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Utilisation of Spent Dairy Yeast Kluyveromyces Marxianus for the Production of Flavoured Yeast Extracts.

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Utilisation of spent dairy yeast *Kluyveromyces marxianus* for the production of flavoured yeast extracts.

Submitted by
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Spent dairy yeast *Kluyveromyces marxianus* (SDYK), a by-product of whey to ethanol fermentations, was investigated as a natural biomass source for the production of alternative flavoured yeast extracts. SDYK which was characterised by a high protein content (55.3% (w/w) ± 0.8) and comparable levels of flavor enhancing amino acids to that of *S. cerevisiae* was prepared for induced autolysis at different pH values in the presence of inductors sodium chloride and the proteolytic enzyme papain. An induced autolysis process was developed for the production of flavoured yeast extracts in which pH and autolytic treatment applied were found to have a significant effect on free amino nitrogen content. Autolysis at pH 6.5 yielded the highest free amino nitrogen content with the addition of papain shown to have a significant effect. Induced autolysis of SDYK at pH 5.5 and 6.5 also provided yeast extracts with increased concentrations of flavour enhancing free amino acids glutamic and aspartic acid and a % DH range of 65%-83%. Other flavour related components identified in the yeast extracts were the flavour enhancing ribonucleotide 5'IMP, reducing sugars, peptides and glycoproteins. Yeast cell wall material a by-product of this process proved to be a good source of functional polysaccharides, ß-glucan and mannan. Descriptive sensory analysis was used to quantitatively describe odour and flavour and resulted in ten attributes being significantly discriminated between SDYK yeast extracts. Further analysis by principal component analysis (PCA) and relative positions on the PCA sensory biplot indicated that pH in association with autolytic inductors, salt and papain, had a significant effect on resultant flavour attributes which could prevent unwanted tastes whilst improving salty and savoury attributes. This could form the basis of an innovative process for the provision of alternative yeast extracts with improved flavour and reduced sodium content for use in dairy based products.

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Declaration

I, Brendan McCarra, declare that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Brendan McCarra

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Glossary of Terms

5’ AMP	5’ Adenine mono phosphate
amu	:Atomic mass unit
APE	Aminopeptidase
CPY	Carboxypeptidase
CWM	Cell wall material
Da	Dalton
DH	Degree of hydrolysis
FAA	Free amino acid
FAN	Free amino nitrogen
5’ GMP	5’ Guanosine mono phosphate
5’ IMP	5’ Inosine mono phosphate
PCA	Principle Component Analysis
SDYK	Spent dairy yeast *K. marxianus*
TDS	Total dissolved solids

List of own Presentations and Papers

Mc Carra, B. and Concannon, M. (2012). Determination of the composition of spent dairy yeast *K. marxianus*, a by-product of the whey to ethanol fermentation process. *International Journal of Food Science and Technology* (in preparation).

Mc Carra, B., Lynch, C. and Concannon, M. (2012). Effect of autolytic inductors on the free amino nitrogen, dissolved solids and enzyme hydrolysis levels of yeast extracts generated from spent dairy yeast. *Bioresource Technology* (in preparation)

Mc Carra, B., Walsh, D. and Bailey, K. (2012). Production of natural flavour enhancers by induced autolysis of spent dairy yeast. *Food Research International* (in preparation)

Mc Carra, B. and Sheehan, L. (2012) Assessment of the impact of induced autolysis on the sensory attributes of developed yeast extracts from spent dairy yeast. *International Dairy Journal* (in preparation).

Sharma, R., Mc Carra, B. and Walsh, D.J. (2012). Development of value added products from spent *Kluveromyces marxianus* yeast waste. In *Smith,C. & Tiwari, B.K. eds International Conference on Food Safety, Quality and Nutrition, Greening the Food Industry,* Manchester Food Research Centre, Manchester Metropolitan University, Manchester UK. pp. 97-110.

McCarra, B. (2006). The characterisation of commercial protease enzymes for the production of protein hydrolysates using soya protein, casein, and wheat gluten as subtrates. Enterprise Ireland & Kerry Food Ingredients funded Innovation Partnership Program (2005-2006) *IP / 2005 / 0664, Final Technical Report.*

McCarra, B. (2005). Bioprocessing of Natural Products, in Irish Scientist, Editor Geraldine Van Esbeck, Oldbury Publishing, Vol. 13, p 58-59.

McCarra, B. (2002). Yeast research at IT Tralee can prevent millions going to waste. Available:http://www.irishscientist.ie/2002/contents.asp?contentxml=02p128a.xml&contentxsl=is02pages.xsl (Last accessed: 13/03/2009)

McCarra, B. (2001). Development of Added value Products from Brewery Wastes. Enterprise Ireland & Dromkeen Food Ingredients funded Applied Research Program (1998-2001), AR /97/182, Final Technical Report, p 1-8.

McCarra, B., (1983). Production of Xanthan Gum from Whey. MSc thesis, National University of Ireland, NUIG.

Abstracts - Conferences

Mc Carra, B. (2009). Research Paper Presentation on Yeast Extracts, at the Shannon Consortium Postgraduate Conference - Mary Immaculate College, Limerick.

Mc Carra, B. and Luz Prieto, Mari (2008). Proceedings of the 38th Annual Food Science and Technology Research Conference, University College Cork 2008, " The development of functional food ingredients by enzymatic treatment of yeast cell wall polysaccharides" p28.

O' Leary, Aine, and Mc Carra, B. (2008). Proceedings of the 38tAnnual Food Science and Technology Research Conference, University College Cork 2008, " Transformation of the food processing waste, apple pommace into a value added product " p27.

Keogh S. , McCarra B. (2005). Proceedings of Science Research Colloquium, IT Carlow, 2005, " The development of functional food ingredients from yeast cell wall beta glucans"

Mc Carra, B. (2003). Proceedings of Science Research Colloquium, IT Tralee, 2003, " The extraction of flavoured biological products from spent yeast" p90.

Chapter 1

Literature Review

Utilisation of spent dairy yeast *Kluyveromyces marxianus* for the production of flavoured yeast extracts.

Introduction

1.1 Yeast extracts and food ingredients

Yeasts have proved to be of immense benefit to mankind, and as one of our oldest microbial associates, they are widely used for the production of a variety of food products, bioingredients, beverages, and nutraceuticals (Aldsworth et al., 2009; Bekatorou et al., 2006, Enfors, 2001; Belem and Lee, 1998a; Walker 1998a; Halasz and Lasztity, 1991a; Peppler, 1979). Indeed, it can be said that yeasts have long been acknowledged as one of the most important microorganisms in biotechnology (National Collection of Yeast Cultures, 2012).

In terms of food ingredients, yeast and yeast derived products can be categorized as food yeasts, feed yeasts, yeast extracts, autolysates and yeast cell wall products (Reed and Nagodawithna, 1991; Bekatorou et al., 2006).

Yeast extracts which consist of the water soluble fraction of the yeast, are commercially available as powders, liquids and pastes and have been used extensively as food ingredients such as flavouring agents (Conway et al., 2001, Champagne et al., 2003, Chae et al., 2001). The Food Chemical Codex defines yeast extract as follows: "Yeast extract comprises the water soluble components of the yeast cell, the composition of which is primarily amino-acids, peptides, carbohydrates and salts". Yeast extract is produced through the hydrolysis of peptide bonds by the naturally occurring enzymes present in edible yeast or by the addition of food-grade enzymes (Noordam and Kortes, 2009). This process is often referred to as autolysis and can be defined as a process that occurs naturally in yeasts at the end of the stationary phase of

growth, when the cell components within the cell are solubilised by activation of the degradative enzymes inherently present in the cell. Developments in bioreactor technology and bioprocessing methods have facilitated both the optimisation of yeast propagation and yeast autolysis in the development of yeast derived products such as yeast extracts (Figure 1.1).

Figure 1.1 Study of yeast autolysis during the course of this work using a five litre automated bioreactor system.

1.1.1 Applications of yeast extracts

Applications of yeast extracts include their use as food ingredients in foods like soups, savoury snacks and packaged foods where they can act as a natural way of intensifying and improving the flavour of the food (Kelly, 1983; West, 1996; Tanguler and Erten, 2007). The flavour effect is attributed to the fact that the soluble fraction of the yeast consists primarily of amino acids, peptides and nucleotides which act synergistically to improve and enhance flavour (Nagodawithana, 1993). Attempts are being made to produce flavoured yeast extracts by effectively controlling the yeast autolysis process

conditions that have an impact on quality and yield of the final yeast extract (Milic et al., 2007; Suphantharika et al., 1997; Chae et al., 2001).

Because yeast extracts are rich in nitrogen, vitamins and other growth stimulating compounds they are also ideal as an ingredient in media for the cultivation of microorganisms (Orberg and Sandine 1984; Selmer-Olsen and Sorhaug 1998; Champagne et al., 2003). Reports also have confirmed that yeast extract can help to prevent bacterial and fungal diseases in treated plants. The active ingredient consists primarily of amino acids, but also includes nutrients such as vitamins, minerals and oligosaccharides (EPA [USA], 2012a). Industrial production of flavoured yeast extracts began in the 1950's. The first commercial yeast extract containing Guanosine 5' Mono Phosphate (5'GMP), a natural nucleotide and flavour enhancer derived from the yeast RNA, was produced on an industrial scale in 1974 (Figure 1.2).

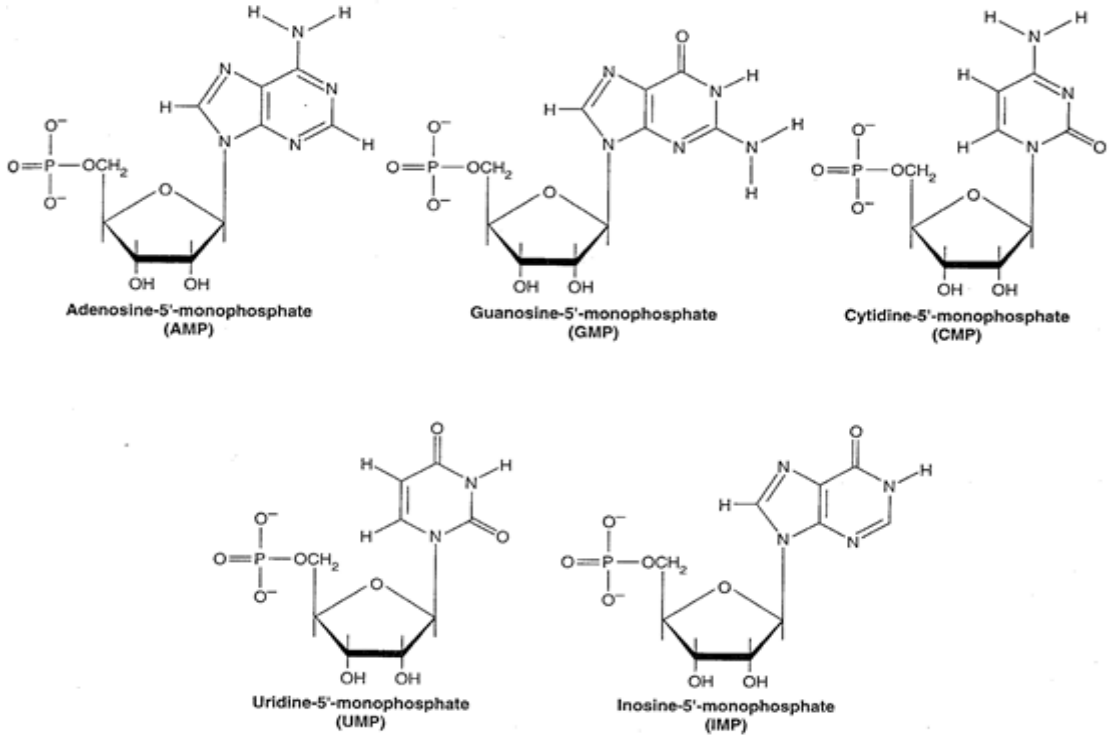


Figure 1.2 Structures of yeast ribonucleotides including Guanosine 5'mono phosphate (5'GMP), and Inosine 5' mono phosphate (5'IMP), natural nucleotides and flavour enhancers.

The market for yeast extracts is expanding worldwide and continues to grow. In a recent report entitled "*Global Yeast Market by Types, Applications, & Geography - Trends & Forecasts*" focussing on data from 2011 to 2016, it was estimated that the worldwide yeast industry will grow 8.6% annually until 2016 to €3.9bn (Market and Markets, 2012). A small number of European suppliers, including BioSpringer, Kerry Ingredients, DSM, and Bel Industries supply two-third's of the world's 100,000 tonnes consumption of yeast extracts. This demand is mainly driven by the growth of convenience food production and by the growing interest of food manufacturers and consumers for clean label and natural savoury ingredients. Growth in the use of yeast extracts in the food industry has thus far been fueled by demand for them as a flavouring ingredient, and it is expected that expanding their use to new sectors of the industry will lead to further growth in the market (Market and Markets, 2012). One such sector is the dairy industry and the utilization of spent dairy yeast *K. marxianus* following its fermentation on whey.

1.2 Advantages of the dairy yeast *Kluyveromyces marxianus*

The growth and predominance of yeast species frequently found in dairy products has led to the term dairy yeasts. *K. marxianus,* also known as dairy yeast, produces the enzyme β-galactosidase and consequently it can ferment or assimilate lactose which is the main carbohydrate of milk. *K. marxianus* also has the ability to grow at temperatures up to 45^{o}C (Kourkoutas et al., 2002; Hack and Marchant, 1998) with thermoduric strains of *K. marxianus* capable of growing at temperatures as high as 52^{o}C (Banat et al., 1996).

In this regard, the characteristics of the yeast *K. marxianus* offers a significant advantage over conventional mesophilic *S. cerevisiae*, by allowing this yeast to grow over a wider range of growth temperatures on a variety of substrates (Lane and Morrissey, 2010; Fonseca et al., 2008; Limtong et al., 2007; Tomas-Pejo et al., 2008; Banat et al., 1996; Gough et al., 1996;). One such substrate is whey permeate, a by-product of cheese production, which is converted to ethanol by the lactose utilizing yeast *K. marxianus.*

1.2.1 Spent dairy yeast and the Carbery process

The whey-to-ethanol plant commissioned in 1978 by Carbery Ireland is believed to be the first modern commercial operation of its kind in the world to produce both potable alcohol and fuel alcohol (Ling, 2008). Since then the Carbery process has been adopted by plants in the United States and New Zealand with the latter commencing production in August 2007.

The decision to build a whey alcohol plant in Ireland by was influenced heavily by the availability of large volumes of whey permeate, a significant waste disposal problem at the time (Sandbach, 1981). This is because of whey's high biological oxygen demand (BOD) and chemical oxygen demand (COD) due mainly to the lactose mass fraction and reported as being 40,000-60,000 ppm and 50,000-80,000 ppm for BOD and COD, respectively (Cristiani-Urbina et al., 2000; Meyrath and Baker, 1977).

In the whey to ethanol fermentation process, whey permeate, a by-product of cheese production, is converted to ethanol by the lactose utilizing yeast *K. marxianus* (Guimarães and Domingues, 2010; Zafar and Owais, 2006; Ghaly and Kamal 2004; Grba et al., 2002; Smithers, 2008; Giec and Kosikowski, 1982). In addition to its high β-galactosidase activity, *K. marxianus* has a good growth yield, and with GRAS

status, it is the only yeast permitted to grow on whey in the U.S. (Lane and Morrissey, 2010; Belem and Lee, 1998a). Other countries utilizing this yeast commercially for the production of alcohol from whey include Ireland and New Zealand (Ling, 2008).

The Carbery whey to ethanol process produces 8.09 million litres of ethanol per annum, using whey permeate as the fermentation substrate for the yeast *K. marxianus.* Yeast specifically produced as the primary product from aerobic cultivation in fermentors are termed primary yeast, while yeast produced as a by-product of alcohol or other fermentations are called secondary or spent yeast (Halasz and Lasztity, 1991a). The anaerobic fermentation of cheese whey by *K. marxianus* utilized in the Carbery process results in significant quantities of secondary (spent) yeast being produced as a by-product of ethanol production. During this anaerobic process, the growth rate is reduced, more sugar is consumed and ethanol is produced with yeast as a by-product. This can be desirable where energy considerations relating to aeration are important and an anerobic fermentation process in which both alcohol and yeast are produced is the process of choice. For this type of production, the raw material whey is required in large amounts and the production facility is normally located close to a large cheese producing area (Ling, 2008; Sandbach, 1981).

1.2.1.1 Microbial utilisation of whey

In view of whey's composition and relative purity, the microbial utilisation of whey for conversion into value added bioingredients has been reported. In addition to ethanol production from whey, (Sandbach, 1981; Silveira et al., 2005; Giec and Kosikowski, 1982), fermentation products such as SCP - single cell protein (Ghaly et al., 1993; Slagle and Zimmerman, 1979) lactic acid (Koutinas et al., 2005), citric acid (Yalcin et al., 2009), enzymes, (Deive et al., 2003; Monti et al., 2008; Singh and

Bhermi, 2008) and xanthan gum (McCarra, 1983) have been investigated using various industrial strains. Many of these primary products utilise the yeast *K. marxianus*.

The fact that the Carbery process utilises a waste stream such as whey permeate and produces large quantities of spent yeast *K. marxianus* as a by-product of ethanol production has provided an additional opportunity to utilise spent dairy yeast as a source of value added food ingredients and further improve the efficiency of the whey to ethanol fermentation process. While many processes are documented relating to the primary fermentation of *K. marxianus* on whey, in producing yeast biomass and single cell protein (Guimarães and Domingues, 2010; Cortes et al., 2005; Cristiani-Urbina et al., 2000; Ben-Hassan et al., 1992), the utilisation of spent dairy yeast *K. marxianus* is not as well documented. One possibility is its utilization as an alternative source of flavoured yeast extracts for use in dairy food products.

1.2.2 Spent Dairy Yeast *K. marxianus* –An alternative source of flavoured yeast extracts

While it is clear that *S. cerevisiae* still remains the primary source of spent yeast for yeast extract production, interest in the utilization of spent dairy yeast *K. marxianus* is growing. This can be attributed to the important advantageous characteristics of *K. marxianus* as described in section 1.2 as well as other factors which can be summarised as follows:

1. Reduction of environmental impacts

The world production of cheese has been estimated to be over twelve million tonnes per year, with the total amount of liquid whey produced in these processes being in the region of 10^8 tonnes per annum (Grba et al., 2002; Ozmihci and Kargi, 2007). New

processing methods have resulted in many new whey products which are now recognized as valuable co-products of cheese and casein manufacture (Herbertz, 2001). However, even with the improvement of processes of recovering whey proteins, the resultant increase in whey protein concentrates has increased the quantities of whey permeate available for processing. Further efforts are therefore required for utilization of lactose, the main component of whey permeate, and thereby reducing its pollution potential significantly (Lane and Morrissey, 2010; Silveira et al., 2005; Ghaly and Kamal, 2004; Grba et al., 2002; Belem and Lee, 1999b; Gough et al., 1996). This by-product of cheese production is a substrate that is readily utilized by the yeast *K. marxianus* and an important advantage over *S. cerevisiae* which cannot ferment the disaccharide lactose because of the lack of the enzyme β-galactosidase (Ozmihci and Kargi, 2007; Cortes et al., 2005).

