presence of residual AITC in the dried, extracted protein will not be problematic when using the complementary process.

6 SUMMARY AND CONCLUSIONS

Thin stillage is a by-product of the ethanol industry. For the purpose of making animal feed, thin stillage has to be evaporated to make a syrup which requires operating cost (amortization, thermal energy, electrical energy, water and sewer, labour, chemicals and supplies, and marketing and overhead). A large amount of power [1,000 kW/44,642.6 kg/hour of thin stillage (44,642.6 kg = 100,000 lb)] is required to evaporate water from thin stillage. If thin stillage could be used right after ethanol distillation for protein extraction, the ethanol industry could save the energy otherwise needed for evaporation. In addition, biodiesel plants can provide glycerol that contains the base-catalyst and defatted meal to the protein industry. Therefore, it will benefit the ethanol, biodiesel, and protein industries if these factories are located near each other. Additionally, organic acids and simple alcohols present in thin stillage might have an inhibitory effect on myrosinase activity, monovalent ions in thin stillage may increase protein extraction efficiency, and thin stillage could be a potential commercial source of GPC and betaine.

Four batches of thin stillage provided by Pound-Maker Agventure Ltd. were analyzed for their physical and chemical characteristics, chemical composition, microbial populations and ion content.

Physical and chemical characteristics

The physical and chemical characteristics of thin stillage were not similar to those of water because of the presence of soluble products from the fermentation of wheat or barley including organic acids, simple alcohols and salts in thin stillage.

Chemical composition

The types and concentrations of compounds in thin stillage were determined using ^{1}H NMR with a water suppression technique. The results showed that the compounds found in thin stillage can be categorized into three groups:

1) Yeast metabolites that include glycerol, ethanol, succinic acid, glycerophosphorylcholine (GPC) and phenethyl alcohol;

2) Bacterial metabolites that include isopropanol, acetic acid, lactic acid and 1,3-propanediol; and

3) Wheat metabolites including betaine.

Furthermore, the results from ^{1}H NMR were compared with results from HPLC. The results from NMR were comparable to those from HPLC for lactic acid and acetic acid. Therefore, the NMR technique can be used for analysis instead of HPLC. NMR has several advantages, including its non-selective nature (it is used to detect organic compounds that contain protons and carbon), little sample preparation is required, and for many relatively abundant compounds, analysis is rapid.

Microbial populations

In this research, bacteria, yeast and fungi were found in commercial thin stillage. The populations of these organisms varied depending on the batch of thin stillage. The microbial populations were not classified in this research.

Ion contents

The results from ion analysis showed that the cations present in industrial thin stillage were potassium, sodium and calcium, in order of concentration. The anions present in thin stillage were sulphate, chloride and nitrate, in order of concentration. The salts present in thin stillage were $CaCl_2$, NaCl, K_2SO_4, $NaNO_3$, $Mg(OH)_2$, Na_2SO_4 and KOH.

It was originally predicted that stillage components could affect protein extraction efficiency and quality of extracted protein and it was reasoned that model thin stillage would allow easy control of stillage extraction efficiency and enable studies of the effects

of stillage components on extraction. It was also reasoned that model thin stillage would enable the design of processes that improve the use of thin stillage for protein extraction. Therefore, model thin stillage was prepared based on compositional information from conventional thin stillage. The results showed that the model thin stillage was similar to thin stillage from the ethanol industry in terms of its physical and chemical properties, chemical composition and ion content. As there were no specific difficulties encountered in extraction of protein using thin stillage as compared to sodium chloride solution, an analysis of the individual components of thin stillage using model stillage was not conducted.

When using thin stillage for protein extraction, it was found that thin stillage can be used to extract protein from defatted *B. juncea* meal. Mixing ground defatted meal with thin stillage was a more efficient method than blending the defatted meal with stillage. To achieve maximum extraction, the isoelectric point was determined for protein extracted with a salt solution. It was found that the extracted protein had an isoelectric point near 6.4. Therefore, maintaining the pH above 7 was studied extensively as a condition for enhancing protein extraction efficiency. In addition to determining the impact of various higher pH treatments on protein extraction, thin stillage to protein ratio, salt concentration and milling conditions were also investigated. In this research, the pH of the protein extraction system was adjusted using a 10 percent solution of KOH in glycerin obtained as a co-product from commercial biodiesel production (Milligan Biotech). Protein extraction efficiency was determined primarily by pH and salt concentration. Furthermore, no optimum conditions were identified for protein extraction. However, within the boundaries of the current research, pH 10 and a salt concentration of 1M afforded the highest protein extraction efficiency. Even though no optimum was discovered, further studies should focus on conditions around pH 10.2 and 0.89 M NaCl concentration (result from Ridge analysis).

The qualities of protein extracted from thin stillage (molecular weight and type of polypeptides, digestibility, lysine availability) were compared to those of protein extracted with model thin stillage or sodium chloride solution. The results showed that the efficiency of extraction and the qualities of protein extracted with thin stillage were comparable to protein extracted with model thin stillage or sodium chloride solution.

Therefore, it can be concluded that the compounds present in thin stillage did not have any significant effect on protein extraction efficiency and the qualities of the extracted protein. However, the quality of extracted protein was affected by the use of thin stillage as an extraction solvent as the colour of protein isolates recovered from thin stillage was considerably darker than those from sodium chloride solution. Therefore, thin stillage might have the effects on the other qualities of extract protein, for instance, *in vivo* digestibility. SDS-PAGE analysis showed discrete protein bands, indicating that the extracted protein had not been extensively hydrolysed. Further analysis of the extracted proteins using mass fingerprinting of trypsin fragments showed that only napin and cruciferin were present in the bands detected by SDS-PAGE. No protein from yeast, bacteria or wheat was found in protein extracted with thin stillage. All extracted protein was subjected to digestion by trypsin, chymotrypsin and pepsin. The levels of essential amino acids in extracted protein were sufficient to meet FAO standards and human requirements. High essential amino acid contents, especially sulfur amino acids and lysine, were observed. Extracted protein had high lysine availability (75%).

Since a ratio of 1:30 of defatted meal:thin stillage is not practical for industry, the ratio of defatted meal:thin stillage was increased to 1:5. As expected, when the ratio of defatted meal:thin stillage was increased, the protein extraction efficiency and the percentage of protein in the extracted protein decreased. The complementary method was compared with a published protocol, and it was found that protein recovery with the complementary method was significantly greater than with a published protocol. Furthermore, AITC was generated and present in protein extracts. NMR results showed that AITC did not bind with protein in the extract, so AITC can be removed/evaporated in further processes such as dialysis and lyophilization.

In this research, the results showed that thin stillage can be used as a solvent for protein extraction without any effects on the efficiency of protein extraction or the important nutritional qualities of the protein extract. Therefore, the potential use of a by-product, thin stillage, as a part of a protein extraction process would increase the financial viability of the industrial process.

Suggested Future Work

- The physical and chemical properties, and the types and amount of compounds present in, thin stillage are dependent on the raw material fermented in ethanol production. Therefore, thin stillage chemical composition from other fermentation feedstocks should be examined if these materials are to be utilized for protein extraction.

- Even though high concentrations of compounds were detected in thin stillage, compounds present at lower concentrations should also be identified to determine if these compounds could have an impact on the use of thin stillage in protein extraction.

- Although a method for GPC and betaine recovery was developed (Yunhua Jia, personal communication), and crude mixtures of GPC and betaine were prepared in this research, methods for separating GPC and betaine would prove valuable in the future.

- Bacteria that consume glycerol and produce 1,3-propanediol were present in the stillage. In the future, isolation and identification of the bacteria endogenous to the ethanol production facility that consume glycerol in thin stillage should be studied. This research could be beneficial for the development of future bioprocesses.

- Thin stillage is a potential commercial source of valuable compounds such as GPC, betaine and 2-phenylethanol. Continued study of methods for maximizing the value of thin stillage through the isolation of these compounds should be pursued.

- The safety of using thin stillage as a solvent for protein extraction is unknown. Research to establish the relative safety of this material must be completed. Such research should determine the presence of potential hazards, including pathogenic microbial, toxins and other materials that might prevent the safe use of thin stillage in protein extraction.

- The isoelectric point of extracted protein in this research was determined with a crude isolate protein. However, the isoelectric point relies on the charges of all proteins. Therefore, the isoelectric point should be determined based on charge of protein.

- Protein extraction may result in either a protein isolate or a protein concentrate. The latter option may reduce the cost of protein recovery.

- The quality and shelf-life of protein concentrates and protein isolates should be studied before being utilized as animal feed.

- Napin protein, a major component of the protein isolate, is resistant to trypsin digestion and may not be as available as cruciferin. The relative concentrations of napin and cruciferin in the extracted protein should be investigated.

- In this research protein, was recovered using dialysis. This method would be difficult to apply at industrial scale. Methods for protein recovery that induce protein precipitation, potentially transglutaminase or tannin, might be investigated as substitutes for dialysis or precipitation at the isoelectric point, as methods for protein recovery.

- *In vitro* digestibility and lysine availability of extracted protein should be tested in animal diets such as swine and fish.

- The solubility of the extracted protein in different pH should be studied as solubility of protein is an important aspect of functionality.

- Glucosinolates and glucosinolate break-down products are known to be highly biologically active compounds. Products of protein recovery might contain glucosinolates or glucosinolate break-down products. Therefore, the concentrations of glucosinolates and glucosinolate break-down products present in the supernatant should be studied. Potentially, glucosinolate-rich products might be isolated from the co-products of protein concentration.

- Myrosinase-free oil seed might be an alternative choice for protein extraction because the glucosinolates could not break down to volatile toxic products. Besides, myrosinases (glucosinolate degrading enzyme) can be found from other sources, such as fungi. Therefore, it is easier for transporting and packing glucosinolate rich products and myrosinases than volatile compounds.