2. Consumer demand

Growth in the use of yeast extracts in the food industry is likely to grow as consumer demand for alternative natural flavoring ingredient increases particularly with respect to clean label and natural savoury food ingredients. The development of new flavours and extracts with light colour is also a requirement for many dairy food applications. The absence of a debittering process for removal of off-flavours when using *K. marxianus,* reduces the unit operations involved in the production of alternative yeast extracts, an important advantage over spent brewers yeast.

3. Requirement for renewable fuels

Rising fossil fuel prices have meant that the development of efficient processes for production of yeast derived ethanol using renewable natural substrates is now a global priority. Ethanol is considered to be one of the most important renewable fuels

contributing to the reduction of negative environmental impacts generated by the worldwide utilization of fossil fuels (Cardona and Sa´nchez, 2007).

With the increasing liklihood of larger volumes of spent dairy yeast being produced as a byproduct of ethanol fermentation processes, it is important to understand how it can be used more effectively as a source of value added bioingredients such as yeast extracts. (Tanguler and Erten 2007; Shotipruk et al., 2005; Sombutyanuchit et al., 2001; McCarra, 2001). In addition to improving efficiencies of existing fermentation processes, this approach will further enhance a greener approach to by-product and waste utilization from large scale yeast fermentation processes.

1.2.3 Spent brewers yeast

A study investigating utilization of spent brewers yeast in Ireland indicated that more than 15,000 tonnes per annum of surplus yeast liquors were produced by the Irish brewing industry with Diagio (Guinness) producing 7,000 tonnes of surplus spent yeast and 2,000 tonnes of inactive yeast and sediment (McCarra, 2001). This spent yeast was used as an animal feed for pigs or as a nutritional supplement after drying because of its high protein level. No processing leading to food ingredients was therefore carried out at the time in Ireland for these waste yeast products arising from the brewery industry or other alcohol producers in Ireland. The production of a value added product such as yeast extract from spent yeast was considered extremely relevant to breweries in terms of efficient disposal of waste, and in the identification of new food related products. Yeasts utilized for yeast extract production can be derived from either primary or spent yeast with baker's yeasts and lactic yeasts including *K. marxianus* being the main primary grown yeasts cultivated specifically for direct processing into speciality yeast products, such as yeast extracts (Dziezak

1987; Lukondeh et al., 2003a; Milić et al., 2007). Spent brewers yeast has been used in the UK since 1902 for the production of meat flavoured yeast extracts. The process developed for the production of brewers yeast extracts in Ireland yielded a product that was quite variable in terms of flavour and was attributed to the fact that spent yeast was derived from different alcohol fermentation processes. However, an important driver for the utilization of spent yeast was the increasing environmental legislation with emphasis being placed on waste utilisation as opposed to waste disposal. Today this is as important as ever, as evidenced by implementation of the National Strategy on Biodegradable Waste, 2006, which proposed the introduction of legal instruments banning food waste from landfill from 2008 and setting targets to be achieved for various sectors e.g. 50% commercial food waste diverted from landfill by 2013 (EPA, 2012b**).**

1.3 Yeast extract process

There have been many reports on yeast extract manufacturing processes, the majority of which use bakers or brewers yeast as the initial raw material (Chae et al., 2001; Reed and Nagodawithna, 1991; Sommer, 1998; Mion-Du Crest, 1998; Thornton, 1992). Yeast extract production can be divided into the following key processing steps, starting always with a reliable source of yeast biomass:

- Controlled autolysis and solubilisation of yeast cell contents
- Removal and utilization of yeast cell walls (separation of yeast autolysate)
- Formation of yeast extract with cell wall material as by-product.

These operations are illustrated in Figure 1.3

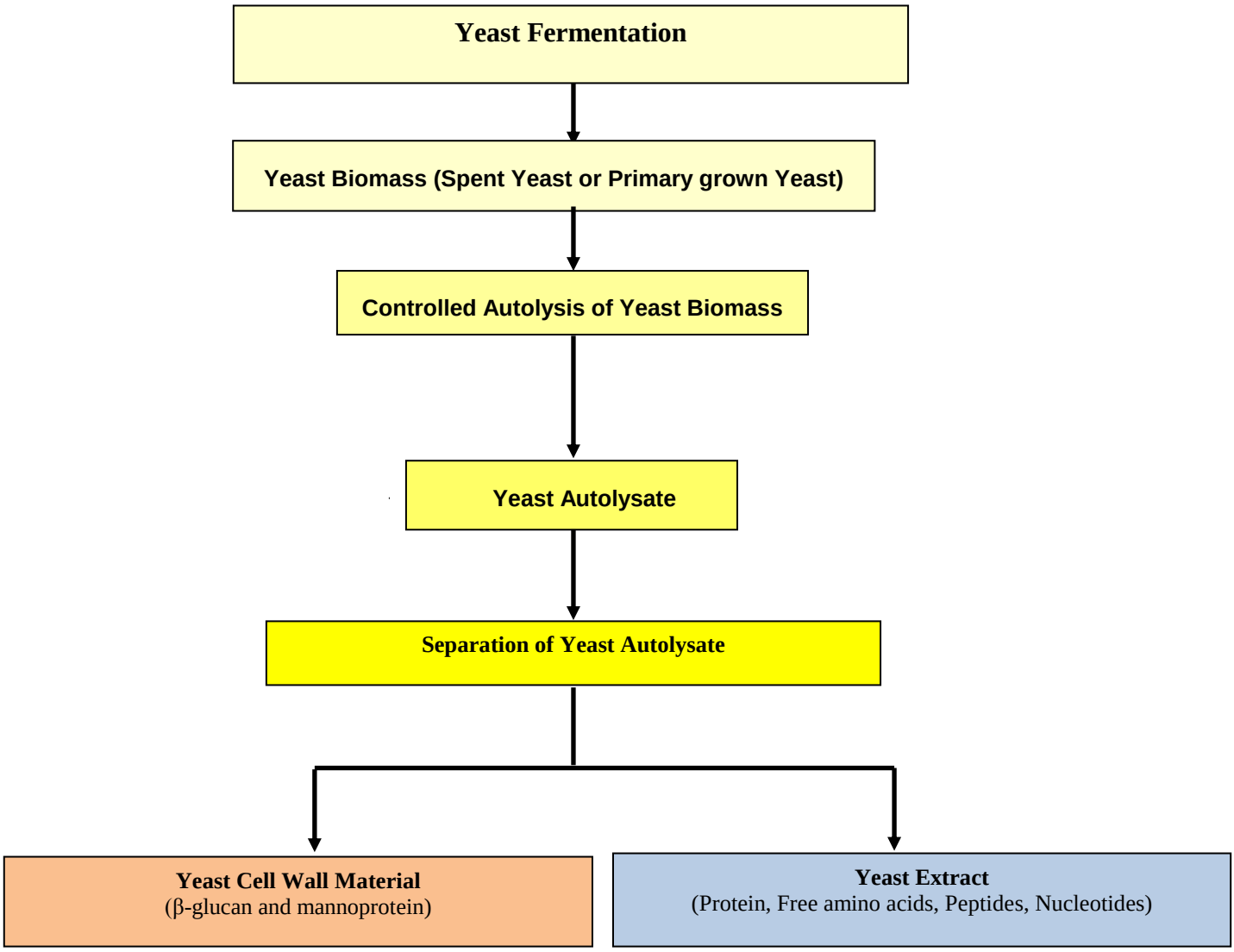


Figure 1.3 Controlled autolysis as a key activity in the yeast extract production process.

1.3.1 Autolysis and solubilisation of yeast cell contents

Yeast biomass, the raw material for yeast extract production, is normally harvested after fermentation, using the process of centrifugation. This has the advantage of recovering yeast at a solids level suitable for further processing. The yeast solids must then undergo solubilisation to facilitate isolation of intracellular materials and in particular the components responsible for flavour improvement. Techniques used for isolation of intracellular materials have been extensively reviewed by Geciova et al., 2002. With respect to yeast extract production, the most popular method utilises the

biological process of yeast autolysis. In addition to being a more natural and environmentally friendly method when compared to chemical hydrolysis, autolysis is believed to be more adaptable to industrial scale-up (Revillion et al., 2003;Tanguler and Erten, 2007; Sommer, 1998).

1.3.1.1 The yeast autolysis process and enzyme hydrolysis

Autolysis is a digestion process in which the cellular enzymes that occur naturally in viable yeasts solubilize the cell components within the cell. (Alexandre, 2011; Nagodawithana, 1992; Hernawan and Fleet, 1995; Martinez-Rodriguez et al., 2001; Chae et al., 2001). Autolysis may be induced by exposing the cells to high temperature, salts, alteration of pH, organic solvents (plasmolysers) and mechanical intervention. This induction process facilitates loosening of the cytoplasmic membrane and activation of lytic cell enzymes which are generally compartmentalised in a normal cell (Babayan et al., 1981; Babayan and Bezrukov, 1985; Hough and Maddox, 1970). The resultant disorderliness within the cell and optimized enzymatic conditions allows the degradative enzymes to cleave their specific substrates more rapidly with release of the required hydrolytic products into the surrounding medium (Hernawan and Fleet, 1995; Suphantharika et al., 1997).

The enzymatic hydrolysis of cellular protein and nucleic acids with resultant release of peptides, amino acids and nucleotides into the autolysate are important reactions of yeast autolysis that are exploited in flavoured yeast extract production. Although the autolysis process inactivates the yeast cell, the yeast's own endogenous enzymes still remain active. Controlled pH, temperature, and duration of the autolysis are decisive factors for an optimal and standardized autolysis process as proteolytic enzymes play a

major role in the process of yeast autolysis. Among the proteases and peptidases that have been identified during autolysis of *S. cerevisiae* are A and B-protease, endopeptidase A and B and carboxypeptidase Y (Halasz and Lasztity, 1991c). Enzymatic hydrolysis also provides several advantages over chemical hydrolysis including fast reaction rates and milder conditions. In addition, the final hydrolysate after neutralisation contains less salts and the functionality of the final product can be controlled by the selection of specific enzymes and reaction factors (Darwicz et al., 2000; Chiang et al., 1999; Madsen et al., 1997).

It is also evident that extensive proteolysis is occuring outside the cell and the proteases involved have been characterised from three different autolysing yeasts of the genus *Saccharomyces* (Hough and Maddox, 1970). Four proteases were identified and appeared to be glycoproteins containing both glucose and mannose residues in various proportions. This suggested that there may be some interaction with the yeast cell wall which is substantially a mannan-glucan-protein complex. Protein and amino acid composition of autolysates therefore tend to be variable because proteinases and peptidases are released into the autolysates and continue their action. Such activity will vary with the conditions of autolysis and yeast species.

1.3.1.2 General mechanism of autolysis

Yeast autolysis has been shown to be a two-step process (Babayan et al., 1981).

The first step in yeast autolysis is characterised by a drop in viscosity of the yeast suspension to a constant value and by the absence of hydrolysis products in the extracellular medium. The second step is characterised by a sharp increase in amino nitrogen and nucleic acid concentrations and by constant viscosity of the system.

These events agree with the theory that the first step consists of the restructuring of cell endo-structures and the activation of lytic enzymes, which is accompanied by reduction of cell volume and system viscosity. The second step which directly follows the first step consists of the hydrolysis of cell components and release of hydrolysis products into the extracellular space. These hydrolytic products are the result of the enzymatic cleavage of proteins, nucleic acids, lipids and polysaccharides (Hernawan and Fleet, 1995). Figure 1.4 shows a schematic representation of the morphological and biochemical changes that occur during yeast autolysis (Alexandre, 2011).

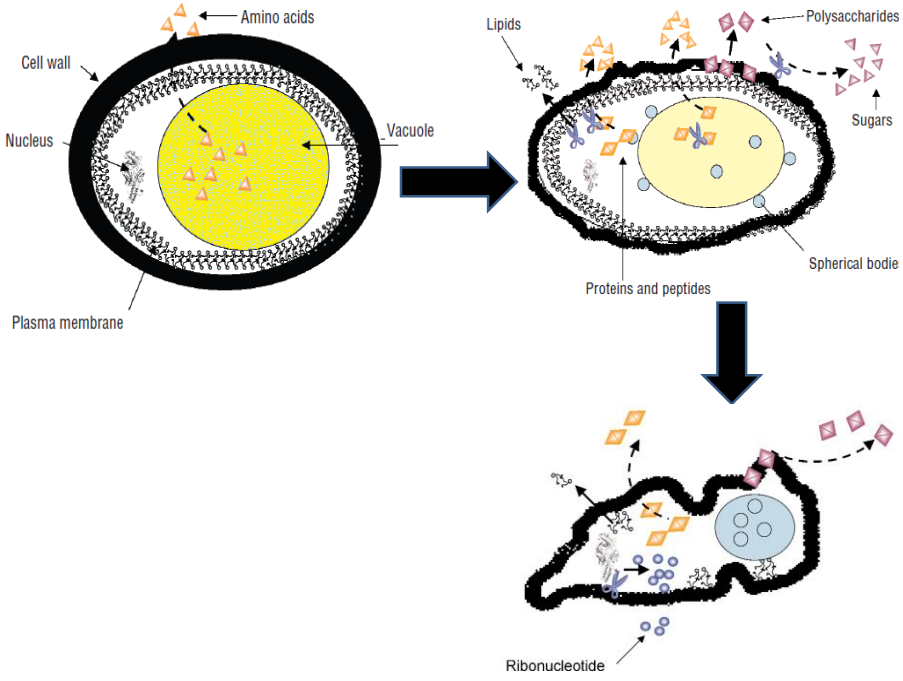


Figure 1.4 Representation of morphological and biochemical changes of yeast during autolysis (Alexandre, H., 2011).

The onset of autolysis in microorganisms also varies depending on the type of microorganism (Babayan and Bezrukov, 1985). Autolysis beginning with loss of the cell wall under the action of its own hydrolases is characteristic of "exo-type" autolysis, which is observed in most bacteria. Autolysis beginning with disturbance of

lipoprotein structures in the cell membrane is called “endo-type” autolysis and is observed in fungi, yeasts and certain kinds of bacteria (Babayan and Bezrukov, 1985). Loss of membrane function and break down in cellular organisation have been reported to be the primary events that trigger yeast autolysis (Hernawan and Fleet, 1995). Conditions of growth also have a major effect on cell disruption kinetics. In the case of *S. cerevisiae*, differences have been observed between disruption of bakers yeast and spent brewers yeast (Phaff, 1977; Mosqueira et al., 1981).

1.3.1.3 Monitoring of autolysis

In order to gain a better understanding of the process of autolysis, the autolytic process can be monitored by measurement of the dynamics of accumulation of autolysis products in autolysate samples after centrifugation. This includes measurement of cellular dry matter, amino nitrogen content, total nitrogen and protein concentration. The concentration of these parameters in the extracellular medium during autolysis, will therefore give an indication of how yeast autolysis is progressing. The movement of autolysis products into the extracellular medium has also been supported by scanning electron microscopy studies where it has been shown, that following autolysis, there were a number of folds on the cell surface and that cells were practically empty (Martinez-Rodriguez, 2001).

Another useful quantitative measurement during the autolysis process is a measurement of enzyme hydrolysis known as degree of hydrolysis (% DH). This is defined as the percentage of peptide bonds cleaved during the reaction.

Different methods are used to evaluate the % DH and are based on one of three essential principals:

1. The determination of the amount of nitrogen released by protein hydrolysis.
2. The determination of free α-amino groups or
3. The titration of the released protons.

A review of these methods is presented in Table 1.1

In a previous study, the effect of various proteases was investigated in relation to producing hydrolysates of different % DH values from various protein sources (McCarra, 2006). Along with other enzymes, the proteolytic enzyme papain was investigated and it was concluded that the % DH could be increased without increasing bitterness if used in cooperation with an exoprotease or by increasing the amounts of papain enzyme used (Figure 1.5).

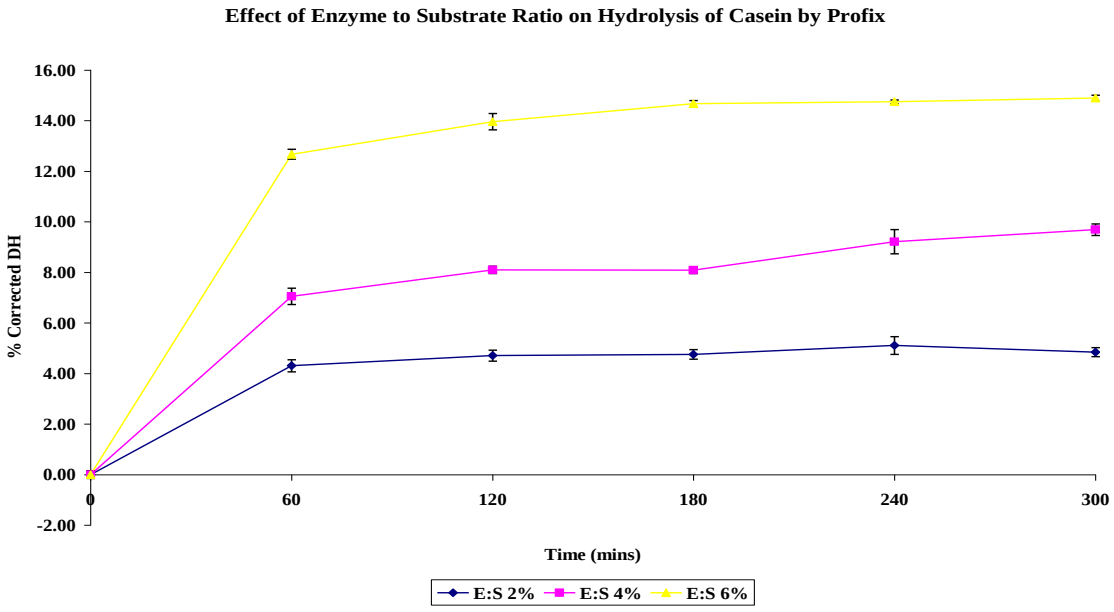


Figure 1.5 Effect of increasing concentration of papain on the degree of hydrolysis (% DH) of the protein substrate, casein (McCarra, 2006).

Table 1.1 Analytical methods used to measure enzyme hydrolysis products.

Procedure		References
Determination of released nitrogen	Kjeldhal technique.	Hrckova et al., 2002; A.O.A.C., 1995; Benton-Jones, 1991.
	Spectrophotometric determination in the UV region of aromatic peptides	Pelisser, 1984.
	Biuret reaction. Lowry Test.	Hung et al., 1984.
Determination of free α-amino nitrogen	Formol titration • Sörensen method • France Pharmacopea Method • American Pharmacopea Method • Method of the Association of Official Analytical Chemists	Nilsang, 2005; Silvestre, 1997.
	Ninhydrin method	Sun et al., 2006; Moore et al., 1948.
	Fluorescamin Method	Weigele et al., 1972.
	Orthophtaldehyde (OPA) method	Penas et al., 2004; Spellman et al., 2003; Goodno et al., 1981.
	Trinitrobenzene sulphonic method (TNBS)	Surowka et al., 2004; Spellman et al., 2003; Alder-Nissen, 1991.
Determination of released prtrotons	pH-stat technique	Kong et al., 2007; Kammoun et al., 2003; Spellman et al., 2003; Adler-Nissen, 1996; Boyce, 1986.

1.3.2 Induced yeast autolysis

In the absence of autolytic inductors, the process of autolysis is very slow and can take from several days (Amrane and Pringent, 1996; Conway et al., 2001) to two weeks or more (Sugimoto, 1974). The fact that the autolysis rate can be increased by physical, chemical and biological inductors is therefore economically significant in processes such as yeast extract production. It has been reported that autolysis incubation times of less than 24 h have been achieved using autolytic inductors (Conway et al., 2001), thereby reducing processing times significantly.

Physical inductors of autolysis (increases in temperature, osmotic pressure, irradiation and mechanical disintegration), chemical inductors (changes in pH and ionic composition, addition of membranotrophic compounds like detergents) and biological inductors, e.g., removal of oxygen and deprivation of growth medium, can be used separately or together to induce autolysis (Babayan and Bezrukov, 1985). As a result of this, the effects of various autolytic inductors during yeast extract production have been examined. These include the effect of temperature (Tanguler and Erten, 2007; Belem et al., 1997), pH (Champagne et al., 1999; Belem et al., 1997), enzyme addition (Chae et al., 2001; Conway et al., 2001), and high pressure homogenization (Liu et al., 2006).

Other studies have also noted interactions between autolysis inductors in yeast extract production. Examples include interactions between the enzymes papain and glucanases (Ryan and Ward, 1985; Ryan and Ward, 1988) as well as ethanol and sodium chloride (Sugimcto, 1974).

The effectiveness of temperature in association with additives such as enzyme, salt or other plasmolysers in promoting autolysis has been reported and temperatures are generally quoted between 50 – 55^{o}C (Moresi et al., 1995)

With respect to initial pH used for the induced autolysis process, the pH used is quite variable, ranging from pH 4.5 – pH 7.0.

A study by Champagne et al., (1999), using the yeast *S. cerevisiae,* indicated an interaction between pH and autolytic inductors with highest yields in terms of total nitrogen obtained at pH 5.5 with the addition of ethyl acetate. This was attributed to the fact that yeast proteinases were more active at pH 5.5 under these autolysis conditions. Although no sensory analysis on the final product was completed, they also reported that the pH can play an important role with respect to levels of free alpha amino nitrogen released, another important parameter in the production of yeast extracts. Babayan and Latov, (2003), reported that autolytic inductors work by accelerating proteinase induced effects and that rate of reactions catalysed by peptidases remained virtually unchanged indicating substrate deficiency.

1.3.2.1 Commercial enzyme preparations

Commercial enzyme preparations can be added at the beginning of autolysis in order to speed up the process of protein degradation, compensate for poor endogenous enzyme activity or modify the properties of the yeast extract. The effect of the addition of proteases and glucanases during yeast autolysis has been investigated (Conway et al., 2001; Chae et al., 2001; Ryan and Ward, 1985).

The proteolytic enzyme papain (extracted from the latex of *Carica papaya* fruits) and belonging to the group of sulphydryl proteases (Sathish et al., 2007; Azarkan et al., 2003; Rao et al., 1998) has been reported to be one of the most effective enzymes in

aiding yeast autolysis (Milic et al., 2007). It has also been commented that many protease preparations also have peptidase activity (Godfrey, 1996). The latex of *Carica papaya* is a rich source of the cysteine endopeptidases, including papain, glycyl endopeptidase, chymopapain and caricain, which constitute more than 80% of the whole enzyme fraction. Papain (EC 3.4.22.2) is a minor constituent (5-8%) among the papaya endopeptidases (Nitsawang et al., 2006). The enzyme is used widely as a meat tenderizer, and has also several other applications including the preparation of highly soluble hydrolysates and the reduction of haze formation in filtered beer. Table 1.2 summarises the specifications of a number of commercial proteases.

Another modification of autolysis used for yeast extract production includes plasmolysis - a modified autolysis process where inorganic salts, such as sodium chloride or in some cases non-polar organic solvents, are used to accelerate autolysis (Chae et al., 2001, Nagodawithana, 1992; Belousova et al., 1995). Plasmolysis accelerates autolysis by causing the cell to lose water and the cytoplasm to separate from the cell wall (Reed and Nagodawithna, 1991; Sugimcto, 1974; Chae et al., 2001; Liu et al., 2006).

Table 1.2 Specifications of commercial protease enzyme preparations.

Enzyme Name	Origin	Appearance	pH Range	Temp. Range	Activity	Main Bonds Hydrolysed
Bioprotease F.V.	*Aspergillus oryzae*	Brown liquid	pH 5-7	45-50 °C	1000 LAP/µg[a]	Exo: N-terminal Leu-Phe,Endo: Leu-Tyr,Phe-Tyr
Bioprotease P. Conc	*Aspergillus oryzae*	Microgranular product (tan)	80% activity, pH 4-7	80% activity 45-60 °C	Min. 400,000 HUT µ/g[b]	Leu-Tyr, Phe-Tyr
Bioprotease N100L	*Bacillus subtilis*	Light-brown liquid	pH 6.0, 70% activity, pH 8	55 °C	100,000 NPU/ml[c]	His-Leu, Ser- His Ala-Leu, Gly-Phe, Arg-Gly
Papain	*Carica papaya*	Pale-yellow liquid	pH 7.5 >80% activity, pH 6.3-8.0	50-70 °C	200 T U/mg[d]	Endo: Glu-Ala, Phe-Tyr, Asn-Gln, Leu-Val-lys

[a]One LAP (Leucine Aminopeptidase) unit is the amount of enzyme which hydrolyses 1 µmol of L-leucine-p-nitroanilide /minute.

[b]Haemoglobin unit on a tyrosine basis. One HUT is the amount that is produced in 1 min under specific conditions. A hydrolysate whose absorbance at 275 nm is the same as that of a solution containing 1.1 µg/ml of tyrosine in 0.006N HCL.

[c]One neutral protease unit is the quantity of enzyme required to produce the equivalent of 1 µg of tyrosine per minute of casein in acetate buffer.

[d]Tyrosine Units

1.3.3 Autolysis in the yeast *K. marxianus*

The fact that there is a general understanding of the biological changes that occur during autolysis is a result of extensive research using *S. cerevisiae.* However, for lactose-utilising yeasts like *K. marxianus,* the data available on the mechanism of autolysis is quite limited. The following findings were reported by Amrane and Pringent (1996), in relation to temperature induced autolysis of primary grown *K. marxianus*.

(i) By monitoring the level of oligonucleotides, amino nitrogen, carbohydrates and cellular dry weight throughout a 25-hour autolysis of primary grown *K. marxianus,* it was concluded that the kinetics of release of these components was slightly faster at 50^{o}C than at 45^{o}C with 40% of initial cellular weight solubilised. During the same conditions, the solubilisation reported for *S. cerevisiae* was 30%.

(ii) Autolysis was proposed as being a two step process similar to that documented for *S. cerevisiae* as described by (Babayan et al., 1981).

Step 1: the cell wall was partly digested and permeabilised, but cells remained intact similar to *S. cerevisiae*.

Step 2: intracellular biopolymers were quickly degraded into peptides, amino acids oligonucleotides and carbohydrates.

(iii) Scanning electron microscopy revealed that no cell burst occurred during or after autolysis but a change in morphology was observed between non-autolysed and autolysed cells the former being ellipsoidal and the latter being cylindrical. The cell wall was partly digested and permeabilised, but its continuity was preserved as is the case for *S. cerevisiae* (Hernawan and Fleet, 1995).

(iv) The dairy yeast *K. marxianus* autolysed at a faster rate than *S. cerevisiae* but with quite similar release kinetics to *S. cerevisiae* for intracellular space markers (total

nucleotide, cell wall components, polysaccharides and amino nitrogen) resulting from the digestion of both cell wall and intracellular proteins.

From these results, the mechanism proposed for *S. cerevisiae* autolysis (Babayan et al. 1981) was also likely for *K. marxianus,* despite the fact that the latter cell autolysis proceeded more quickly than the former.

In another study of *K. marxianus* as a source of yeast autolysates, Lukondeh et al, 2003a, compared the composition of yeast extracts from primary grown *K. marxianus* with the traditional *S. cerevisiae*, focusing on nucleic acid, carbohydrate, protein and amino acid composition. Autolysis of primary grown yeast was initiated by incubating the yeast suspension (3.3% w/v wet weight) at 40^{o}C with orbital shaking at 180 rpm for ten days. Values for the recovery of protein was 8.2 % - 12% in the yeast extracts approximating those reported by Hough and Maddox, (1970) and by Hernawan and Fleet, (1995). The amino acid glutamic acid was detected in low amounts but still important for the production of flavour profiles as the threshold value for flavour enhancement is 0.01-0.03%. *K. marxianus*, therefore had potential as a source of yeast extract but a shorter autolysis time would be required. This could be achieved by the addition of autolysis promoters such as NaCl which would help to keep the autolysis time under 24 h, prevent the development of contaminants and limit the actions of proteinases and peptidases to yield the desired components.

1.3.3.1 Induction and acceleration of autolysis in the yeast *K.marxianus*

The effect of some physio-chemical treatments on the kinetics of autolysed yeast extract production from primary grown *K. marxianus* has been studied (Moresi et al., 1995). It was found that maximum yield of yeast-whey protein extracts was achieved when the endogenous enzymes of ground cells were induced by 5% (w/v) salt addition at a temperature of 54^{o}C and with no pH adjustment. However the effects of these treatments on the flavour profile of the final yeast extract obtained from *K. marxianus* has not been reported..

With respect to the endogenous enzymes of *K. marxianus,* the purification and characterization of a serine carboxypeptidase (CPY) and a lysine aminopeptidase (APE) from *K. marxianus* have been described (Ramirez-Savala et al., 2004a; Ramirez-Savala et al., 2004b). In another study, Carboxypeptidase Y was isolated from autolysed *K. marxianus* cells and shown to have maximum activity at pH 6.0 (Transfiguracion et al., 1998). The food processing industry uses carboxypeptidases to treat protein products to reduce their bitter taste. Aminopeptidases have been studied in yeast where they participate in generating the characteristic texture and taste of the products in which they are found, such as meats and cheeses (Ramirez-Savala et al., 2004a; Ramirez-Savala et al., 2004b).

Regarding the temperature of the autolysis process, it is generally accepted that 50^{o}C is optimal for both *K. marxianus* and *S. cerevisiae* (Belem et al., 1997; Tanguler and Erten, 2007). However, the pH of autolysis utilised appears to vary depending on the properties required from the final yeast extract product. Belem et al., (1997), have studied the effects of temperature and pH on yield of the 5'-nucleotide flavour enhancers in autolysates of primary grown *K. marxianus*. The yield of 5'-nucleotides

in the autolysates varied from 100 – 600 µg/g of biomass, dry weight, which was lower than those reported for *S. cerevisiae*. The yield of 5'-nucleotides was influenced by temperature with highest levels of 5'-GMP obtained at 50^{o}C. The effect of pH on yield of free 5'-nucleotides was not obvious with levels of 5'-IMP not appearing to be influenced by pH while a pH of 6.5 resulted in the highest concentration of 5'-GMP. These autolysis trials were carried out in the absence of salt, a plasmolyser and commercially used autolysis inductor.

Chemical hydrolysis can be used to increase and accelerate solubilisation of yeast but acid and alkali hydrolysis tend to be difficult processes to control. They are also not desirable for food applications because of the harsh reaction conditions, non-specific chemical reagents and the difficulties of removing residual reagents from the final product. Chemical hydrolysis can also destroy L-form amino acids, produce D-form amino acids and can form toxic substances such as lysino-alanine (Lahl and Grindstaff, 1994). With acid hydrolysis, the production of toxic products such as monochloropropanol and dichloropropanol can also result. As a result of this, the use of microbial enzymes in accelerating protein hydrolysis has gained prominence (West, 1996; Guadix et al., 2006; Bisswanger, 2004; Fersht, 1999).

1.3.4 Yeast cell wall material

1.3.4.1 Composition of yeast and yeast cell wall

Although the intracellular contents of spent brewers yeast are used to produce flavoured yeast extracts using the process of controlled autolysis, there still remains a significant quantity of unused cell wall material, which makes up 20-25% of the dry weight of yeast biomass (Liu et al., 2006; Klis and Hellingwerf, 2002**;** Walker 1998a; Kath and Kulicke, 1999; Phaff, 1977). Information on the composition of yeast cell wall has proved useful in development of value added products from this material (Thammakiti et al., 2004; Prieto, 2009)

About 45-55% of the dry weight of yeast is protein, 7-15% is total lipid and 5-10% is nucleic acids. The balance comprises a range of complex carbohydrates located in the cell wall. The wall of yeast cells represents quite a thick structure (generally,

100 – 200 nm) and is therefore a prominent distinguishing feature of all yeasts (Osumi, 1998; Kollar et al., 1997; Kollar et al., 1995; Klis, 1994; Walker, 1998a). The yeast cell wall is composed largely of carbohydrate with polysaccharides accounting for 80-90% of the cell wall (Aguilar-Uscanga, 2003; Keogh, 2006), some of which is covalently linked to protein. These polysaccharides are mainly β-(1-3), β-(1-6) glucans and mannan. These comprise of (1,3) and (1,6)- β-linked glucose residues and α-mannan as highly glycosylated mannoproteins (Figure 1.6).

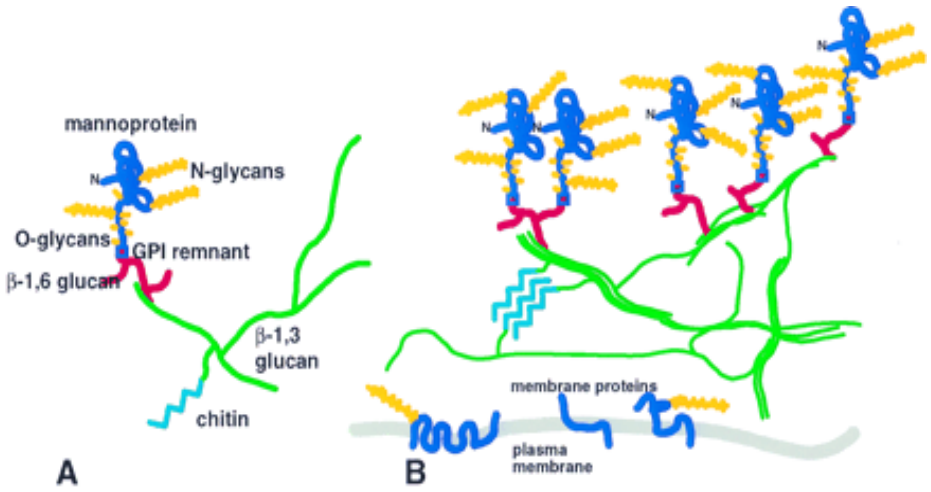


Figure 1.6 Yeast cell wall components. Polysaccharides are mainly β-(1-3), β-(1-6) glucans and mannan as highly glycosylated mannoproteins located on the outside of the wall. Chitin near the plasma membrane may also be linked to the β-1,6 glucan. (Lipke and Ovalle, 1998).

β-glucans and α-mannans each comprise some half of the dry weight of the cell wall. There is also a small percentage of chitin, linear chains of (1,4)-β-linked N-acetyl glucosamine, and lipid in the yeast cell wall (Lipke and Ovalle et al., 1998).

Stratford, 1994 has made an interesting analogy between yeast cell wall structure and reinforced concrete. Steel reinforcing rods are represented by enmeshed alkali-insoluble (1,3) –β -glucan fibrils composing some 35% of the wall. The reinforcing is surrounded by concrete pebbles in a sand/cement matrix; secreted mannoproteins represent pebbles, some 20-50% of the wall, encased and bonded to the reinforcing fibrils by a matrix of amorphous β glucan and chitin.

Table 1.3 shows the major components of the yeast cell wall of *S. cerevisiae*. These figures may vary between yeast strains with *K. marxianus* reported to have a higher percentage of soluble β-1,6-glucan than S. *cerevisiae* (Belem and Lee, 1998a). Similar figures have also been reported by Halasz and Laszitiy, (1991b) and Dallies et al., (1998).

Table 1.3 Major components of *S. cerevisiae* yeast cell wall (Klis et al., 2002).

Yeast cell wall components	Mean molecular mass (kDa)	Degree of Polymerization (DP)	% of cell wall mass
Mannoprotein	100-200	Highly variable	30-50
β1,6-glucan	24	150	5-10
β1,3 glucan	240	1500	50-55
Chitin	25	120	1.5-6

The β-glucans in yeast cell walls have been divided into three fractions; 1. Alkali-soluble (1,3)-β-glucans with 10% (1,6)-β-linkages; 2. Alkali-insoluble (1,3)-β-glucans with rare (1,6)-β-branches; 3. Highly branched (1,6)-β-glucan containing 20% (1,3)-β-linkages. The two (1,3) fractions are now regarded as one, varying only in the extent of cross-linkage with chitin. The other major component of yeast cell wall, α-mannan, consists of α-1,6 linked mannose residues with α-1,2 and α-1,3 side chains and exist as glycoproteins anchored in the wall structure (Walker,1998a). The chemical structures of these components are shown in Figure 1.7 (a-c).