- The results of this research did not indicate significant binding between AITC and protein. Nevertheless, a more thorough investigation of AITC binding would be needed to prove if this phenomenon is occurring during extraction.

- Thin stillage may be used to extract other materials and potentially produce concentrates or isolates of mucilage, beta-glucan or starch. Consequently, future research could be focus on using thin stillage as a solvent in other extractions.

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APPENDIX A - ^{1}H NMR SPECTRA OF COMPOUNDS IN THIN STILLAGE

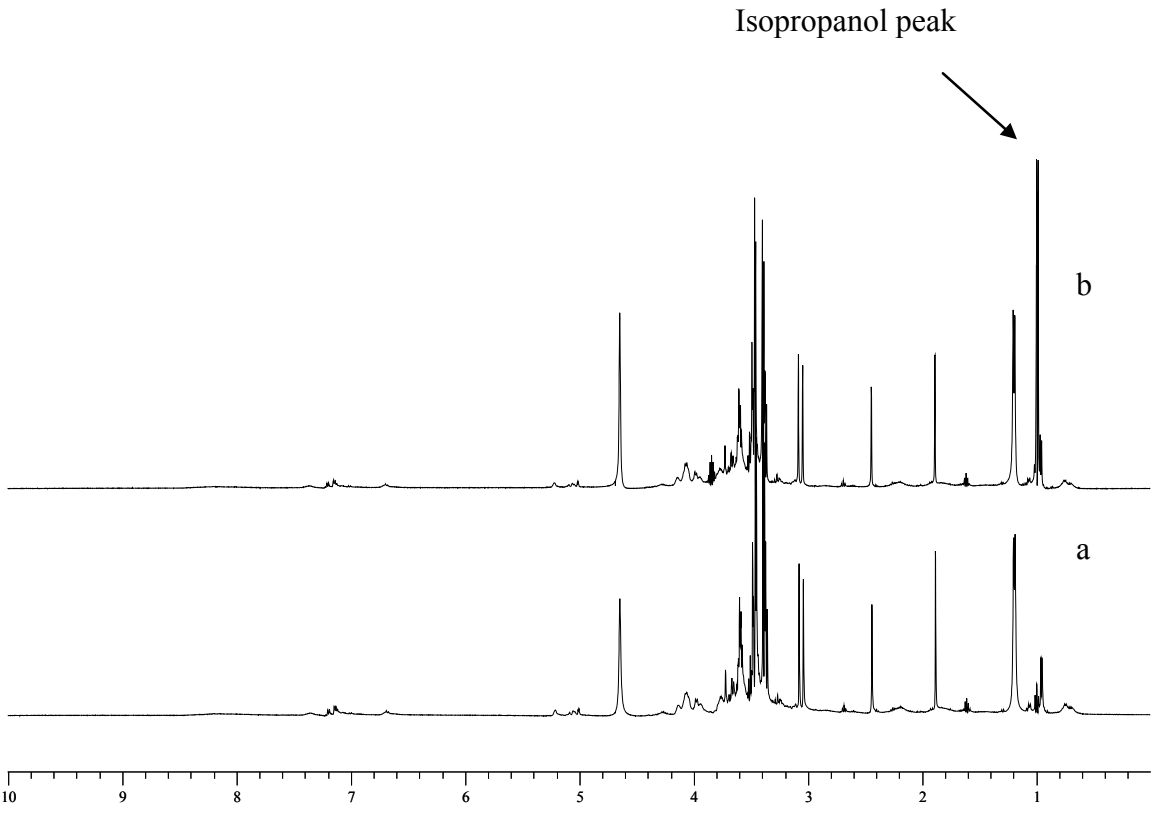

Fig. 8.1 ^{1}H NMR spectra at 500 MHz of thin stillage (May 18, 2007 sample)

a) without added isopropanol;

b) with added isopropanol.

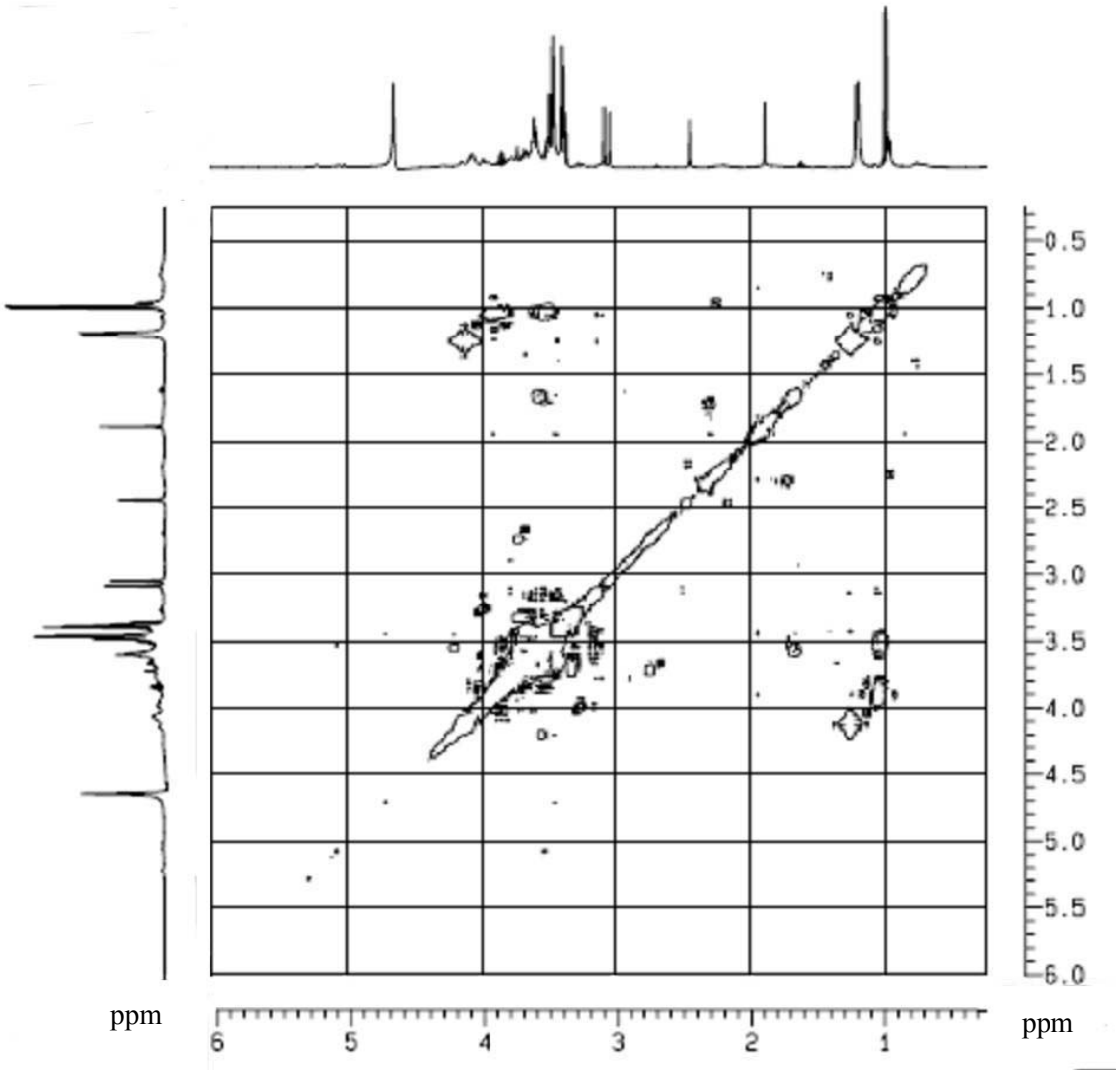

Fig. 8.2 2D ^{1}H NMR spectra of thin stillage (May 18, 2007 sample) with added isopropanol.