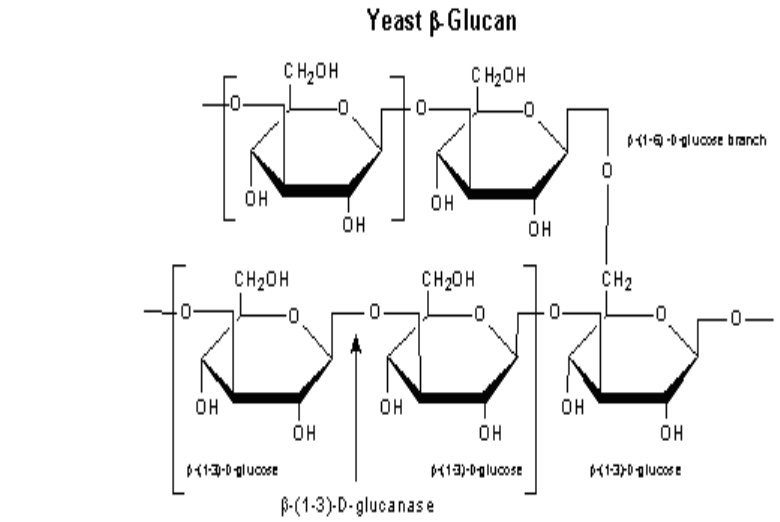


Figure 1.7(a) Chemical structure of yeast β-glucan.

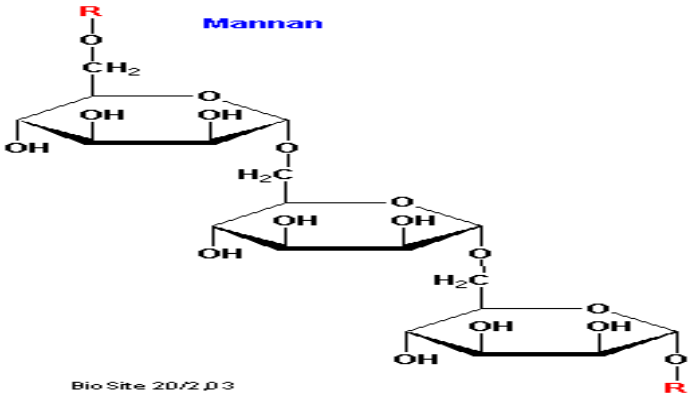


Figure 1.7 (b). Chemical structure of mannan.

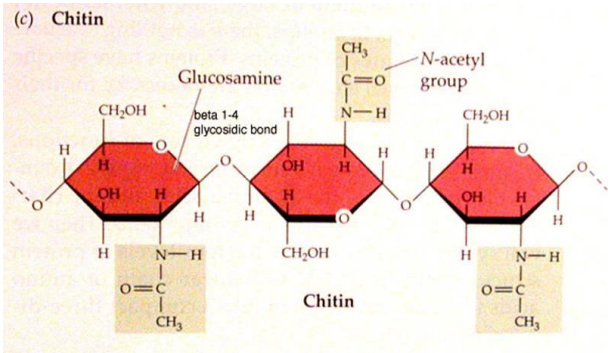


Figure 1.7(c) Chemical structure of chitin.

The mannan fraction of the yeast cell wall is an interesting polysaccharide as it is reported to have prebiotic activity after being converted into oligosaccharides and selectively consumed by beneficial bacteria in the gut. This yeast derived product which is called "MOS" or mannanoligosaccharide (Van Hai and Fotedar, 2009) can also bind to pathogenic bacteria and assist in their removal from the intestinal tract. Mannoproteins in the yeast cell wall have also been described as natural bioemulsifiers (Lukondeh et al., 2003b; Vasallo et al., 2006; Cameron et al., 1988).

Chitin is present in small quantities in *S. cerevisiae* (approx. 2-4%), mainly in bud scars (Walker, 1998a). Chitin is a polymer of (1,4) linked N-acetly glucosamine (Halasz and Lasztity, 1991b) and can be found attached to the non-reducing ends of β-1,3 glucan and β-1,6 glucan (Lipke and Ovalle, 1998; Hong et al., 1994; Kollar et al., 1997; Popolo et al, 1997.; Molano et al., 1980).

Chitin is also located in smaller amounts throughout the cell wall where it serves various functions as a killer toxin receptor and in the maintenance of osmotic and morphological integrity (Stratford, 1994).

1.3.4.2. Functional properties of yeast cell wall polysaccharides

In a recent publication relating to functional foods (Gormley and Holm, 2010), the importance of oat and barley grains as a source of dietary fibre has been reported. As a rich source of β-glucan, these grains also have interesting functional properties, such as thickening and stabilizing, emulsifying and water-binding effects (Lawther, 2010; Santipanichwong and Suphantharika, 2009; Temelli, 1997; Wikstrom et al., 1994). Similar to cereal derived β-glucan, desirable properties of extracted cell wall β-glucan from *S. cerevisiae* includes its ability to function as a noncaloric food thickener and

fat replacer while its inert nature makes it an excellant dietary fibre (Thammakiti et al., 2004; Van Hai and Fotedar, 2009; Sedmak, 2006; Banchathanakij and Suphantharika, 2009). Details regarding the extraction, functionality and food applications of yeast cell wall polysaccharides from the dairy yeast *K. marxianus* have also been reported (Lukondeh et al, 2003b; Lukondeh et al, 2003c).

In addition to their functional properties, it has been claimed that yeast cell wall polysaccharides are bioactive with associated health promoting effects (Belem et al., 1998a; Kogan and Kocher, 2007; Leung et al., 2006; Jaehrig et al., 2008; Suphantharika et al., 2003; Thanardkit,et al., 2002; Hromádková et al., 2003; Kogan, 2000; Sedmak, 2006). A report by Kogan and Kocher (2007) described three different mechanisms by which yeast β-glucan and mannan produce beneficial effects when they are added to animal feed. These included inhibition of pathogen adhesion to epithelial tissue, stimulation of immunocompetent cells and absorption of mycotoxins.

1.4 Yeast derived flavour enhancers

Controlled autolysis of yeast has resulted in established flavours associated with commercial yeast extracts today and as such these extracts are the main component of many savoury food products. Important flavour effects are attributed to the fact that compounds in the yeast extract can act synergistically to improve and enhance flavour (Chae et al., 2001; Mion-Du Crest, 1998; Nagodawithana, 1992). These compounds which improve continuity of flavour, meatiness, and mouthfeel of certain foods are termed flavour enhancers or flavour potentiators (Charpentier et al., 2005). These normally include compounds that have little taste themselves but are capable of

enhancing the taste of other foods. This is called the "umami" effect or sometimes called the fifth taste (Festring and Hofmann, 2010; Yamaguchi, 1991). The term comes from the Japanese word meaning 'savoury'or 'delicious' and is used to indicate a palatable and flavour enhancing taste. The discovery of the molecules responsible for those properties is relatively recent but condiments such as seaweeds, black mushrooms, or soya sauce containing them have been traditionally used in Asia for centuries. In a review by Nakao, (1979), it is claimed that the Japanese scientist, Ikeda, first discovered the flavour enhancer monosodium glutamate when extracting glutamic acid from seafood in 1908-1909, where it was found in the seaweed *Laminaria japonica* and identified it as the source of the umami taste. In addition to glutamic acid, the amino acid aspartic acid is another well-known amino acid contributing to "umami" in food. Nandakumar, (2003) proposed the following two step reaction (acylation – deacylation) in relation to the enzymatic formation of L-glutamic acid from L-glutamine, catalysed by the enzyme glutaminase.

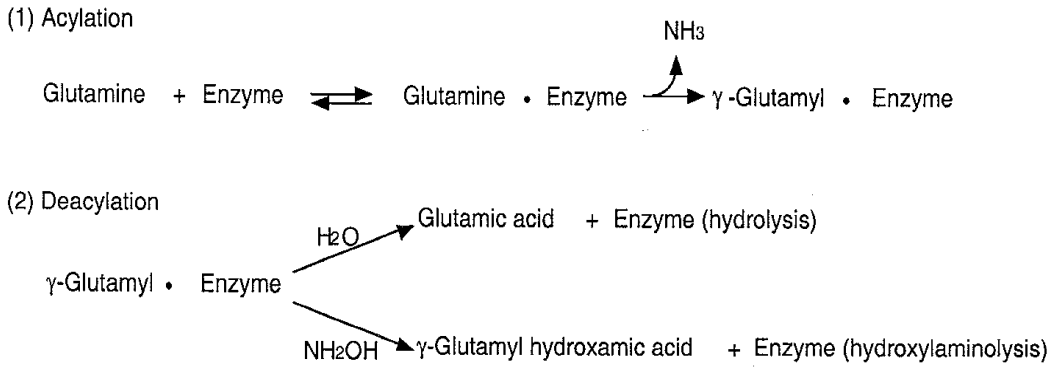


Figure 1.8 Mechanism for the conversion of L-glutamine to L-glutamic acid, a reaction catalysed by the enzyme glutaminase (Nandakumar, 2003).

The best known flavour enhancers that are in commercial use worldwide, are monosodium glutamate (MSG), and yeast RNA derived free nucleotides, inosine 5'-monophosphate (5'IMP) and guanosine 5'-monophosphate (5'-GMP). For 5'-nucleotides, such as 5'-GMP, the prime indicates the number on the ribose ring, to which the phosphate is attached (Elliott and Elliott, 2001).

While dried bonita and black mushrooms were the initial sources in which the flavour enhancers 5'IMP and 5'-GMP respectively were first discovered (Mion-Du Crest, 1998), yeast's economic production along with its high ribonucleic acid (RNA) content (2.5 - 11%) has made it the organism of choice for the production of extracts rich in these 5' ribonucleotides (Charpentier et al., 2005). The industrial production of flavoured yeast extracts which began in the 1950's, was further accelerated by developments in manufacturing methods of 5'IMP and 5'-GMP using the enzymatic hydrolysis of yeast RNA. By 1974 the first commercial yeast extract containing 5'-GMP, a natural nucleotide and flavour enhancer derived from the yeast RNA, was produced on an industrial scale (Nakao, 1979).

1.4.1 Free ribonucleotides

In yeasts, the nucleic acid fraction consists mainly of RNA and a relatively small proportion of DNA. *Candida utilis* contains 10-15% RNA, *S. cerevisiae* has 8-11% RNA while *K. marxianus* has 10 % RNA (Lukondeh et al., 2003a).

In addition to protein degradation, RNA degradation is a characteristic feature of yeast autolysis and products of this degradation, e.g., nucleotides, nucleosides and bases, can be measured in the autolysate (Zhao and Fleet, 2005;Hernawan and Fleet, 1995). Although the autolytic fate of nucleic acids, carbohydrates and lipids is not as well understood as that of protein degradation, methods of optimising release of the flavour

enhancing components, particularly free nucleotides, have been reported. Towards the end of autolysis, the autolysates derived from *K. marxianus* can be enzymatically treated to optimise the level of 5'-nucleotides which are used as flavour enhancers (Belem et al., 1997). The enzyme 5'-phosphodiesterase (Figure 1.9) hydrolyses RNA efficiently to a mixture of ribonucleotides, from which the flavour enhancers, 5'-GMP and 5'-IMP can be isolated (Deoda and Singhal, 2003; Steensma et al., 2004). With the incubation of commercial adenylic deaminase, 5'-AMP in the autolysate can also be converted to the flavour enhancer 5'-IMP (Belem and Lee, 1998a).

Many of the flavour improving properties of yeast extracts are the result of the interaction of various amino acids (the most important being glutamic acid) in combination with the 5' prime ribonucleotides, 5'IMP and 5'-GMP (Yamaguchi, 1991; Bellisle, 1999). It is said that the 5' prime nucleotides in combination with glutamic acid and cysteine contribute to a foods typical meat flavour (Revillion et al., 2003). The techniques utilised to quantify nucleotides are mainly chromatographic, with many of these techniques requiring quaternary gradient systems to separate the free nucleotides effectively, particularly 5'GMP and 5'IMP (Belem and Lee, 1997; Charpentier et al., 2005, Zhao and Fleet, 2005). To date, no satisfactory isocratic separation has been reported.

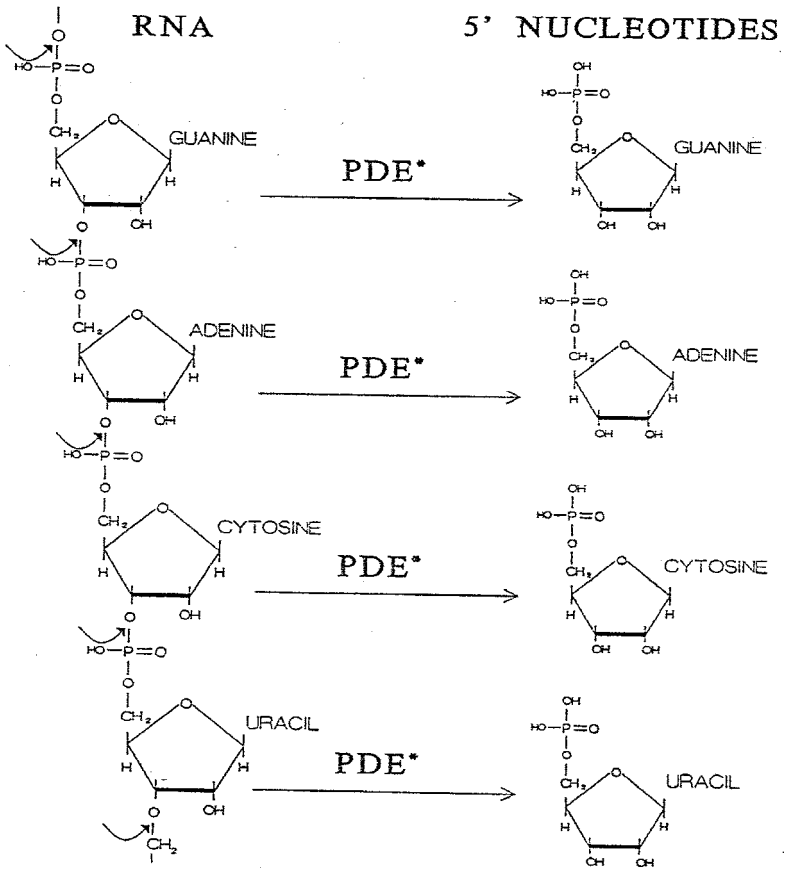


Figure 1.9 The conversion of RNA to 5'-ribonucleotides by the enzyme phosphodiesterase (PDE*) (Nagodawithana, 1992).

1.4.2 Yeast derived flavours and free amino acids

Initial studies by Halasz and and Lásztity, (1991c) investigated the amino acid composition of brewers yeast hydrolysate using gel filtration chromatography. Six fractions were separated using a gel filtration Biogel-P4 column. Fractions having a meaty flavour were found to contain high quantities of the amino acids, glutamic acid and aspartic acid (Figure 1.10).

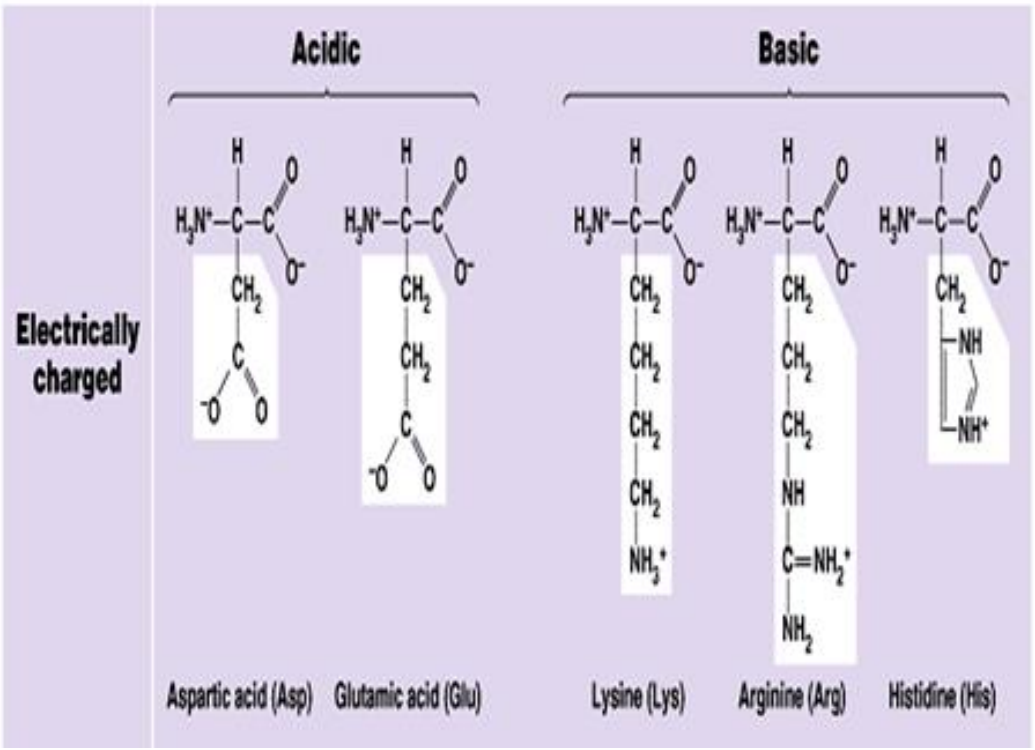


Figure: 1.10 Chemical structure of acidic amino acids (Aspartic acid and Glutamic acid) and basic amino acids (Lysine, Arginine and Histidine).

Removal of undesirable flavoured constituents can be an important step particularly with respect to brewers yeast extracts (McCarra, 2001). The taste of untreated yeast extract can in many cases be extremely bitter and this bitterness is reported to be associated with peptides characterized by the presence of hydrophobic amino acids (Figure 1.11). The intensity of bitterness has been found to be proportional to the number of hydrophobic amino acids and the size of the peptide (Ney, 1979). This requirement of debittering when using spent brewers yeast as a source of yeast extracts (Shotipruk et al., 2005; Fitzgerald and O'Cuinn, 2006), has led to growing

interest in the use of *K. marxianus,* as removal of bitter hop components is not a necessary step when using this yeast (Belem and Lee, 1998).