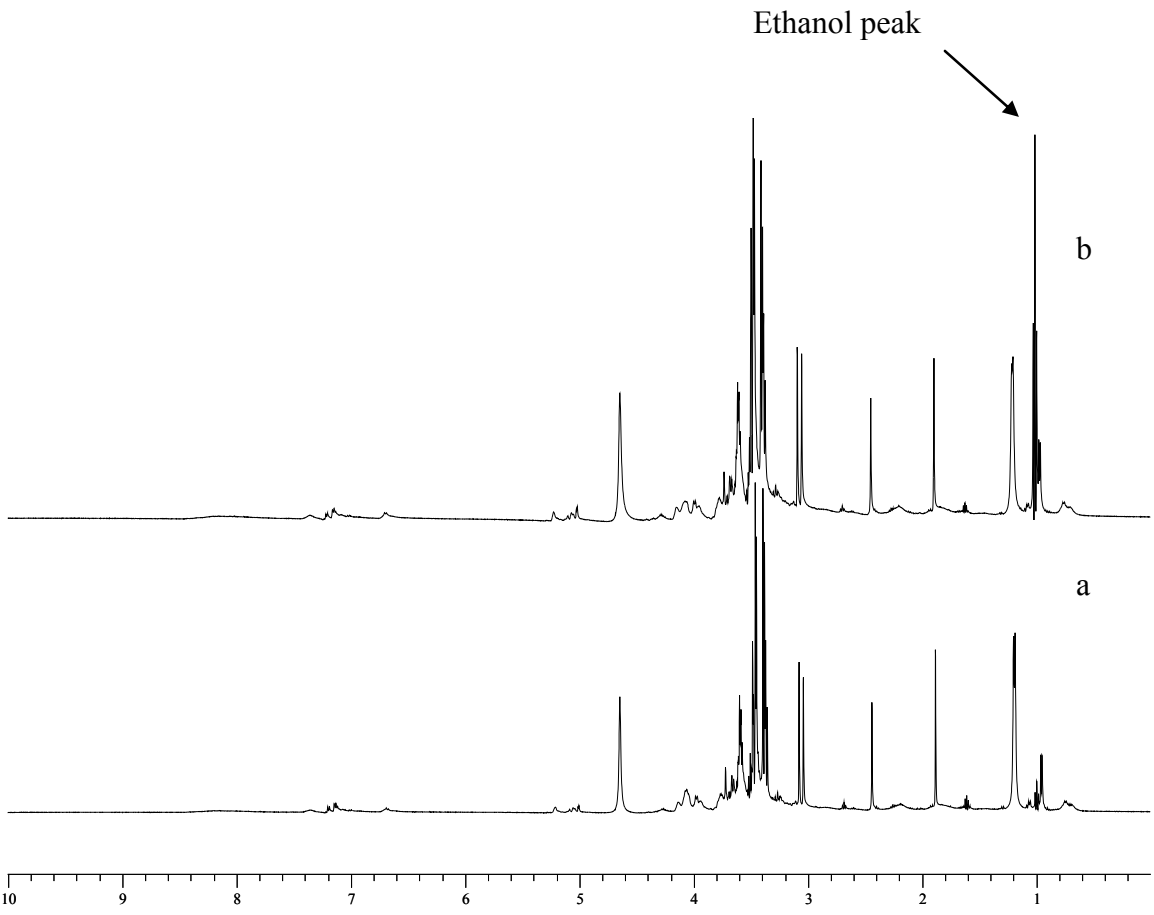

Fig. 8.3 ^{1}H NMR spectra at 500 MHz of thin stillage (May 18, 2007 sample)

a) without added ethanol;

b) with added ethanol.

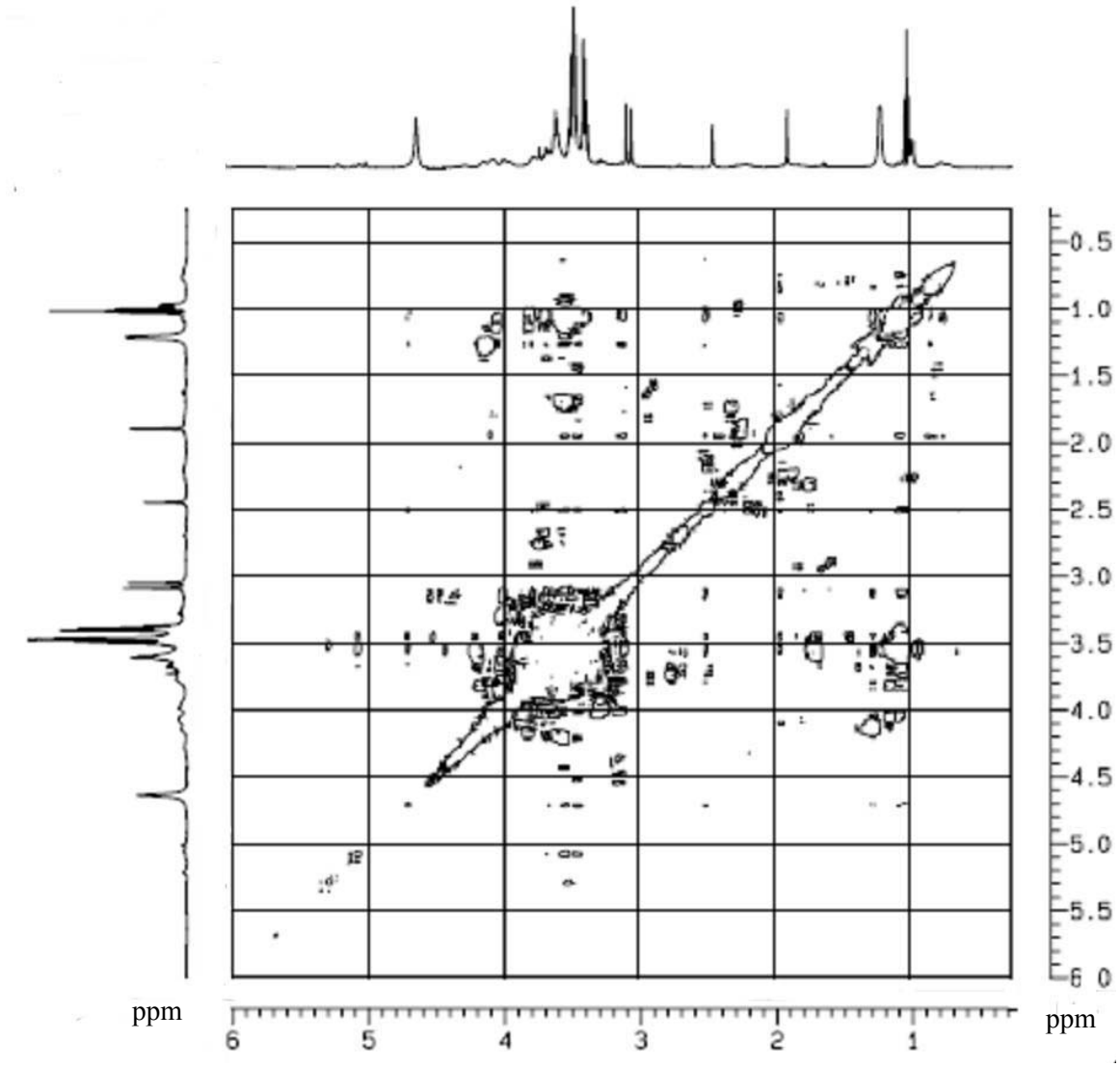

Fig. 8.4 2D ^{1}H NMR spectra of thin stillage (May 18, 2007 sample) with added ethanol.

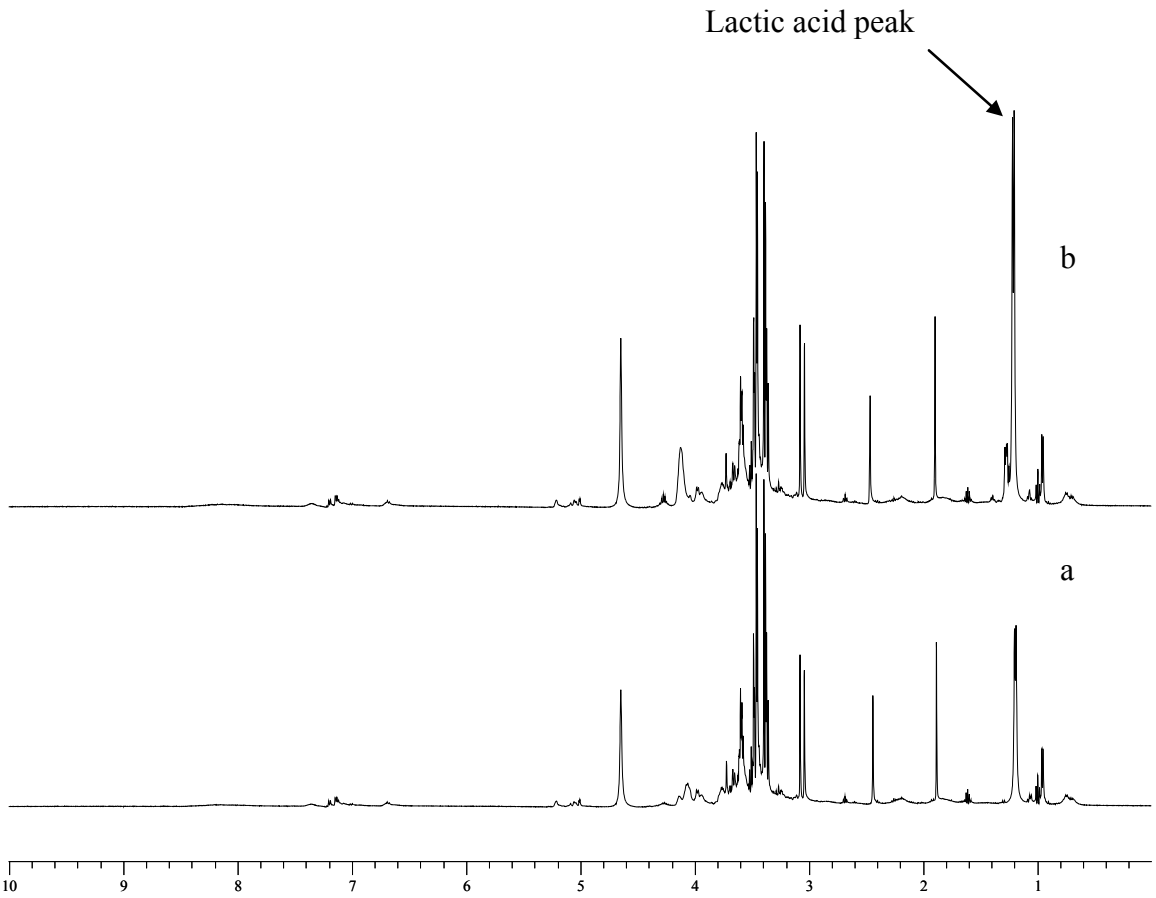

Fig. 8.5 ^{1}H NMR spectra at 500 MHz of thin stillage (May 18, 2007 sample)

a) without added lactic acid;

b) with added lactic acid.

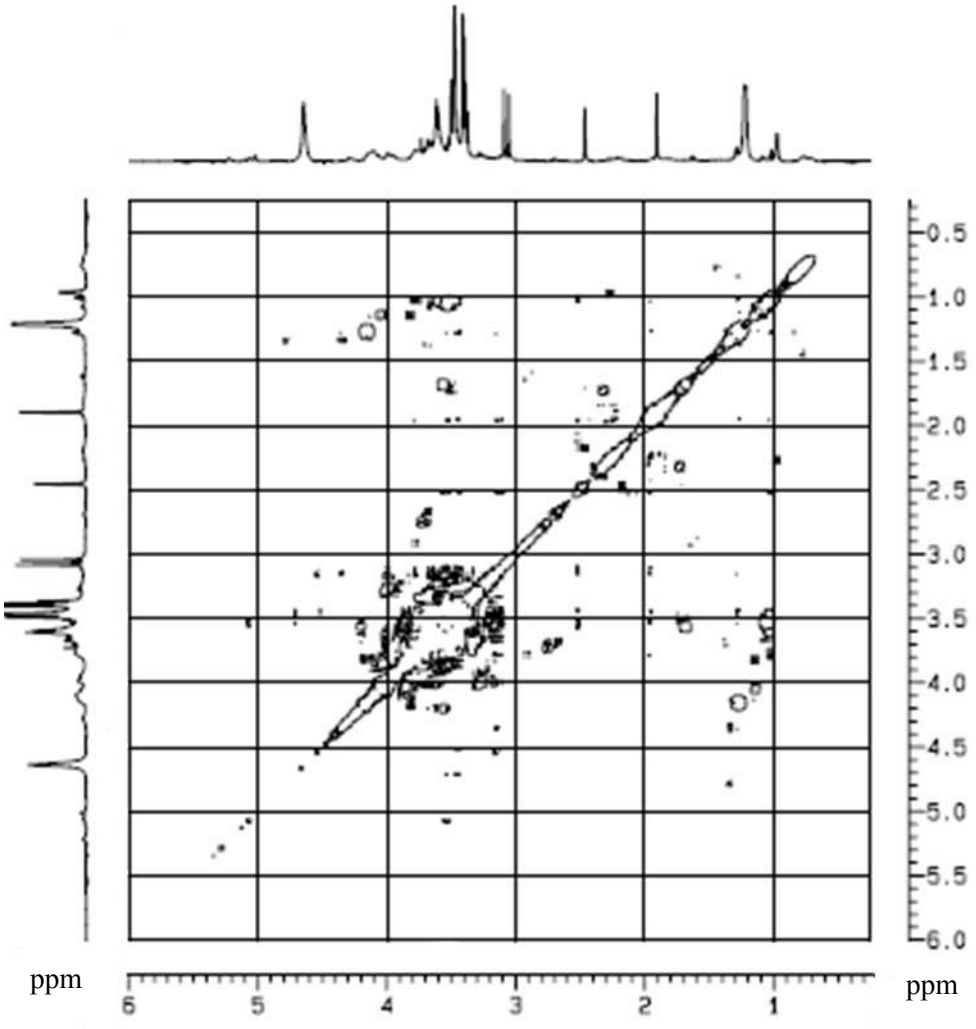

Fig. 8.6 2D ^{1}H NMR spectra of thin stillage (May 18, 2007 sample) with added lactic acid.

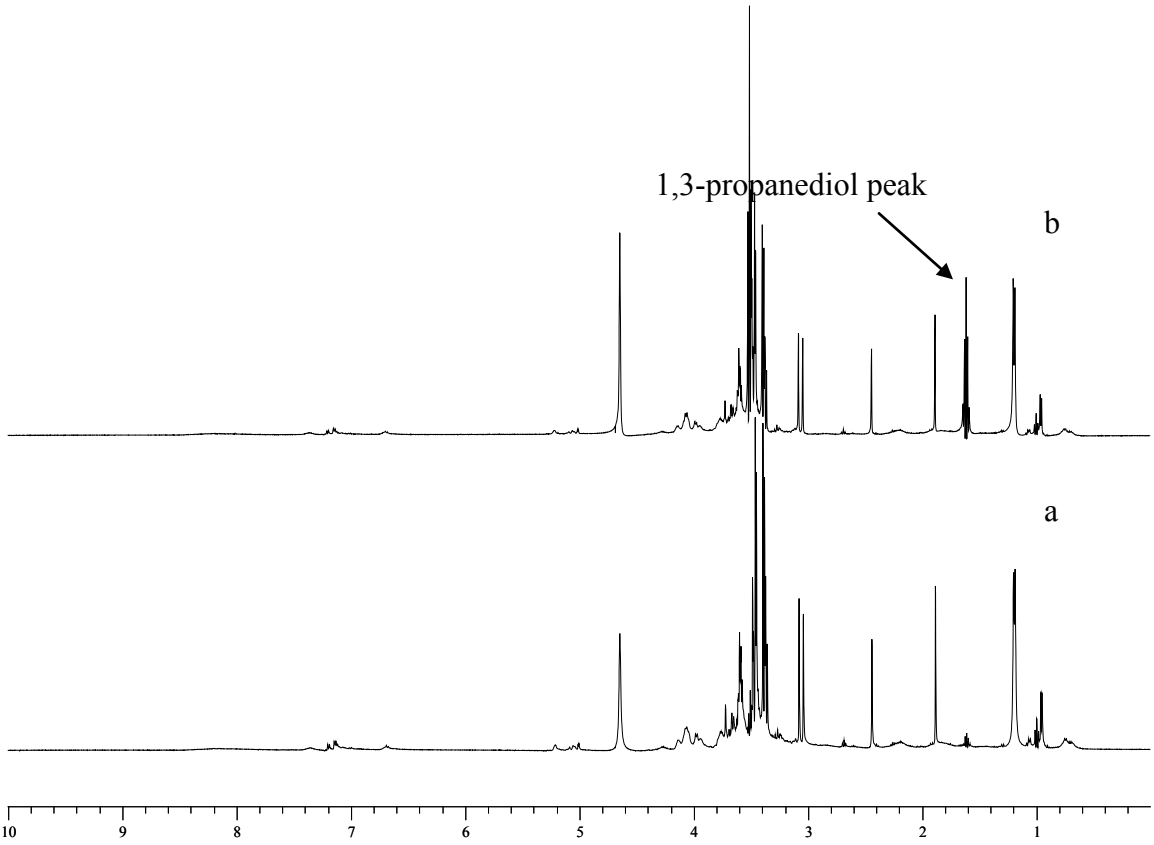

Fig. 8.7 ^{1}H NMR spectra at 500 MHz of thin stillage (May 18, 2007 sample)

a) without added 1,3-propanediol;

b) with added 1,3-propanediol.

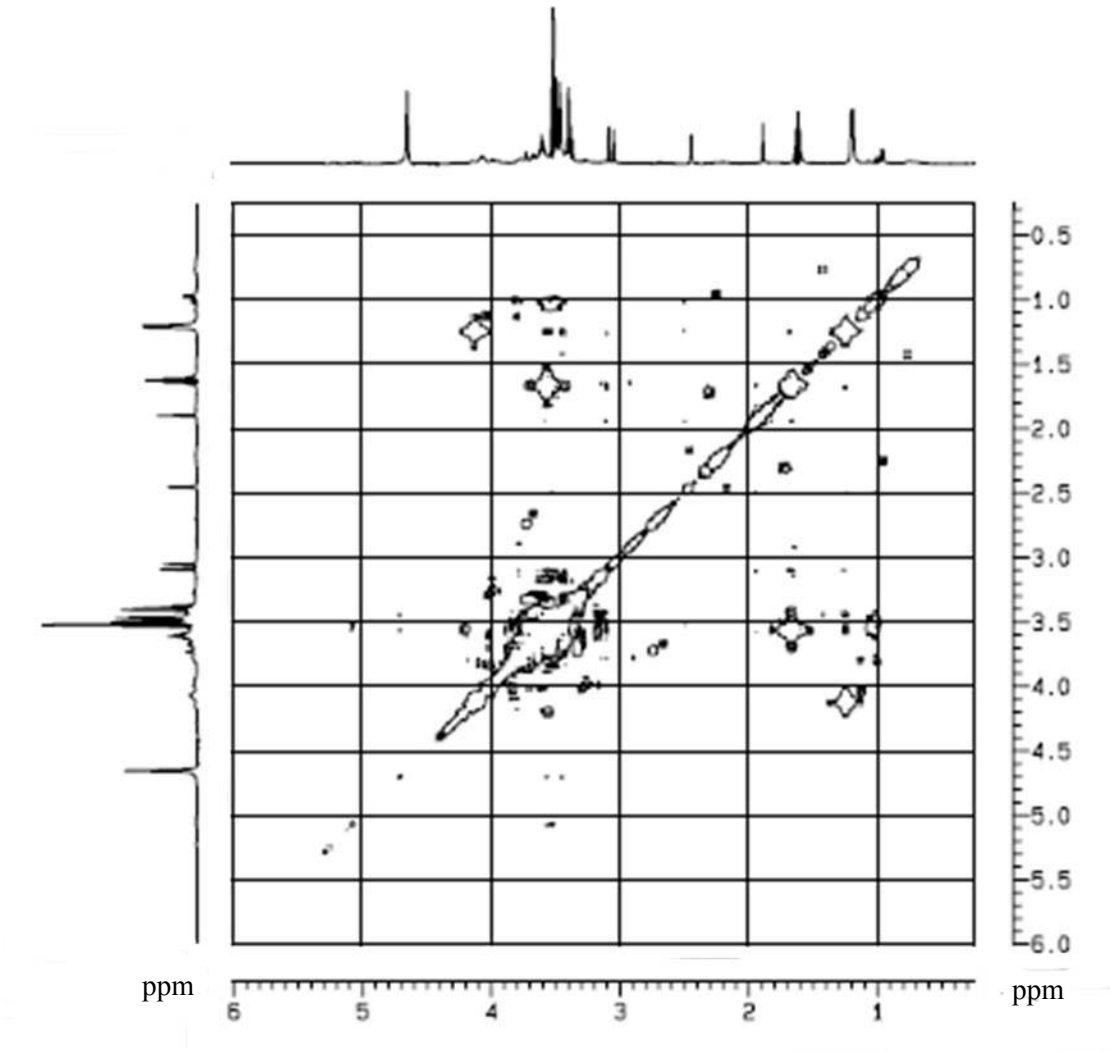

Fig. 8.8 2D ^{1}H NMR spectra of thin stillage (May 18, 2007 sample) with added 1,3-propanediol.