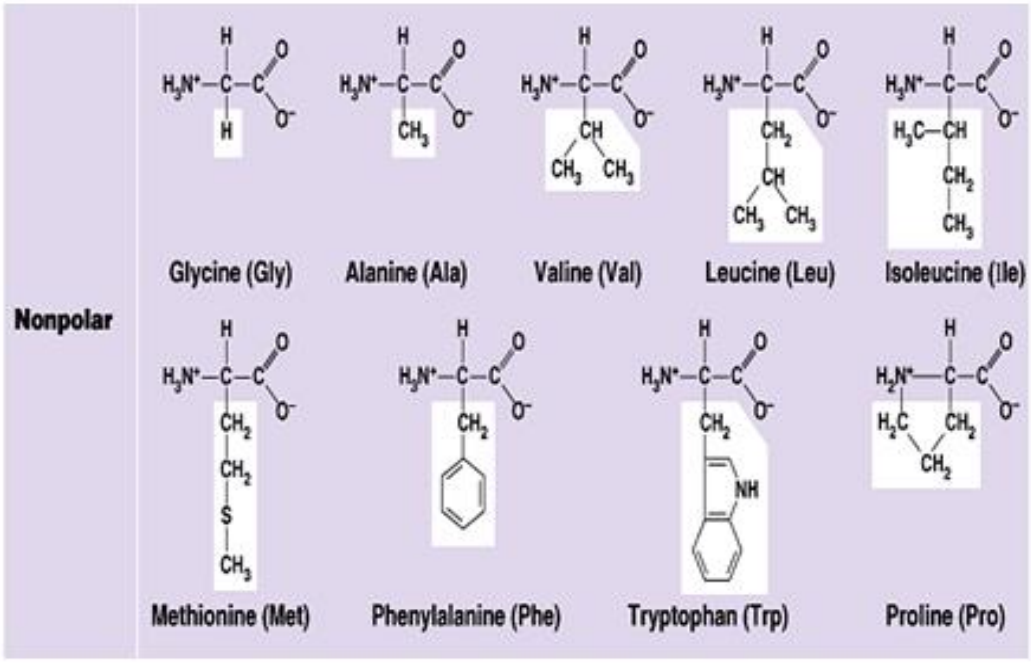


Figure 1.11 Chemical structure of hydrophobic (non-polar) amino acids.

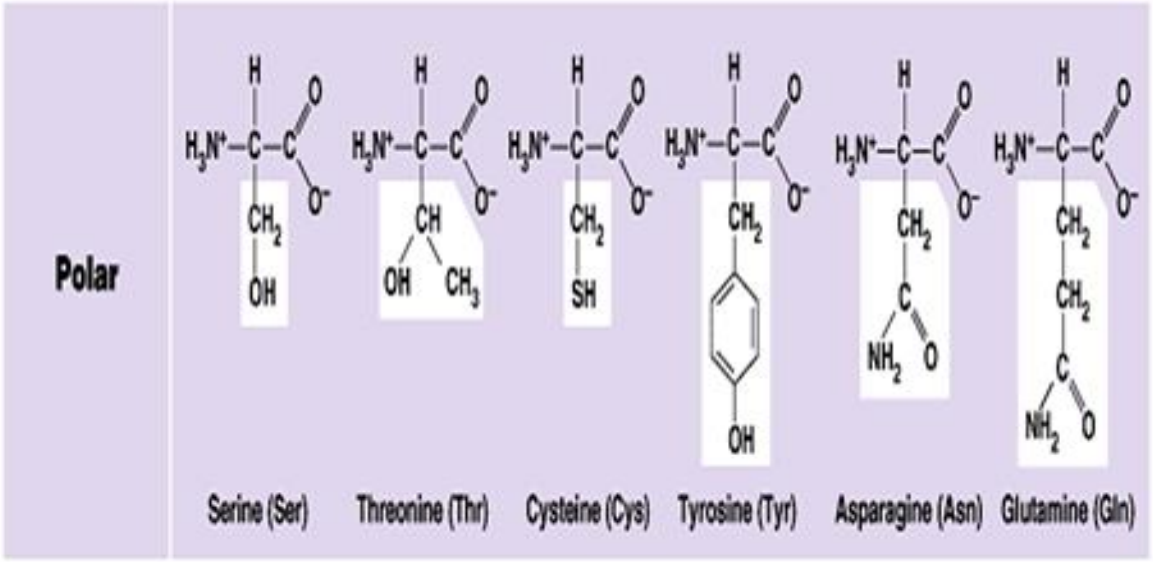


Figure: 1.12 Chemical structure of polar type amino acids.

The food industry has employed a range of methods to overcome bitterness of unhydrolysed proteins including masking with other flavourings, extraction with charcoal and limiting the hydrolysis reaction (Pawlet and Bruce, 1996). The use of peptidase enzymes to reduce or eliminate bitterness has increased significantly in recent years (Saha and Hayashi, 2001; Raksakulthai and Haard, 2003; Fitzgerald and O'Cuinn, 2006; Nishimura and Kato, 1988). Peptidases act by removing a single or pair of amino acids from the terminal end of a peptide chain: carboxypeptidases acting from the C-terminal and aminopeptidases from the N-terminal (Figure 1.13). The effectiveness of peptidases in debittering will largely depend on their specificity, with peptidases cleaving hydrophobic amino acids and proline being the most valuable.

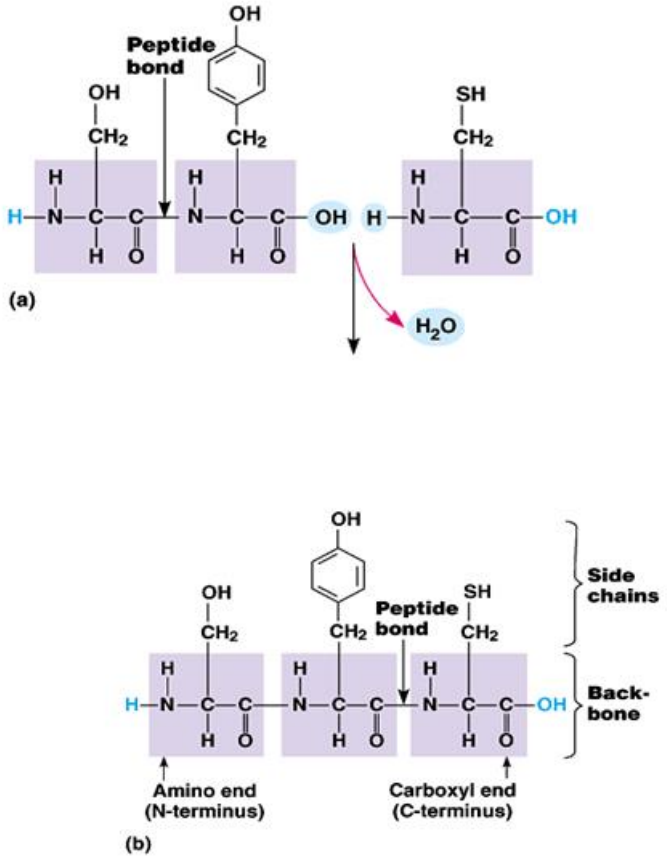


Figure: 1.13 Conversion of a dipeptide (a) to a tripeptide (b), containing the amino acids serine, tyrosine and cysteine. In this example, a peptidase can act by hydrolyzing the peptide bond at either the carboxyl or amino end of the tripeptide (b).

When evaluating yeasts for their ability to produce flavoured yeast extracts, the amino acid composition of whole yeast protein can be compared with amino acid composition in the yeast extract. It has been found that the amino acid pattern of whole cells of *Hansenula* yeast were similar to that of the protein extract (Halasz and

Lasztity, 1991c). However, the amino acid concentrations of the protein extract were slightly higher than the whole cells excluding Methionine, Alanine, Glycine, and Proline. This is consistent with the report of Vananuvat and Kinsella, (1975) who found that the concentration of most amino acids in the proteins precipitated with acid or heating from the alkaline extracts of *K. marxianus* was similar to that of whole cells, even though there might be slight degradation of amino acids by alkali extraction. This demonstrated that major proteins contained in the whole cells of yeast could be extracted with little losses. The quality of the original protein was also related to its amino acid composition, most notably the essential amino acids (Friedman, 1996).

1.4.3 Descriptive sensory analysis of yeast extracts

Descriptive sensory tests involve the discrimination and description of both the qualitative and quantitative sensory components of a food product by a trained panel of accessors (Murray et al., 2001). The qualitative aspects of a food product include aroma, appearance, flavour, texture and aftertaste which distinguish it from others. Sensory panel members quantify these product aspects in order to facilitate description of the perceived product attributes using a consensus vocabulary for odour, flavour and other attributes of the food product. Modern sensory analysis techniques, such as descriptive sensory analysis, when combined with multivariate statistical methods, allow the key attributes of food products to be objectively determined and described. Principal component analysis (PCA) is a multivariate statistical method useful for studying correlation in a set of measurements of a given number of variables for a determined number of accessors. It has been described as a way of identifying patterns in data, and expressing the data in such a way as to highlight their similarities and

differences. Initially, the ability of descriptive vocabulary to discriminate between flavour attributes of a product such as a yeast extracts are first tested using one-way analysis of variance (ANOVA). Attributes which discriminate significantly ($p < 0.05$) between the tested samples are averaged across replicates, standardized and analysed using PCA. How each principle component descriminates between the sensory characteristics of yeast extracts can also be investigated using ANOVA (Hannon et al., 2001).

The main advantage of PCA is that once patterns have been found in the data, the data can be compressed, i.e. by reducing the number of dimensions, without much loss of information. This technique can therefore simplify complex and diverse relationships of observed variables by contracting information into a smaller number of principal components based on correlation among them. Variables which contribute significantly to important characteristics of a product are retained. The sample distribution based on the resulting principal component score plot can be used to examine the mutual relationships among samples (Ragasso et al., 2001).

PCA has therefore the capability to separate food products on the basis of a particular characteristic. While there have been many reports on the chemical composition of yeast extracts, there is however a need for more comprehensive descriptive sensory analyses of these products in order to understand the full effect of autolysis parameters on flavour of yeast extracts. In particular, this could prove to be a very useful tool in assessing flavour differences in yeast extracts arising from different yeast autolysis treatments

1.4.3.1 Yeast extracts and flavour

Taste is an essential component of flavour. Although taste carries fewer distinctive qualities than odour, it provides an essential base on which aroma builds to generate the widely varying flavour of our foods and beverages (Breslin, 2001). The perception of flavour is comprised of the sensory combination and integration of odours, tastes, thermal sensations and mouthfeels that arise from a particular food. In this study, the focus was on the non-volatile chemical components of flavour in dairy yeast extracts. Although volatiles and non-volatiles function together in the construction of flavour, it is believed that it is the non-volatiles which allows us to identify a food as edible or not (Breslin, 2001).

The sense of taste is characterized by qualitative descriptors such as sweet, sour, salty, bitter and umami, a term associated with savoury, meaty or brothy (Eurasyp, 2012). Controlled autolysis of spent brewers yeast has resulted in this recognised fifth taste which is the strong savoury flavour associated with commercial flavoured yeast extracts. Extracts produced from spent brewers yeast generally impart flavours that are strong, robust and bitter while the flavour of extracts from primary grown yeast are milder ranging from delicate brothy to browned roasted and meaty depending on the processing conditions.

Another interesting application of yeast extracts is the masking of bitter flavours in foods. An example includes the autolysis of *Candida utilis* using enzymes for improving flavour of foods containing chicken and pork and masking the bitterness of KCl in low sodium foods. A similar yeast based product was produced by mixing plasmolysed yeasts *K. marxianus* and *K. lactis* with crystallized whey ultrafiltration permeate. The dried product contained 5% moisture, 30% protein, 9.5% minerals and

1% calcium with recommended uses as ingredients for meat products, sauces, soups and pates (Halasz and Lásztity, 1991c). Although less is known concerning dairy yeast extracts, they are reported to deliver a mild but effective flavour enhancement when applied to dairy–themed sauces and cheese-based snacks (http://www.carbery.com).

Improvement in flavour can also be attained with advances in enzyme technology. Protein structure may now be modified by enzymatic hydrolysis to improve the functionality of the protein which includes improving flavour and texture or removing off flavours and preventing undesirable interactions (Kong et al., 2007; FitzGerald and O'Cuinn, 2006; Popineau et al., 2002; Drago and Gonzalez, 2001; Lahl and Grindstaff, 1994; Vegarud and Langsrud, 1989; Chobert et al., 1988).

1.5. Bioreactors for study of yeast bioprocesses

Laboratory-scale automated bioreactors have proved invaluable in the study of cheese whey fermentation for both alcohol and biomass production using *K. marxianus* (Belem and Lee, 1999b; Belem et al., 1998c). Such studies have been facilitated by the use of sensors for the measurement of on-line parameters such as dissolved oxygen, pH, temperature, aeration and agitation. Improvements in yields of yeast biomass or yeast derived products can therefore be made not only through strain and media selection but also through process-related modifications (Walker, 1998b). These latter considerations relate to the design, operation, monitoring and control of yeast fermentations. Bioreactors have also been utilized in the study of yeast autolysis processes for the production of yeast extracts (Champagne et al., 1999; Belem et al., 1997).

Recent advances in hardware and software technology have ensured that bioreactors are now fully validatable for current Good Manufacturing Practie (cGMP) production. Process data can now be stored digitally on a single computer for multiple reactor systems. This effectively means that multiple processes can be controlled automatically from a single computer (Figure 1.14). Computer supervisory control and data acquisition (SCADA) software, also enable independent monitoring of multiple bioreactor vessels (Kakes, 2008). For scale-up studies, industry still embraces traditional glass and steel stirred tank technology such as benchtop, autoclavable bioreactor systems as illustrated in Figure 1.15 and stainless steel bioreactor systems as illustrated in Figure 1.16. The latter system was commissioned in 2012 at the Shannon Applied Biotechnology Centre (SABC) where this current study was carried out.

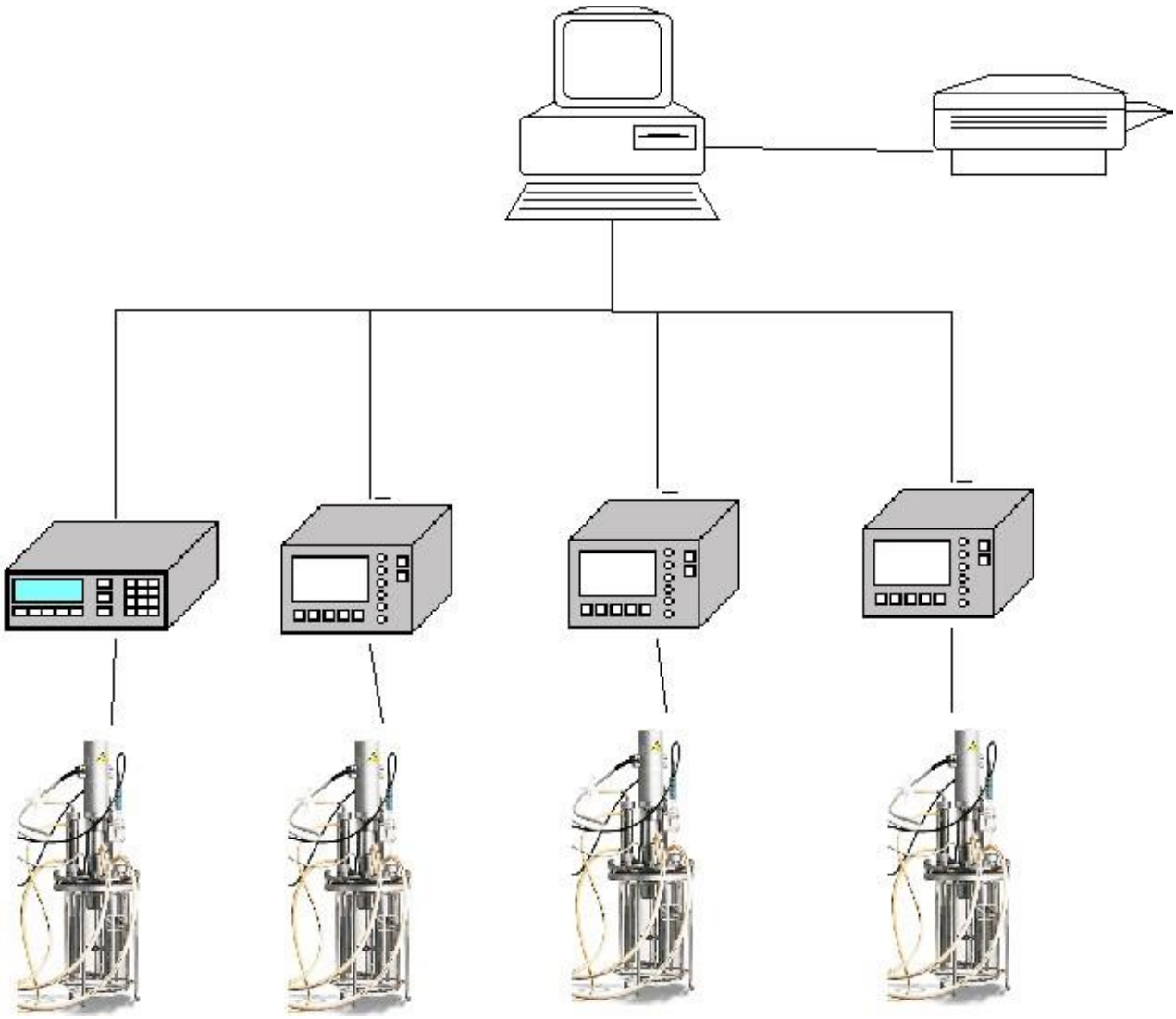


Figure 1.14 Multiple bioprocesses can be controlled automatically from a single computer.

Figure 1.15 Benchtop, autoclavable bioreactor system controlled by supervisory control and data acquisition (SCADA) software.

These bioreactor systems are extremely versatile particularly in scale-up studies when transitioning from shaker flask studies and are currently been utilised at the SABC for fermentation and yeast autolysis optimisation. Sensors for temperature, dissolved oxygen, pH and fill level control ensure accurate on-line monitoring of biotechnological processes (Hayward, 2008). Recent developments include culture vessels with optional redox and turbidity monitoring. As a result, the new benchtop bioreactor systems are also now designed for flexibility and use in a broad range of applications. These validatable units allows up to eight or more external devices, such as analyzers, sensors, pumps, or scales to be directly connected to the controller for process optimization. The preference today is either towards off-the-shelf, modular systems or a custom-engineered approach for systems in the volume range of 100 -

500 litre as shown in Figure 1.16. The latter approach is implemented only when process requirements are fully understood.

Figure 1.16 The custom-engineered approach for a 100 litre bioreactor or fermentor system together with on-line sensors, touch-screen technology and advanced process control software. (Commissioned at Shannon Applied Biotechnology Centre, May, 2012).

1.6 Objectives of this study

1. To determine the composition of spent dairy yeast, a by-product of whey to ethanol fermentations.

2. To develop an induced autolysis process for the generation of alternative yeast extracts.

3. To investigate if induced autolysis of spent dairy yeast can provide yeast extracts with desirable flavour enhancers.

4. To assess the impact of induced autolysis on the sensory attributes of the developed yeast extracts.

Figure 1.17 is a flow diagram of the current study.

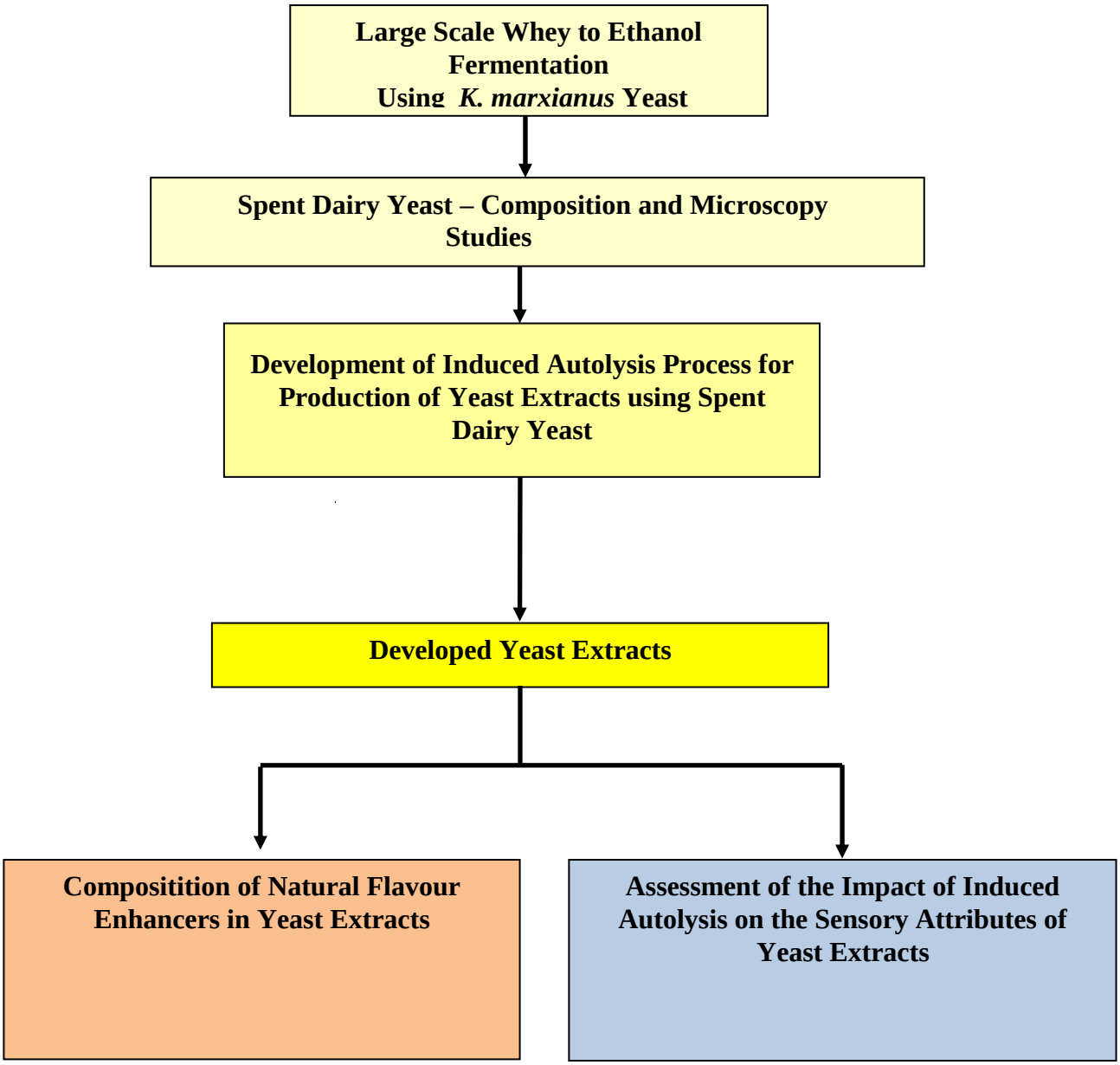


Figure 1.17 Flow diagram of the current study with induced autolysis of spent dairy yeast as a key step in yeast extract development.

Chapter 2

Composition and characteristics of spent dairy yeast *Kluyveromyces marxanius*, a by-product of whey to ethanol fermentations.

2.1 Introduction

Yeast biomass is primarily comprised of macromolecules which are assembled into the structural components of the cell. The macromolecules in question normally are comprised of proteins, polysaccharides, lipids and nucleic acids (Alexandre, 2011; Moresi et al., 1995; Walker, 1998a). The relative concentrations of these constituents can vary from species to species and are also influenced by conditions of growth (Lipke and Ovalle, 1998; Nguyen et al., 1998). While the use of spent brewers yeast as source of biomass for the production of food grade flavoured yeast extacts has been well documented (Tanguler and Erten, H, 2007; Chae et al., 2001; Thammakiti et al., 2004; Liu et al., 2006;), considerably less information is available with respect to spent dairy yeast *K. marxianus,* a by-product of whey to ethanol fermentations.

The large scale anaerobic fermentation of a waste stream such as whey permeate by *K. marxianus* for the production of ethanol is of particular relevance to this study as in addition to producing a valuable commodity such as ethanol, it also produces significant quantities of secondary (spent) dairy yeast. The production of value added products such as flavoured yeast extracts from spent dairy yeast could therefore result in the more efficient utilisation of dairy waste streams while also providing alternative flavoured products for use in food products. The objective of this study was to evaluate the composition and microbiological characteristics of spent dairy yeast *K. marxianus*, prior to it being utilized for the production of flavoured yeast extracts**.**

2.2 Materials and methods

2.2.1 Yeast

Spent dairy yeast, *K. marxianus* (SDYK), a by-product of whey to ethanol fermentations was kindly provided by the food ingredient company Carbery Group, Ballineen, Co. Cork, Ireland, as a liquid yeast suspension with a solids content of 18.7 % ± 1.2 (w/v) and pH 4.5 ± 0.25, n = 10.

2.2.2 Microscopy

Cell morphological characteristics of SDYK were studied using differential interference contrast microscopy, compound light microscopy and scanning electron microscopy. Differential interference contrast microscopy was carried out using a BX51 light microscope (Olympus, Germany) with an attached Progres® CT3 camera (Jenoptik Optical Systems Inc, USA). Analysis was completed using Progres® CapturePro Software Version 2.7.7 (Jenoptik Optical Systems Inc, USA). The compound light microscope utilised was a Nikon Y32-T microscope. Scanning electron microscopy was carried out using a Carl Zeiss Supra 40VP Field Emission SEM, filtered with an Alto 2500 cryo stage (Gaton, UK.). Use of this equipment was kindly facilitated by Teagasc, Moorepark, Co. Cork (Figure 2.1).

2.2.3 Yeast cell viability

Yeast cell viability of SDKY was determined using the spread plate total viable plate count technique on YEPL agar which consisted of lactose monohydrate (Merck), 2% (w/v); yeast extract (Difco), 1% (w/v); Bacto peptone (Difco), 2% (w/v); agar (Difco), 2% (w/v). Incubation conditions were at 30^0C for 48 h. The growth profile in YEPL inoculum flasks was monitored by following optical density at 600nm using a UV/VIS Shimadzu spectrophotometer.

Figure 2.1 Carl Zeiss Supra 40VP Field Emission SEM, (Teagasc, Moorepark)

2.2.4 Analytical Methods

The following analytical methods were used on suspensions of spent dairy yeast SDYK as received from Carbery, Ireland.

Total Nitrogen measurement:

Total Nitrogen of SDKY suspensions was determined using the Kjeldahl method (AOAC, 1980) on an Auto Kjeltec system and % protein calculated using the formula, total nitrogen x 6.25.

Amino acids measurement*:*

Amino acid composition of SDKY was determined by hydrolysing 0.5g in 6M HCl at 110°C for 23 h (Hill, 1965). The resulting hydrolysates were analysed for total individual amino acid content using a Jeol JLC-500/V amino acid analyser (Jeol UK Ltd., Garden city, Herts, UK) fitted with a Jeol Na^+ high performance cation exchange column, using known concentrations of amino acid standards (Fenelon et al., 2000). Samples were deproteinised by mixing equal volumes of 24% (w/v) tri-chloroacetic acid (TCA) and sample, which were allowed to stand for 10 min before centrifuging at 14400g (Microcentaur, MSE, UK) for 10 min. Supernatants were removed and diluted with 0.2M sodium citrate buffer, pH 2.2 to give approximately 250 nmol of each amino acid residue. Samples were then diluted with the internal standard, norleucine, to give a final concentration of 125 nm/ml. Colourimetric detection of amino acids was achieved by postcolumn ninhydrin derivatisation. Data analysis was carried out using a Minichrom data handling system (VG Data Systems, Altrincham, Cheshire, UK).

Total carbohydrate measurement:

Total carbohydrate of SDYK suspensions was measured by the phenol/sulphuric acid method (Dubois et al.; 1956).

Yeast β-glucan and mannan measurement:

Yeast β-glucan and mannan concentrations in yeast cell wall after autolysis were measured using the yeast β-glucan kit K-YBGL and D-mannose, D-fructose and D-glucose kit K-MANGL, respectively (Megazyme, Ireland). The concentration and pH of the SDYK suspension for analysis was adjusted to 10 % (w/v) and pH 5.5 respectively. Sodium chloride was added to give a final concentration of 5% (w/v) followed by a 0.2% (v/v) addition of the the proteolytic enzyme, papain (Kerry Ingredients & Flavours) and incubated in an orbital shaker (200 rpm) at 50°C for 24 h. Autolysis was completed by finally heating the yeast solution to 85°C for 30 minutes, at which temperature all enzymes were inactivated. Yeast cell wall material was isolated by centrifuging at 5000 x g for 30 min. Total solids of the cell wall residue was estimated and adjusted to 7%-10% (w/v) prior to freeze drying using a Modulyo Freeze Dryer equipped with an Edward's vacuum pump.

Ribonucleic acid (RNA):

The RNA content of SDYK suspensions was determined by the Orcinol method (Herbert et al., 1971) with yeast RNA as standard (Sigma).

Chitin:

The chitin content of SDYK was measured on the basis that glucosamine reacts with acetylacetone to form a chromogenic complex with p-dimethylaminobenzaldehyde (Popolo et al., 1997). The standard used was N-acetyl glucosamine (Sigma).

***Mineral Composition**:*

Mineral composition of SDYK was determined using an inductively coupled plasma atomic emission spectrometer (McKinstry et al., 1999). A 0.5 g aliquot of dried and ground material was placed into a burning cup and 15 ml of pure HNO_3 was added. The sample was incinerated in a MARS 5 Microvawe Oven at 200 °C, and the solution was diluted to the desired volume with water.

Measurement of total solids and total dissolved solids:

Total solids content of SDYK was measured by drying a sample overnight in an oven at 105^{o}C. Total dissolved solids content was measured using a refractometer (Atago, Japan).

pH measurement:

pH was measured using a laboratory pH meter (Mettler Toledo).

2.3 Results and Discussion

2.3.1 Microscopic examination of SDYK

In general, *Kluyveromyces* yeasts are unicellular, nucleated, non-motile microorganisms. They are Ascomycetes that can reproduce sexually when nutrients are limited (sporulation and conjugation) or asexually (multilateral budding and fission). Primary *K. marxianus* grown on whey are typically ovoid or ellipsoidal in shape (Belem and Lee, 1998a). Figures 2.2 and 2.3 show images of SDYK, using differential interference contrast and phase contrast microscopy, respectively. The yeast SDYK was observed to be ellipsoidal in shape. The subcellular organelles, some of which comprised of vacuoles, were of particular importance to this study as they contain a variety of hydrolytic proteases which are utilised effectively in

commercial yeast extract production. *K. marxianus* is a dimorphic yeast that can grow vegetatively in either single cell or filamentous (hyphal or pseudohyphal) form. In the budding yeast *K. marxanius*, limitation of nitrogen and the presence or absence of O_2 can influence dimorphism (O'Shea and Walsh, 2000; Walker, 1998a). Yeast cells range in size from 5 – 8 µm and from Figures 2.2 and 2.3 it can be observed that the size of SDYK was falling within this range.

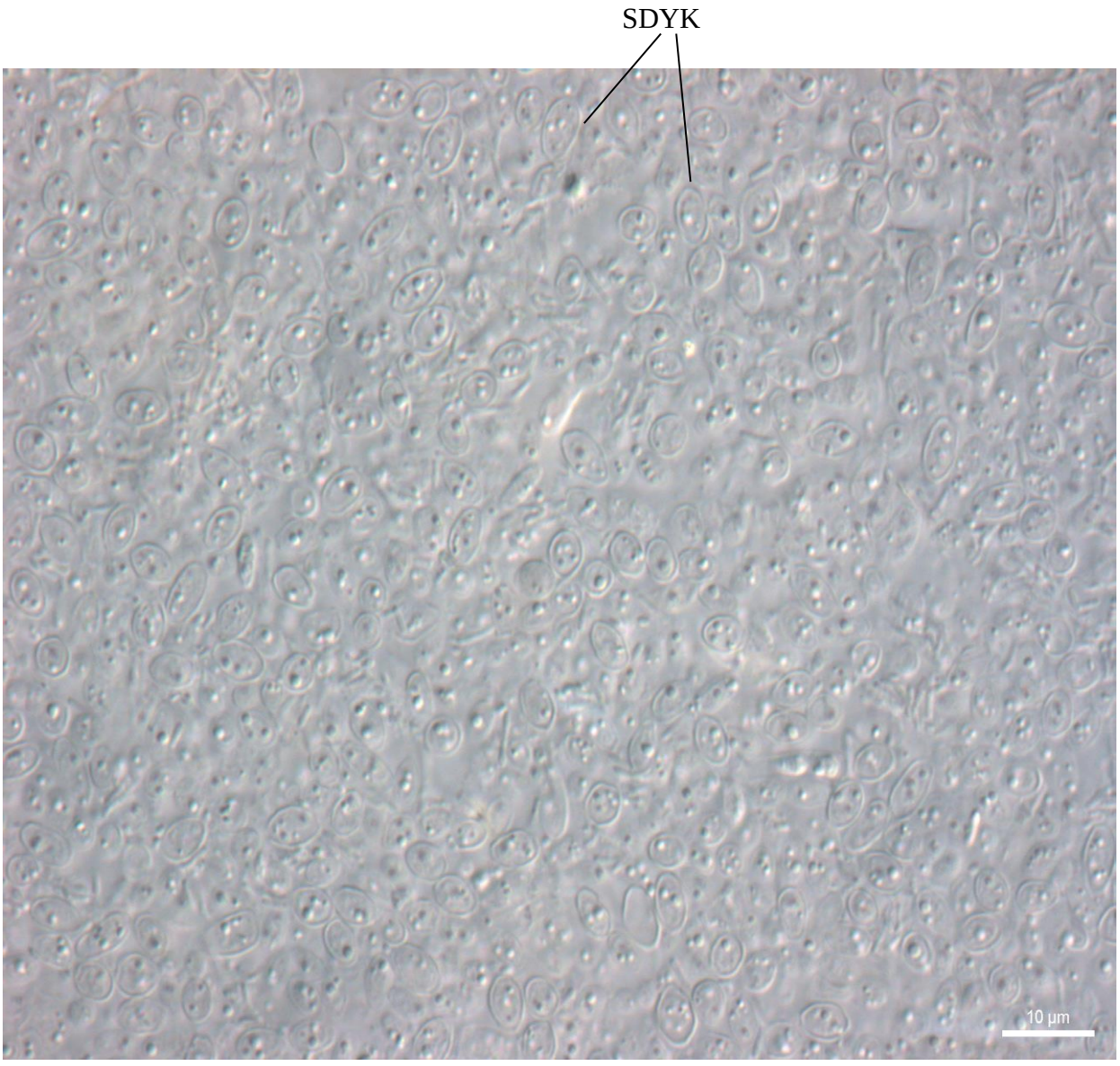


Figure 2.2. Differential interference contrast microscopy of SDYK (magnification, 1000x).

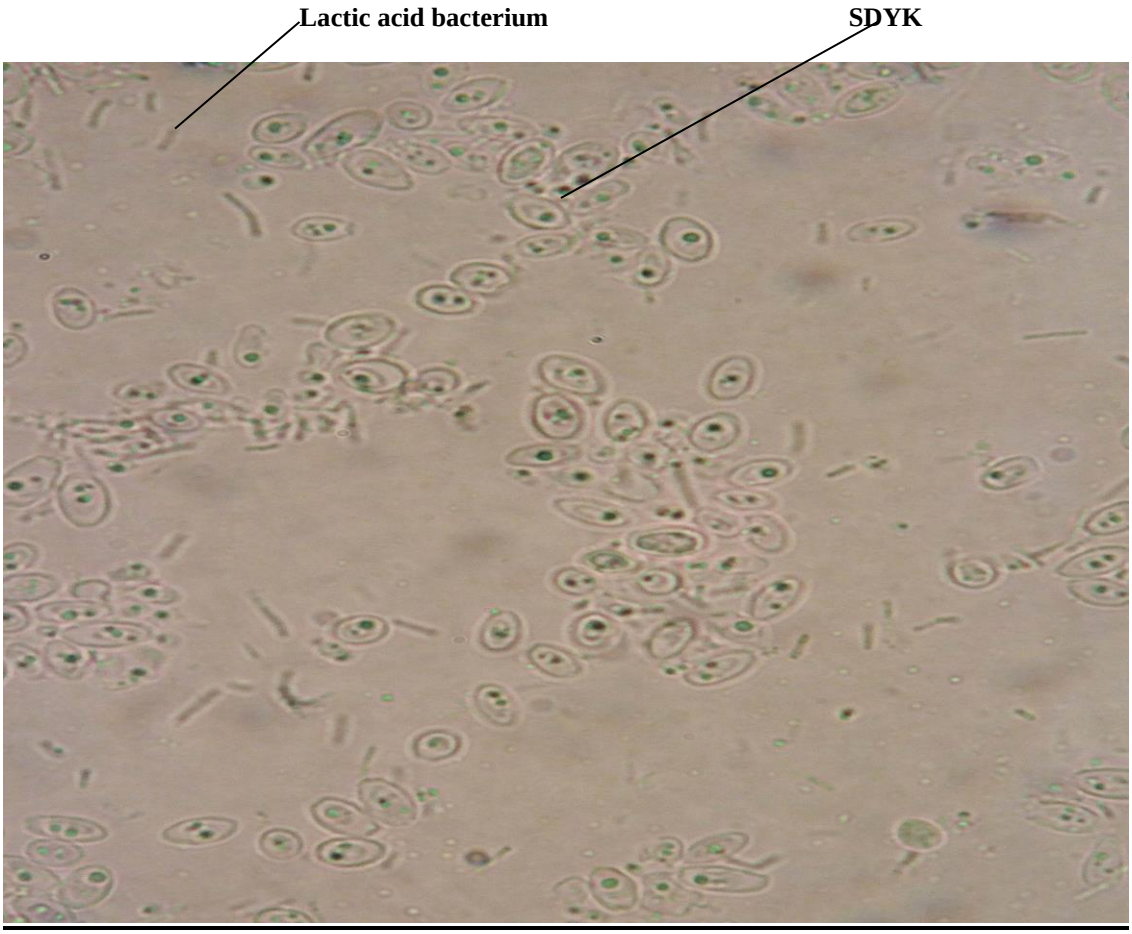


Figure 2.3 Phase contrast microscopy of SDYK in the presence of lactic acid bacteria (magnification, 1000x).