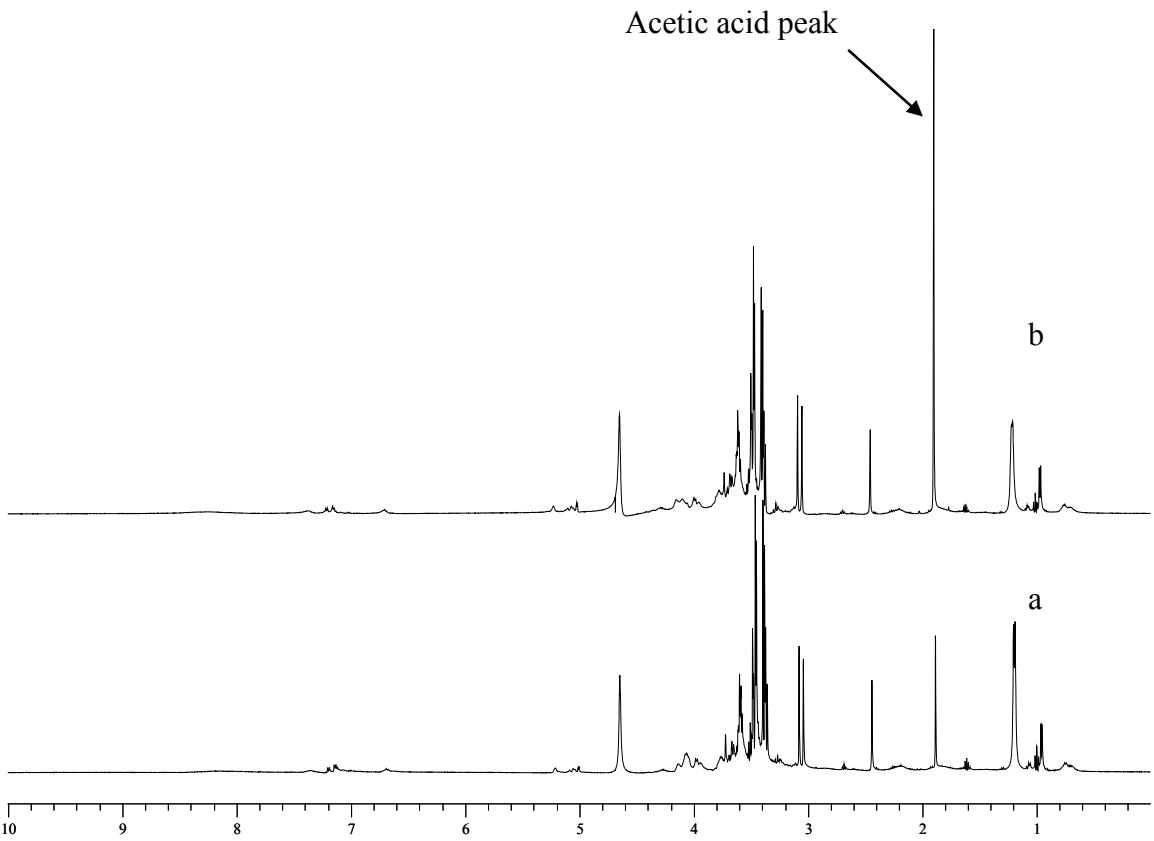

Fig. 8.9 ^{1}H NMR spectra at 500 MHz of thin stillage (May 18, 2007 sample)

a) without added acetic acid;

b) with added acetic acid.

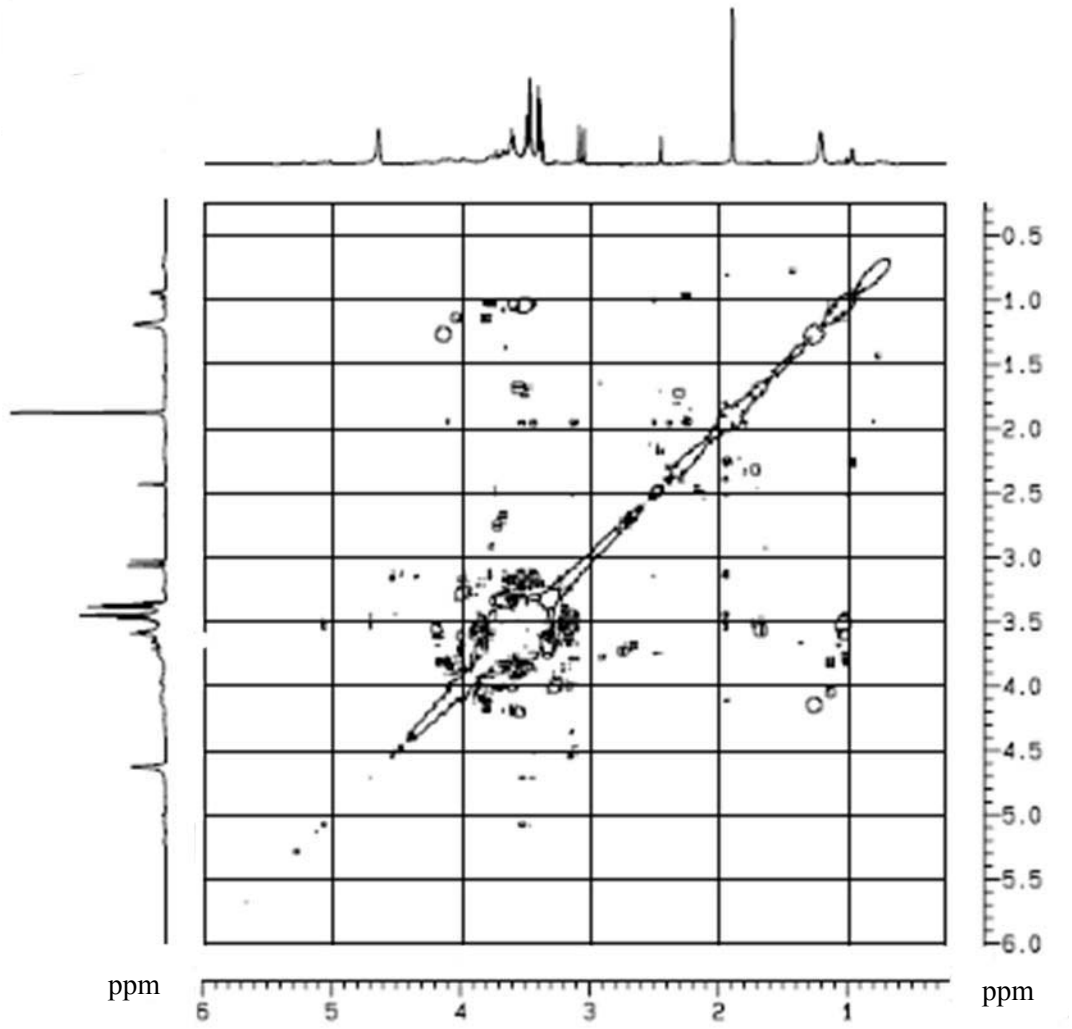

Fig. 8.10 2D ^{1}H NMR spectra of thin stillage (May 18, 2007 sample) with added acetic acid.

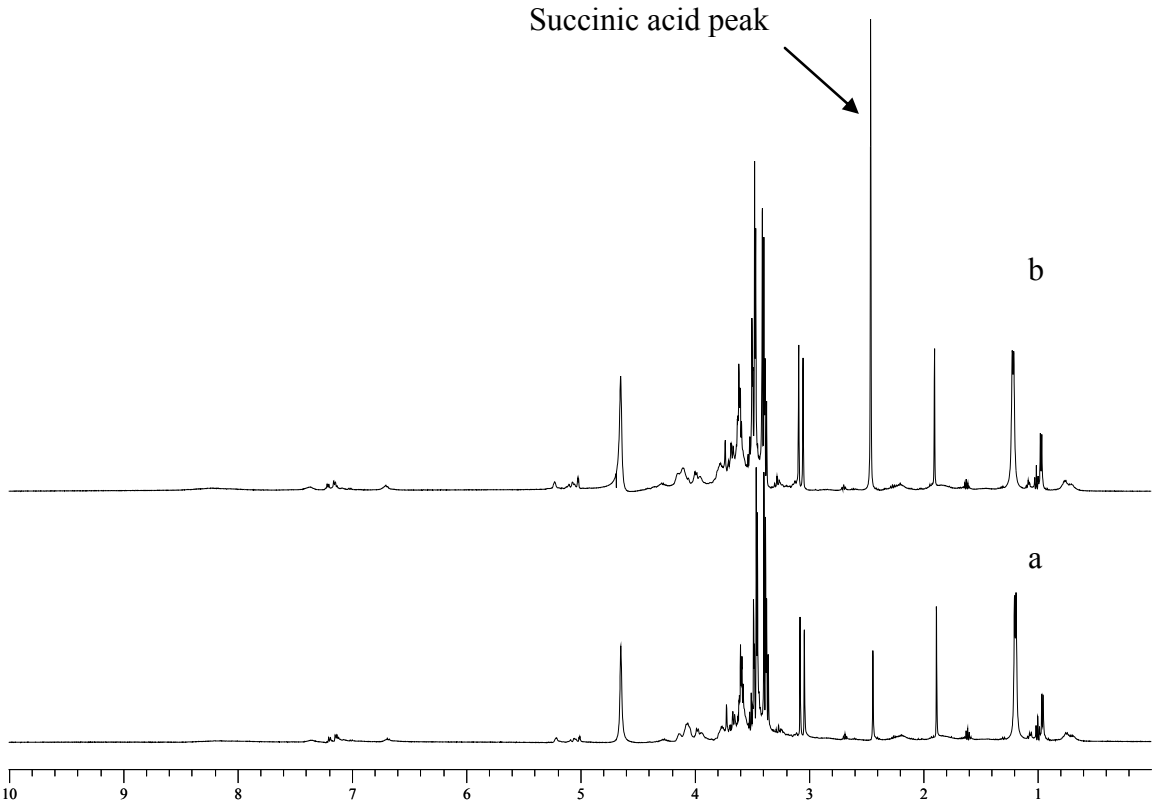

Fig. 8.11 ^{1}H NMR spectra at 500 MHz of thin stillage (May 18, 2007 sample)

a) without added succinic acid;

b) with added succinic acid.

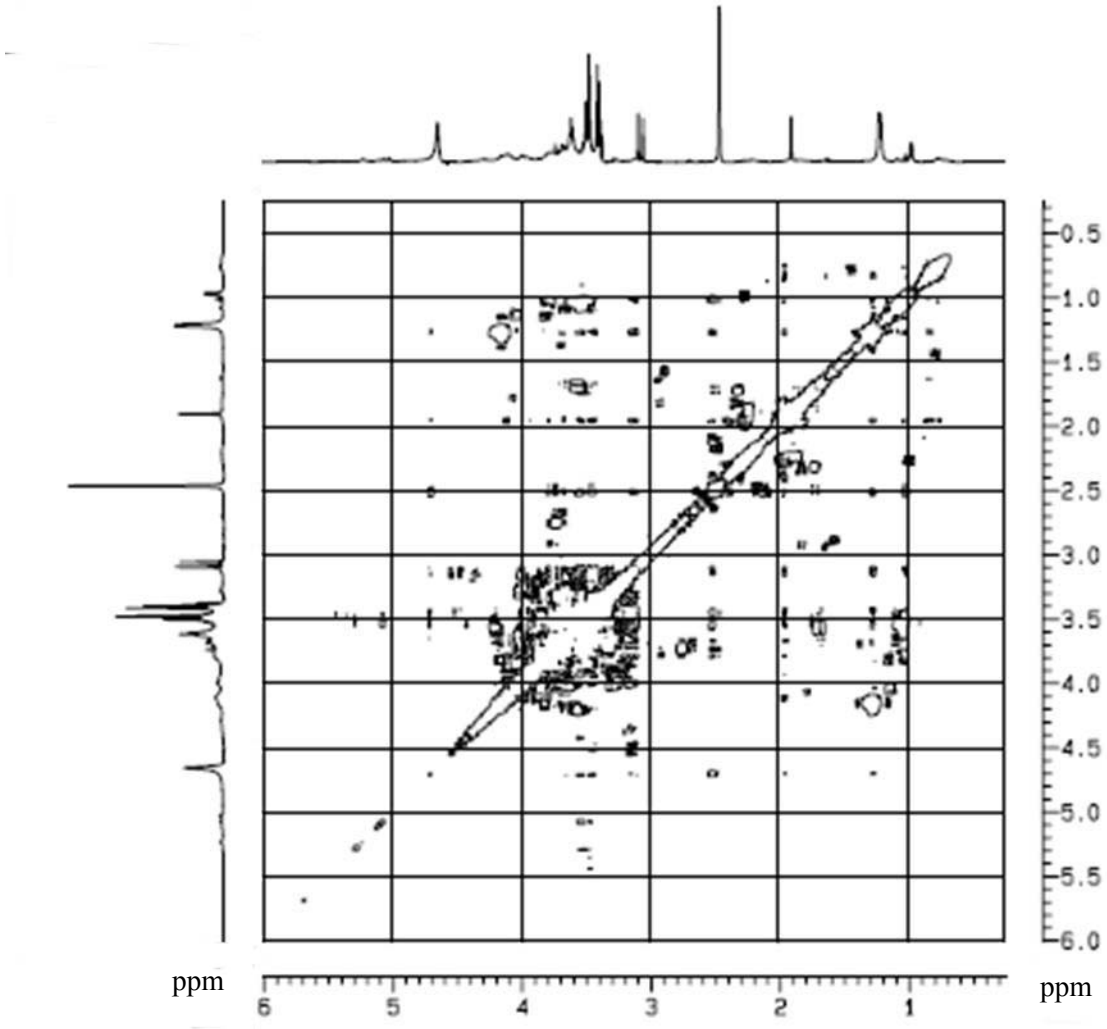

Fig. 8.12 2D ^{1}H NMR spectra of thin stillage (May 18, 2007 sample) with added succinic acid.

.

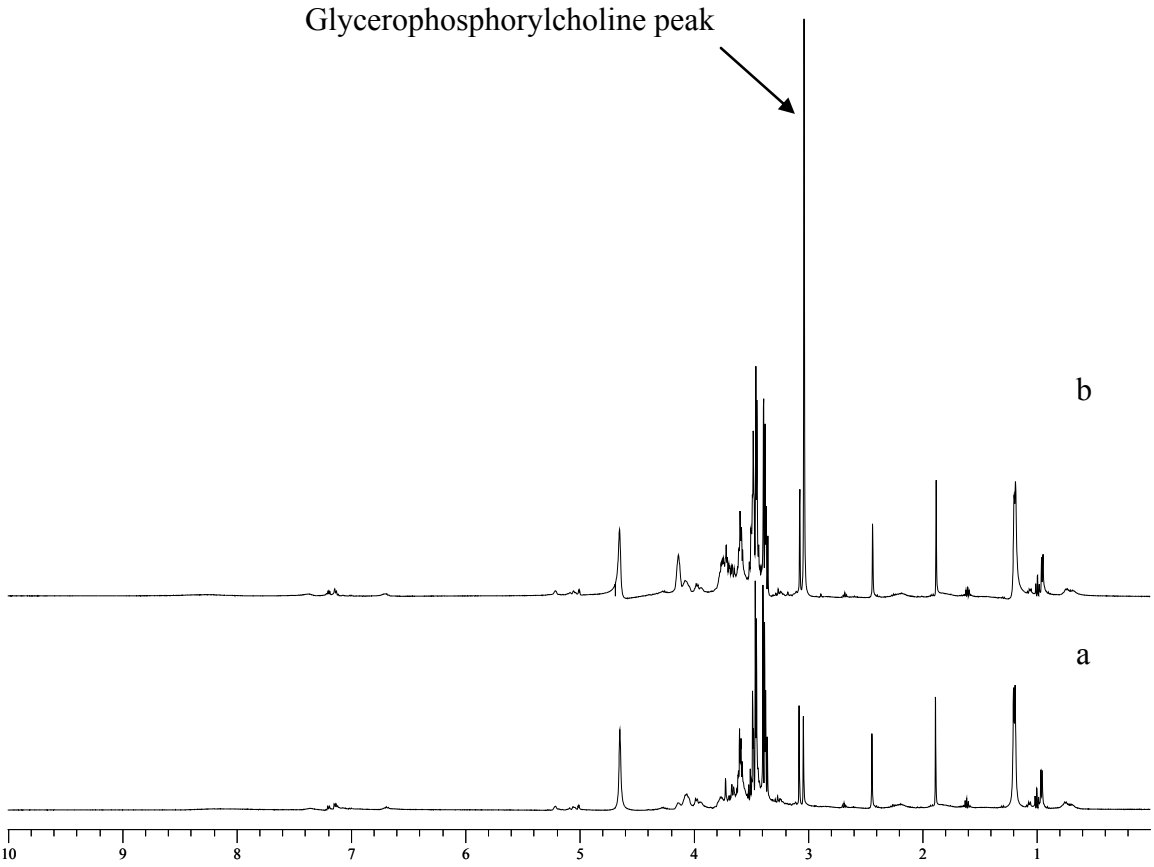

Fig. 8.13 ^{1}H NMR spectra at 500 MHz of thin stillage (May 18, 2007 sample)

a) without added glycerophosphorylcholine;

b) with added glycerophosphorylcholine.