In addition to SDYK, the presence of rod shaped lactic acid bacteria was evident (Figure 2.3) and this is referred to later in section 2.3.1.1.

In order to observe the ultrastructural features of SDYK, this spent dairy yeast was also observed using scanning electron microscopy (Figure 2.3a). While the yeast shape was obvious as ellipsoidal, the spent yeast appeared to have folded inwards indicating that natural autolysis of the yeast had already commenced.

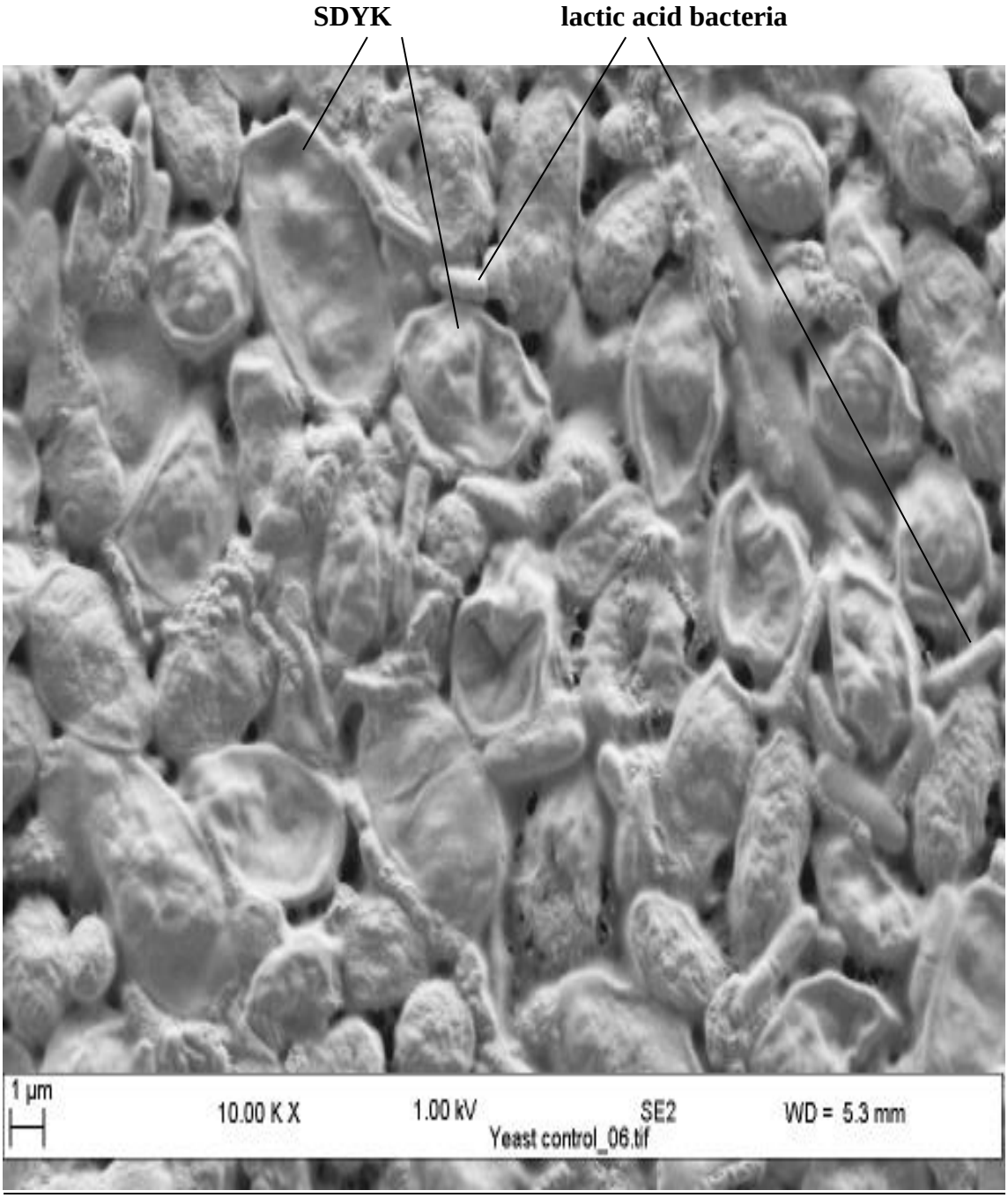


Figure 2.3a Scanning electron micrograph of SDYK in the presence of lactic acid bacteria (magnification, 10,000x).

The cell wall of SDYK was observed as a relatively thick structure (0.5 µm) and this is a prominent distinguishing feature of all yeasts. The main structural components of the yeast cell walls are polysaccharides, accounting for 85-90% of the cell wall and

from which yeast derive their strength and shape (Aguilar-Uscanga, 2003). These polysaccharides are mainly β-(1-3), β-(1-6) glucans, and mannan, while most of the cell wall protein is covalently linked to the mannan component forming mannoproteins. Chitin, a polymer of (1,4) linked N-acetly glucosamine, consists of 1 – 2% of the yeast cell wall and is linked to both the non-reducing branches of β-1,3 glucan and β-1,6 glucan resulting in a further strengthening of the cell wall framework (Halasz and Laszitiy, 1991b; Lipke and Ovalle, 1998).

Another notable feature in the scanning electron micrograph was the presence of rod shaped lactic acid bacteria which are highlighted in Figure 2.3a. The significantly larger size of SDYK (5-8 μm) when compared to lactic acid bacteria was quite evident. Lactic acid bacteria have also been reported to be present in other yeast fermentations (Champagne et al., 1999).

2.3.1.1 Lactic acid bacteria

In whey the main carbon source is lactose and microorganisms which can utilise lactose will have a competitive advantage over other organisms present.

Microscopic examination of SDYK suspensions showed clearly the presence of lactic acid bacteria (Figure 2.3-2.3a). As SDYK is in this study was derived from whey to ethanol fermentations, the presence of lactic acid bacteria such as *lactobacilli* could be expected as these fermentations were carried out under conditions which were also suitable for the growth of this bacterium. These fermentation conditions were typically 30^{o}C and initial pH 5.5 using whey media that was pasteurised and not sterilised as for many industrial fermentations. Extensive contamination was controlled by utilisation of a strong inoculum of yeast and favourable conditions for

ethanol production. Although it is inevitable that bacterial contamination will occur, this does not have a serious adverse effect on this yeast fermentation process. This view is supported by Christensen et al., (2011), who reported that *K. marxianus* was able to compete effectively with lactic acid bacteria during ethanol production. Although some lactic acid was produced prior to ethanol fermentation, *K. marxianus* was capable of taking over the fermentation and producing ethanol from lactose before it was converted to high lactic acid concentrations by *lactobacilli*.

There are also reported advantages to fermentations involving yeasts and lactic acid bacteria (Wood, 1985). These fermentations are anaeobic, acidic, saturated with carbon dioxide and produce alcohol, a combination of conditions that are inhibitory to many spoilage microorganisms. In relation to the Carbery process, it is believed that because lactic acid bacteria are known flavour contributors, yeast-lactic acid bacteria associations can also result in synergistic relationship with respect to flavour development (Wood, 1985). This association has also been reported by Hay (1993) when bakers yeast extract was combined with lactic acid and succinic acid from *lactobacillus* fermentations resulting in improved meaty and savoury attributes.

2.3.2 Viability of SDYK

The yeast viability count for SDYK received during this study indicated good viability with an average value of 1.3×10^8 colony forming units /ml (log cfu/ml = 8.7 ± 0.63). This high viability was necessary to promote autodegradation during future autolysis

trials. SDYK also had a higher initial viability when compared to other yeast strains (Hernawan and Fleet, 1995). The high viability of SDYK was most likely related to the high solids level of the concentrated yeast suspension. Reducing initial yeast solids has been shown to significantly increase the rate of autolysis by Champagne et al., (2003).

SDYK suspensions obtained were normally stored and refrigerated at 4^{o}C on arrival. Stability of the spent yeast obtained in terms of cell viability at a storage temperature of 4^{o}C was investigated by monitoring cell viability over time. It was observed that a reduction of 19 % of cell viability of SDYK occurred over a 12 week period (Figure 2.4). This can be attributed to "natural autolysis" whereby autolysis by endogenous enzymes occurs naturally in yeast as the cell becomes aged or where suitable growth conditions are not sustained (Chae et al., 2001).

Low yeast cell viability results in poor activity of the yeast's endogenous degrading enzymes. During yeast extract production, these degradative enzymes inherently present in the cell are required for effective autolysis resulting in the cell components within the cell being solubilised and forming yeast extracts. Reduced activity of these enzymes therefore makes the yeast cell less susceptible to autolytic activity and yeast extract production (Reed and Nagodawithana, 1991).

Total dissolved solids was monitored as an indicator of natural autolysis and the consequent release of hydrolysis products. The % total dissolved solids increased during this period, indicating the release of intracellular material due to natural lysis of cells. For this reason, and in order to obtain a standardised induced autolysis process for future trials, SDYK would not be used after four weeks storage at 4^{o}C.

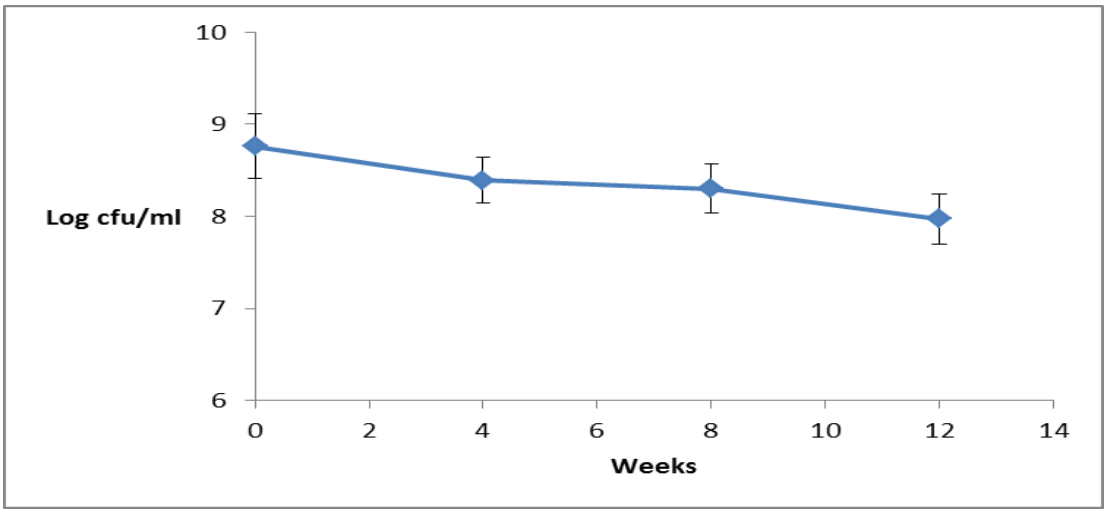


Figure 2.4 Viability of SDYK during storage temperature of 4ºC. Samples of SDYK were analysed in triplicate at selected times during the storage of this yeast.

The pH of SDYK suspensions was relatively stable during storage, rising slightly from an initial pH of 4.51 ± 0.20 to a final pH of 4.91 ± 0.25. The initial pH of SDYK is consistent with the pH of fermentation broths of *K. marxianus* grown on whey lactose after 20-24 h (Belem, 1999b).

2.3.3 Chemical composition of SDYK

Although the chemical composition of yeast is greatly affected by changes in the medium and culturing conditions, Halasz and Lasztity, (1991b), summarized the basic characteristic of the dry matter of yeast biomass composition as having a high protein, nucleic acid and ash content with a moderate carbohydrate concentration. The composition of primary grown *K. marxianus* has been reported (Lukondeh et al., 2003a; Revillion et al., 2003 and Moresi et al., 1995) but there is no detailed information with respect to SDYK produced as a by-product of whey to ethanol fermentations. Compositional analysis was therefore carried out on SDYK, with

emphasis on components of this yeast associated with yeast extract flavours. Equally important would be any unique features of the yeast that could be exploited in yeast extract product development in terms of delivering new benefits.

2.3.3.1 Protein composition of SDYK

The protein concentration of SDYK was determined to be 55.3 ± 0.8 % on a dry weight basis which was similar to that obtained by Moresi et al., (1995) and Lukondeh et al., (2003a), who obtained 57% and 56% respectively when using primary grown *K. marxianus*. However values in the range 40% - 42% have also been reported when *K. marxianus* was grown on various whey media indicating the important effect of growth medium on protein content of yeast (Ghaly et al., 1993; Ghaly and Kamal, 2004). The presence of residual whey nitrogen will also contribute to the variation in protein concentrations obtained. This was found to be the case by Moresi et al., (1995), when primay grown dairy yeast was analysed for both Lowry (soluble) protein and crude protein, resulting in values of 48.4% and 57.5%, respectively.

Yeast protein can be divided into three basic groups - cytoplasmic including enzymes, storage proteins and cell wall protein. The protein content of yeast biomass which on average varies from 40-57% on a cell dry matter basis, has traditionally been measured as crude protein (total nitrogen x 6.25). Table 2.1 describes the results obtained with respect to chemical composition of SDYK which was received as a post-fermentation concentrated yeast solution.

Table 2.1 Chemical composition (% w/w, dry basis), of SDYK. All values were reported as means ± standard deviations of triplicate determinations.

Macro-Constituents of SDYK	
Protein (Crude)	55.3 ± 0.8
Total Nitrogen	8.85 ± 0.75
Total Carbohydrate	31.5% ± 4.4.
RNA	7.5 ± 0.75
Ash	5.97 ± 0.2

2.3.3.2 Comparison of amino acid composition of SDYK and spent brewers yeast

As no data was currently available with respect to amino acid composition of SDYK, total amino acid analysis was completed and the amino acid profile obtained is shown in Figure 2.5**.** For comparative purposes, total amino acid analysis was also completed for spent brewers yeast *S. cerevisiae*. Using analysis of variance, the amino acid profiles of both yeasts were characterised by having significantly different concentrations of the individual amino acids determined (Table 2.2).

Table 2.2 Analysis of variance of total amino acid content of SDYK and brewers yeast, *S. cerevisiae*

Dependent Variable: % Amino acids

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	375.859	1	375.859	7345.850	P<0.001
Yeasts	2.862	1	2.862	55.932	P<0.001
Amino acid (aa)	99.408	16	6.213	121.428	P<0.001
yeasts * aa	24.468	16	1.529	29.888	P<0.001
Residual	1.740	34	.051		
Total	504.337	68			
Corrected Total	128.478	67			

Glutamic acid and alanine were found to be in significantly higher amounts when compared to the other amino acids determined ($p < 0.001$). The amino acid aspartic acid was also found to be present in significantly higher amounts than the other amino acids in both yeasts with the exceptions of valine and leucine. This has important implications in flavour development of food products, as both glutamic acid and aspartic acid are recognized flavour enhancers. While the amino acids aspartic acid and glutamic acid were found to be present in relatively high amounts in both yeasts there was no significant difference in concentrations of these amino acids ($p > 0.05$) between spent brewers yeast and SDYK.

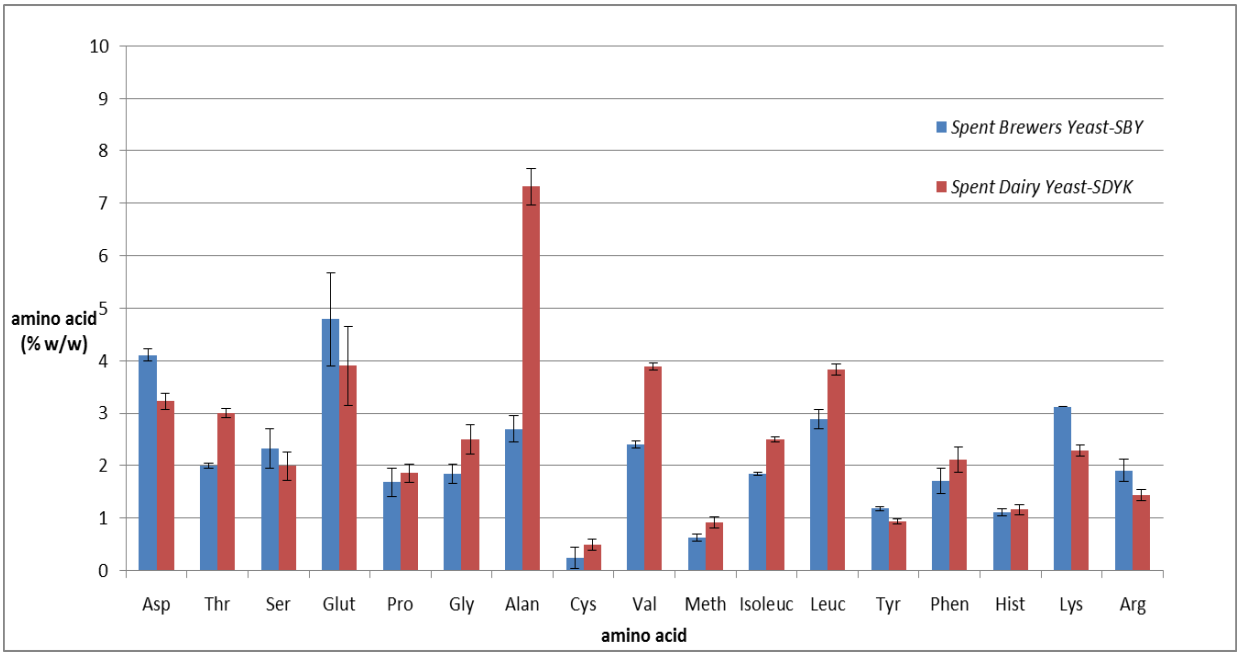


Figure 2.5 Comparison of amino acid profile (% w/w, dry basis), of SDYK and spent brewers yeast *S. cerevisiae*. All values were reported as means ± standard deviations of duplicate determinations.