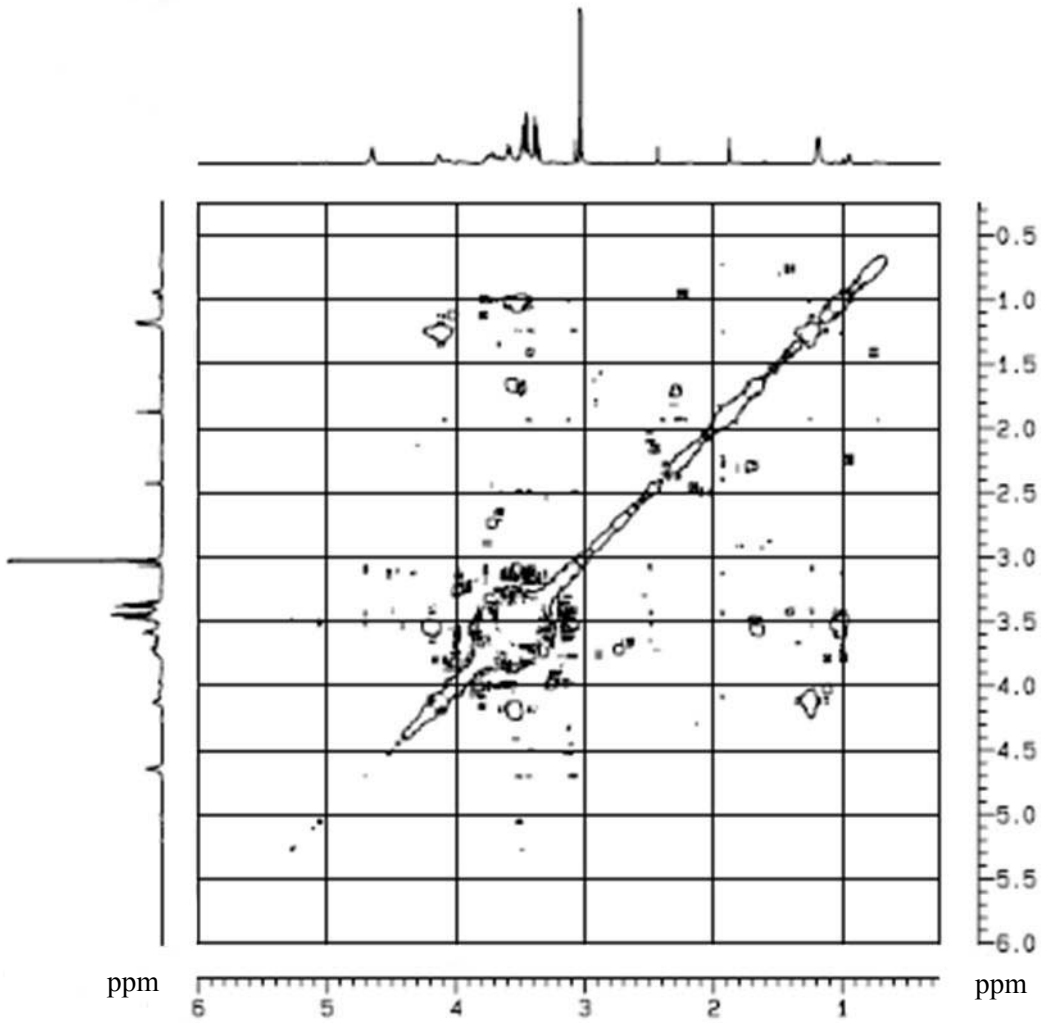

Fig. 8.14 2D ^{1}H NMR spectra of thin stillage (May 18, 2007 sample) with added glycerophosphorylcholine.

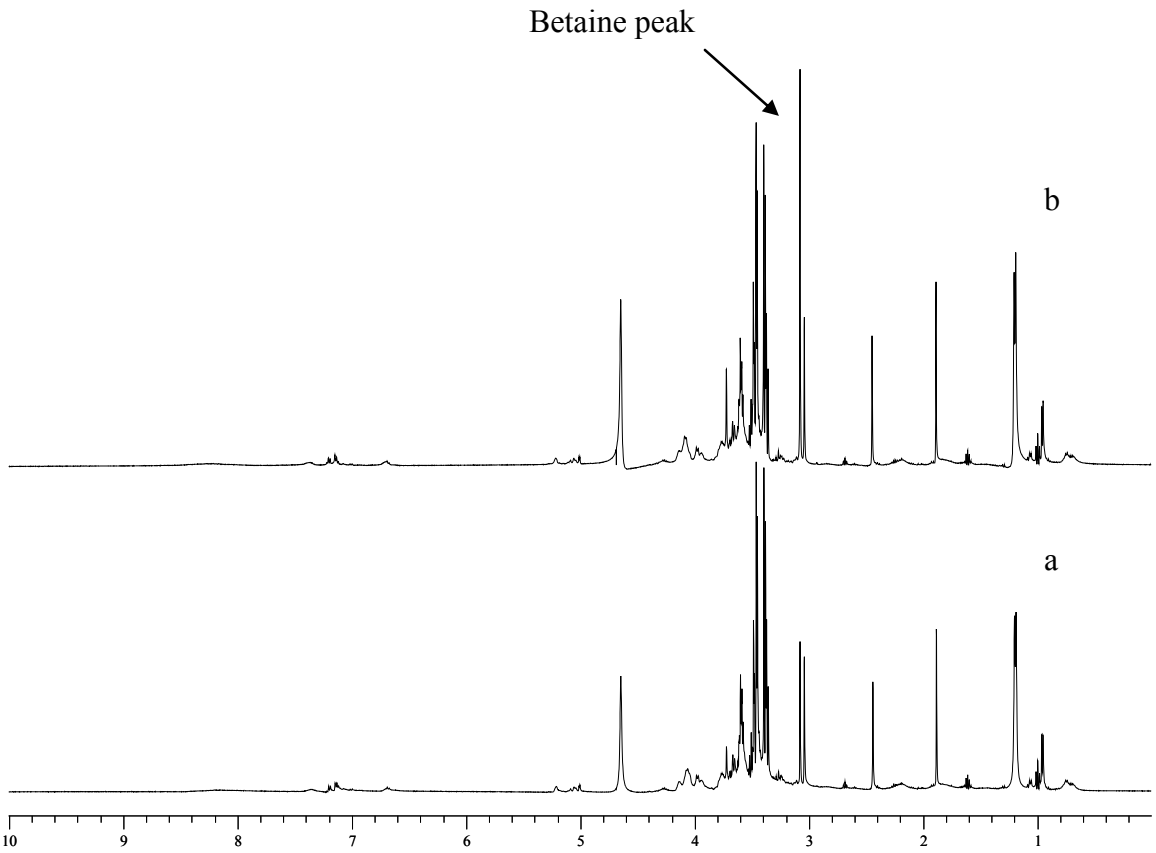

Fig. 8.15 ^{1}H NMR spectra at 500 MHz of thin stillage (May 18, 2007 sample)

a) without added betaine;

b) with added betaine.

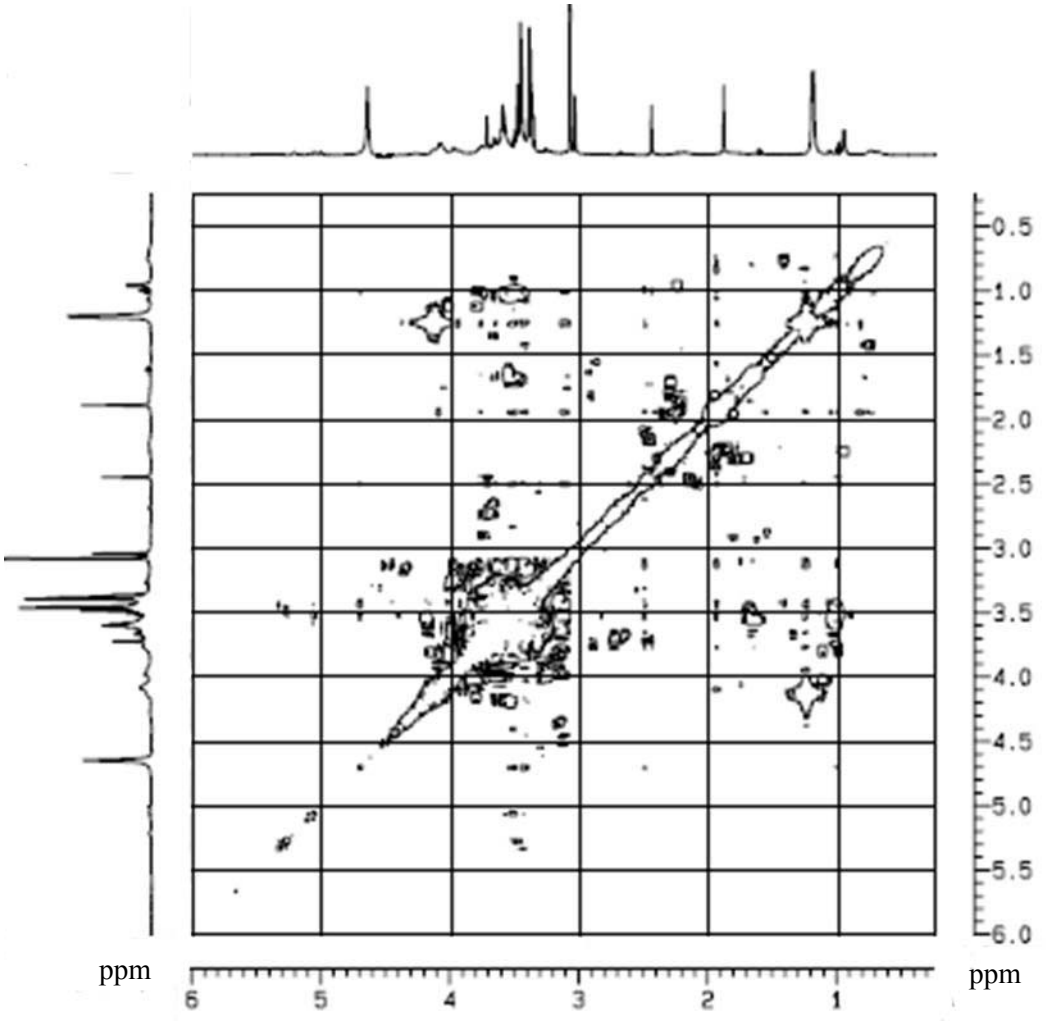

Fig. 8.16 2D ^{1}H NMR spectra of thin stillage (May 18, 2007 sample) with added betaine.

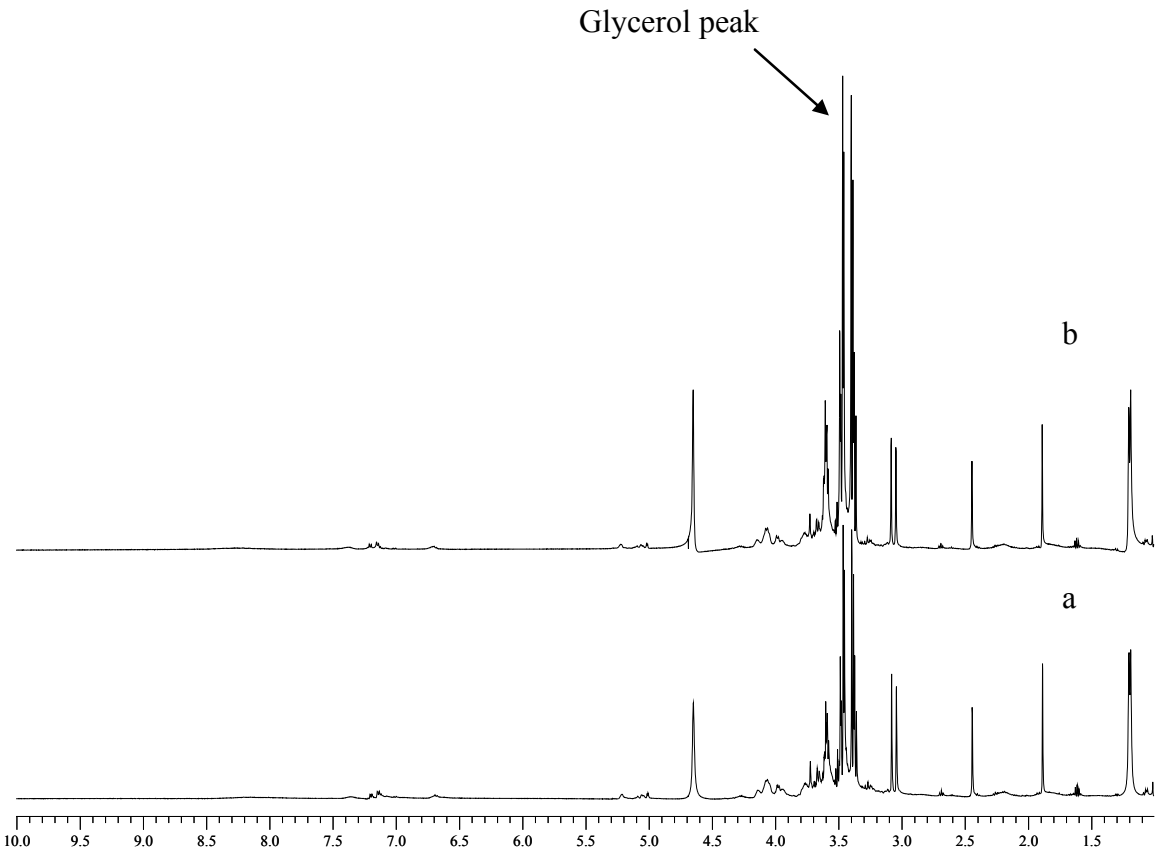

Fig. 8.17 ^{1}H NMR spectra at 500 MHz of thin stillage (May 18, 2007 sample)

a) without added glycerol;

b) with added glycerol.

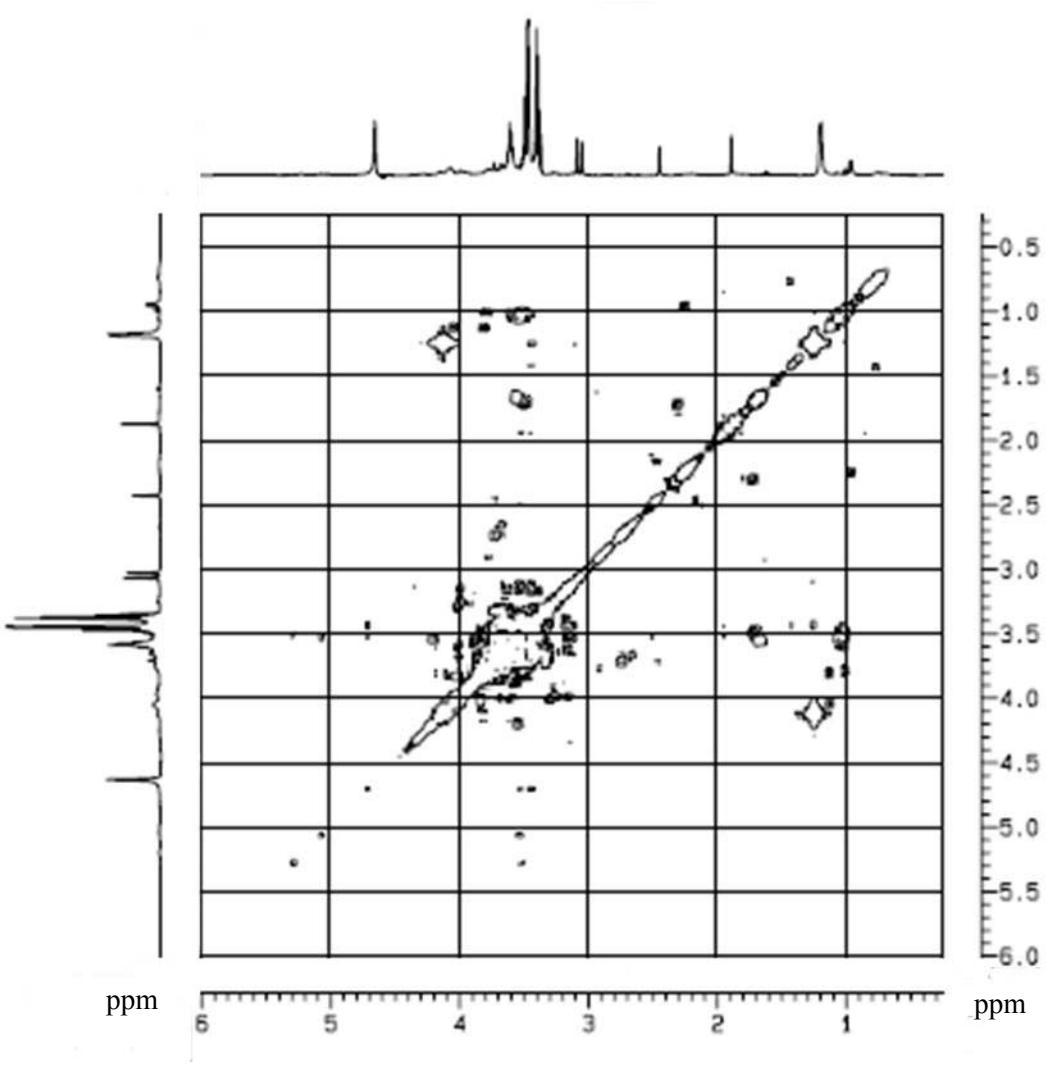

Fig. 8.18 2D ^{1}H NMR spectra of thin stillage May 18, 2007 sample) with added glycerol.

APPENDIX B – LIST OF CHEMICALS

Purchased from EMD chemicals Inc (Gibbstown, NJ)

H_2SO_4	GR ACS grade
NaOH	GR ACS grade
Boric acid	GR ACS grade
Titristar N point indicator	
Hexane	HPLC grade
N,N dimethylformamide	Spectrophotometry and liquid chromatography grade
Isopropanol	HPLC grade
Glycerol	GR ACS grade
Acetic acid	GR ACS grade
$Na_2(SO)_4$	GR ACS grade
$NaNO_3$	GR ACS grade
KOH	GR ACS grade
NaCl	Reagent grade
Methanol	HPLC grade
Acetone	HPLC grade
$AgNO_3$	Analytical grade
HNO_3	GR ACS grade
$FeNH_4(SO_4)_2.12\ H_2O$	GR ACS grade

Purchased from Sigma-Aldrich (St. Louis, MO)

DL lactic acid	Analytical grade
1,3 propanediol	99.6% ACS grade
Succinic acid	minimum 99.0%
L-α glycerophosphorylcholine from soybean	approximately 98%
Betaine phosphate	Analytical grade

Phenyl ethanol	Analytical grade
$Mg(OH)_2$	minimum 95%
K_2SO_4	minimum 99%
Maltotriose	minimum 95% HPLC, plus amperometic detection
Maltose monohydrate	minimum 95%

Dextrin from corn type I powder reducing sugar 5% or less

Bromophenol Blue salt for electrophoresis

Trypsin type IX-S from porcine pancreas 13100 units/mg protein

α-chymotrypsin type II from porcine pancreas 66 units/mg solid

Peptidase from porcine pancreas 102 units/mg solid

OPA	minimum 99% HPLG grade
Sodium tetraborate	99%

Casein bovine milk after dephosphorylation

Purchased from VWR International (West Chester, PA)

Kjeldahl digestion mixture # 200

HCl	1 ± 0.002N
Trichloroacetic acid	10% (w/v)

Purchased from Commercial Alcohol Inc (Toronto, ON)

Ethanol	100%
Ethanol	95%

Purchased from Bio-Rad laboratories (Hercules, CA)

1.5 Tris-HCL buffer pH 8.8

10x Tris/Glycine/SDS buffer

Purchased from Becton Dickinson and Company (Franklin Lakes, NJ)

Tryptic soy broth (soybean-casein digest)

Agar technical

Purchased from HiMedia Laboratories Pvt, Ltd (Mumbai, India)

Rose Bengal agar

Purchased from JL. Baker Chemical Co. (Phillipsburg, NJ)

$CaCl_2$ purified

Purchased from GE Healthcare Bio Science AB (Princeton, NJ)

SDS minimum assay 99%

Purchased from Cambridge Isotope Laboratories, Inc (Andover, MA)

Deuterium oxide 99.9%

Purchased from Mallinkrodt Baker, Inc (Phillipsburg, NJ)

Coomasie blue-G250

Purchased from Amersham Bioscience (Pittsburgh, PA)

β-mercaptoethanol minimum assay 98%

Purchased from Invitrogen (Carlsbad, CA)

SeeBlue®plus 2 prestained standard

Purchased from Fisher Scientific (Ottawa, ON)

Sodium phosphate dibasic hetahydrate Analytical grade

Purchased from BDH Chemicals Canada Ltd (Toronto, ON)

NH_4OH Analytical grade

Purchased from Merck Chemicals Ltd (Beeston, Nottingham, UK)

NH_4SCN GR ACS grade

Purchased from Fluka Chemical Corp. (St. Louis, MO)

Ally isothiocyanate	> 98% GC grade