The Food and Agriculture Organization together with the World Health Organization (FAO/WHO) have established a table of essential amino acids with estimations of amino acid requirements for humans based on nitrogen balance (WHO/FAO/UNU, 2007). Figure 2.6 indicates that the amino acid profile of SDYK compared very well with FAO/WHO guidelines for most of the essential amino acids. The lower values obtained for methionine and cysteine was not surprising as low levels of these amino acids are typical of yeast (Dziezak, 1987).

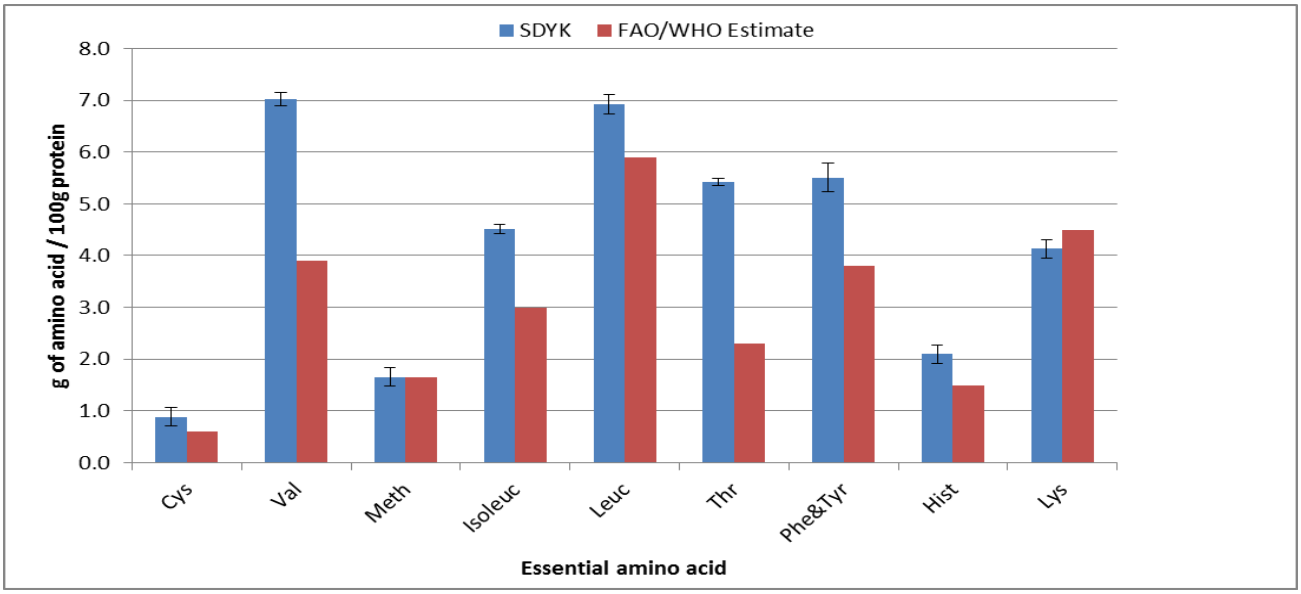


Figure 2.6 Comparison of essential amino acid profile of SDYK with recommended FAO/WHO estimates.

2.3.3.3 Carbohydrate composition of SDYK

The carbohydrate content of SDYK, measured as total carbohydrate, was 31.5% ± 4.4%. Carbohydrate content of yeast also varies depending mainly on growing conditions in the fermenter. Normally one fifth to one third of the dry matter of yeast consists of different carbohydrates. From a morphological point of view, yeast carbohydrate can be divided into intracellular (cytoplasmic) and cell wall types (Halasz and Lasztity, 1991b). The latter is of particular interest to this study, as yeast cell wall is a by-product of yeast extract production and another source of value added food ingredients. These include β-glucans for improvement of physical properties of food such as water-holding capacity and emulsifying stability (Thammakiti et al., 2004). Published data for chemical composition of the cell wall of *S. cerevisiae* shows large variability with carbohydrate and protein values quoted at 60-91% and 6-13% respectively (Halasz and Lasztity, 1991b). Carbohydrate content of the yeast cell wall of SDYK was found to be 61.0 ± 1.8 % and similar to spent brewers yeast

(65.23% ± 1.1), as reported by Thammakiti et al., (2004). These cell wall carbohydrates of SDKY consisted mainly of the polysaccharide β-glucan and mannan and were determined to be 41.5% ±0 3 and 18.65% ± 0.25 respectively. The composition of the cell wall components of SDYK are summarised in Table 2.2.

Table 2.3 The composition (% w/w, dry basis) of cell wall of SDYK. All values were reported as means ± standard deviations of triplicate determinations.

Constituents of cell wall of SDYK	
Total Carbohydrate	61.0 ± 1.8
Protein	13.0 ± 0.95
β-glucan	41.5 ±0 30
Mannan	18.65 ± 0.25
Chitin	0.70 ± 0.05
Ash	7.65± 1.5

Thammakiti et al., (2004) obtained a value of 55.21% ±1.4 β-glucan in cell wall extracts of spent brewers yeast. However, cell wall polysaccharides can vary considerably between yeast strains (Lipke and Ovalle 1998; Nguyen et al., 1998) in addition to variations due to environmental and growth conditions. Similar to β-glucan composition, mannan and chitin composition of yeast will vary depending mainly on environmental and growth conditions (Nguyen et al., 1998: Lipke and Ovalle; 1998: Lukondeh et al., 2003c).

2.3.3.4 Nucleic acids in SDYK

In this study, the amount of RNA in SDYK was found to be 7.5 % (w/w) ± 0.75 (Table 2.1). Depending on the yeast strain, there appears to be some variation in nucleic acid content. The yeast *Candida utilis* can contain up to 10-15% RNA, while *S. cerevisiae* and *K. marxianus* when grown aerobically were reported to have 8-11% RNA and 10 %, respectively (Lukondeh et al., 2003).

A high content of nucleic acid is typical for yeasts (Kockova-Kratochvilova, 1990; Halasz and Lasztity, 1991b). The amount of nucleic acid in any microorganism is dependent on its rate of growth, and generally high growth rates are associated with high nucleic acid content and protein biosynthesis. The nucleic acid fraction of yeasts consists mainly of RNA and a relatively small proportion of DNA (Zhao and Fleet, 2005). Because of their high RNA content, yeasts are the preferred source of nucleic acids for the enzymatic production of 5'IMP and 5'-GMP nucleotide flavour enhancers.

While the anaerobic fermentation of cheese whey by *K. marxianus* resulted in significant quantities of ethanol being produced (Gough et al., 1996; Walker, 1998b), the growth rate of the microorganism was reduced to facilitate ethanol production. SDYK derived from the anaerobic fermentation of cheese whey had therefore a lower RNA concentration than primary grown yeast.

2.3.3.5 Mineral content of SDYK

The mineral content determined for SDYK is shown in Table 2.4. The mineral content of yeast has been reported to be quite variable and the average ash content of yeast cells tend to be relatively high with typical values being 8 - 10% of dry matter (Kockova-Kratochvilova, 1990; Thornton, 1992; Halasz and Lasztity, 1991b). It is

now believed that growing conditions of the yeast have a significant effect on the quantity and the composition of the ash. As the whey fermentation substrate contains minerals such as calcium and phosphorous, these minerals are macro-element components of SDYK.

Table 2.4 Mineral content of SDYK. All values were reported as means ± standard deviations of triplicate determinations.

Macro-Elements	**(% w/w, dry basis)**
Calcium	0.53 ± 0.01
Magnesium	0.14 ± 0.001
Potassium	3.23 ± 0.16
Phosphorous	1.95 ± 0.05
Sodium	1.51 ± 0.035
Micro-Elements	**(ppm)**
Copper	4.03 ± 0.008
Iron	48.1 ± 0.275
Manganese	7.85 ± 0.01
Zinc	40.1 ± 0.12
Molybdenum	< 0.25
Selenium	< 0.25

Table 2.5 Comparison of the composition (% w/w, dry basis) of SDYK in this study with that reported for spent brewers yeast and primary grown *Kluyveromyces.*

Yeast Type	Protein	Carbohydrate	RNA	Ash	Lipid	References
Spent brewer's yeast	43.4 ±0.30	59.6 ± 0.51	-	10.3± 0.17	1.2 ± 0.57	Thammakiti et al., 2004
Spent brewer's yeast	48	36	-	3.8	-	Nigam et al., 2009
Spent brewer's yeast	48.0	36.0	9.5	8.0	1.0	Halász and Lásztity, 1991
Spent brewer's yeast	59.7	35.1	-	7.1	4.9	Liu et al., 2008
Spent brewer's yeast	39.6	-	9.0	4.6	0.5	Yamada and Sgarbieri,2005
Primary *K. fragilis* on sugarcane molasses	50.8 ± 1.83	30.2 ± 1.65	7.5 ± 0.61	6.0 ± 0.43	-	Vasallo et al., 2001
Primary *K. fragilis* on lactose	49.75 ± 1.81	40.96 ± 2.89	7.67 ± 0.65	6.54 ±0.37	0.65 ±0.06	Orban et al., 1994
Primary *K.fragilis* on whey	57.57±0.33	40.96±2.89	9.46±0.44	8.09± 0.32	0.763± 0.10	Moresi et al., 1995
Primary*K. fragilis*	50.76±1.83	31.21±1.65	7.54±0.61	5.98± 0.43	-	Otero et al., 2002
Primary *K. fragilis* on whey	37.0	34.3	4.82	16	7.8	Paul et al., 2002
Primary *K marxianus*	56.0	26.0	10.0	-	-	Lukondeh et al., 2003a
Spent dairy yeast *K.marxianus* (SDYK)	55.3 ± 0.8	31.5% ± 4.4.	7.5± 0.75	5.97 ± 0.2	-	This Study

2.4 Conclusion

The composition of SDYK was characterised by a high content of protein, carbohydrate and minerals with moderate levels of RNA. The protein concentration of this yeast (55.3%) along with its amino acid composition indicated its potential as a suitable alternative source of flavoured yeast extracts. SDYK biomass had comparable levels of the flavour enhancing amino acids, glutamic acid and aspartic acid, to that of spent brewers yeast and a significantly higher concentration of the amino acid alanine. The amino acid profile of SDYK also compared favourably with FAO/WHO guidelines for most of the essential amino acids with the lowest values obtained for methionine and cysteine which are typical of yeast. The carbohydrate composition of the yeast cell wall consisted mainly of the functional polysaccharides, β-glucan and mannan. SDYK had high levels of macroelements associated with the fermentation substrate whey, while microscopic examination of SDYK suspensions showed clearly the presence of lactic acid bacteria which are known flavour contributors.

Chapter 3

Effect of pH and autolytic inductors on the percentage free amino nitrogen, degree of hydrolysis and total dissolved solids in yeast extracts derived from spent dairy yeast *Kluyveromyces marxianus.*

3.1 Introduction

When active, yeast's endogenous enzymes play an important role in yeast autolysis, the main process used for production of commercial flavoured yeast extracts (Conway et al., 2001; Hough and Maddox, 1970; Babayan et al., 1981; Sommer, 1998; Milic et al., 2007). However, yeast autolysis is slow and can extend to 72 h if not accelerated in some way. The rate of release of autolysis products can be increased by the addition of physical, chemical and biological inductors with most production processes utilizing both plasmolysis and yeast autolysis to further accelerate the process (Kelly, 1983; Ryan and Ward, 1988). Plasmolysis is achieved by addition of sodium chloride, while the proteolytic enzyme papain is frequently added to increase the rate of proteolysis during yeast extract production. Plasmolysis, in cooperation with hydrolytic enzymes also helps to to improve the flavour attributes of yeast extracts derived from spent brewers yeast (McCarra, 2001; West, 1996; Reed and Nagodawithna, 1991). While some efforts have been made to understand the mode of action of autolytic inductors during autolysis of spent brewers yeast *S. cerevisiae* and primary grown *K. marxianus,* there is considerably less information available for SDYK.

The aim of this study was to obtain a better understanding of the induced autolysis process for the generation of yeast extracts s by evaluating the effects of the autolysis inductors pH, salt and the proteolytic enzyme papain on free amino nitrogen, total dissolved solids and degree of hydrolysis in yeast extracts derived from spent SDYK.

3.2 Materials and methods

Spent dairy yeast *K. marxianus*, (SDYK) a by-product of whey to ethanol fermentation with a solids content of 18% ± 1.2 w/v (n = 10) and pH = 4.55 ± 0.25 (n = 10) was kindly provided by the Carbery Ireland, Ballineen, Co.Cork, Ireland, as a concentrated yeast solution.

3.2.1 Induced autolysis of SDYK

Autolysis conditions were selected with the aim of evaluating initial pH of autolysis in the presence and absence of inductors. This involved pH adjustment and sodium chloride addition followed by the proteolytic enzyme papain. Induced autolysis conditions are outlined in Table 3.1. The concentration of SDYK biomass utilised was 10 % (w/v) based on previous optimisation studies (McCarra, 2001) and reports in the literature (Chao et al., 1980; Orban et al., 1994; Moresi et al., 1995).

Table 3.1 Induced yeast autolysis conditions at 50 °C and 10% (w/v) SDYK solids, with varying initial pH.

Autolysis treatment	Inductor
A, Control	No sodium chloride and no papain enzyme
B, Salt	With 5 % (w/v) NaCl
C, Enzyme	With 0.2% (w/v) papain enzyme
D, Salt and Enzyme	With 5% (w/v) NaCl and 0.2% (w/v) papain enzyme

The pH of 150 ml quantities of SDYK solutions placed in 250 ml conical flasks was adjusted with 2 M NaOH (Merck) or 10% orthophosphoric acid (Merck) to pH 4.5,

pH 5.5, pH 6.5 and pH 7.0 respectively. Sodium chloride was added to give a final concentration of 5% (w/v) followed by a 0.2% (v/v) addition of the the proteolytic enzyme, papain (Kerry Ingredients & Flavours) and incubated in an orbital shaker (200 rpm) at 50°C. The time course of the induced yeast autolysis process was followed by sampling during a 24h period starting from a zero point coincident with the instant at which the yeast slurry had reached the reaction temperature of 50°C and and continuing for times 3h, 8h and 24h. Autolysis was completed by finally heating the yeast solution to 85°C for 30 min, at which temperature all enzymes were inactivated. Yeast cell wall material was removed by centrifuging at 5000 x g for 30 min. Autolysis trials were scaled up from laboratory shake flasks using an automated five litre bioreactor (Biostat A, Sartorius) with Microsoft Fermentation Control System (MFCS/win) SCADA software, version 2.1 or one litre stainless steel beakers with automated stirring (Heidolph) in a temperature controlled waterbath.

3.2.1.1 Extraction of yeast cell wall polysaccharides from SDYK.

After induced autolysis, the yeast cell wall of SDYK was recovered by centrifugation (5000 x g for 30 min). The yeast cell walls were resuspended in distilled water to obtain a suspension containing 10% (w/v) solids content and adjusted to pH 8.0 with 2M NaOH. Yeast cell wall polysaccharides were then enzymatically extracted using 0.01% (v/w) of the alkaline protease, alcalase for 24 h at 60C (Sedmark (2006). The products isolated after centrifugation were an insoluble fraction consisting of β-glucan, and a soluble fraction which consisted mostly of mannan. Total solids of these fractions were estimated and adjusted to 7%-10% (w/v) prior to freeze drying using a Modulyo Freeze Dryer equipped with an Edward's vacuum pump.

3.2.2 Biochemical analyses of SDYK autolysates

During yeast autolysis, the concentration of free amino nitrogen in the extracellular material serves as an indication of the extent of enzyme hydrolysis of available protein to free amino acids (Nagodawithana, 1992).

Free α-amino nitrogen (FAN) concentration of SDYK yeast extracts was determined using the method as described by the European Brewing Convention, Analytica (1975). The standard used was glycine (Riedel-de Haën) from which a standard solution containing 2 mg amino nitrogen per litre was prepared.

The total nitrogen content of an autolysate corresponds to hydrolysed proteins, peptides, free amino acids and non-protein nitrogen (Chae et al., 2001). Total nitrogen was determined using the Kjeldahl method (AOAC, 1980) on an Auto Kjeltec system and % protein calculated using the formula total nitrogen x 6.25.

The % DH is an analytically determined ratio of soluble free amino nitrogen (AN) divided by total nitrogen (TN) and yields information on the % degree of protein hydrolysis (percentage of peptide bonds cleaved) and resultant flavour attributes (Alder-Nissen,1996). Measurement of % DH was determined using the formol titration method (U.S. Pharmacopeia Method, 1985).

Total dissolved solids in the extracellular medium was calculated using a refractometer (Atago, Japan). Dry solids (total solids) content was measured by drying a sample overnight in an oven at 105^{o}C.

Reducing sugars concentration was determined using the dinitrosalicyclic acid (DNS) method (Miller,1959).

3.2.3 Microscopy

The morphological characteristics of autolysed SDYK were examined using differential interference contrast microscopy and phase contrast light microscopy as described previously (section 2.2.2). Differential interference contrast microscopy was carried out using a BX51 light microscope (Olympus, Germany) with an attached Progres® CT3 camera (Jenoptik Optical Systems Inc, USA). Analysis was completed using Progres® CapturePro Software Version 2.7.7 (Jenoptik Optical Systems Inc, USA).

3.2.4 Statistical methods

Results were analysed for mean, standard error and statistical significance and were then evaluated using analysis of variance (ANOVA). A value of $p < 0.05$ was considered statistically significant. Experimental design consisted of carrying out yeast autolysis trials (replicates of four) and applying four pH conditions and four autolytic treatments (Table 3.1). Sampling took place at four sampling intervals or at 24h for selected trials. All statistical analysis was performed using the program SPSS for Windows, Version 20, SPSS Inc., Chicago, IL.

3.3 Results and Discussion

3.3.1 The effect of induced autolysis condition on % free alpha amino nitrogen, degree of hydrolysis and total dissolved solids of yeast extract derived from SDYK

3.3.1.1 Free alpha amino nitrogen

The dynamics of accumulation of autolysis products derived from the hydrolysis of SDYK's intracellular protein was monitored by the measurement of the hydrolysis products, free amino nitrogen content and total dissolved solids released in the autolysate (Tanguler and Erten, 2007). Figure 3.1 illustrates the free amino nitrogen released during autolysis of SDYK at various initial pH values in the presence and absence of salt and enzyme papain. No inductors were added to the control. Both pH and autolytic treatment applied were found to have a statistically significant on release of free amino nitrogen ($p < 0.001$) and these results are summarised in Table 3.2. Multiple comparisons of the means indicated that pH 6.5 yielded the highest free amino nitrogen content and was found to be significantly different to pH 4.5 and pH 5.5 ($p < 0.05$) indicating optimal enzyme activity in the pH range of 6.5-7.0.

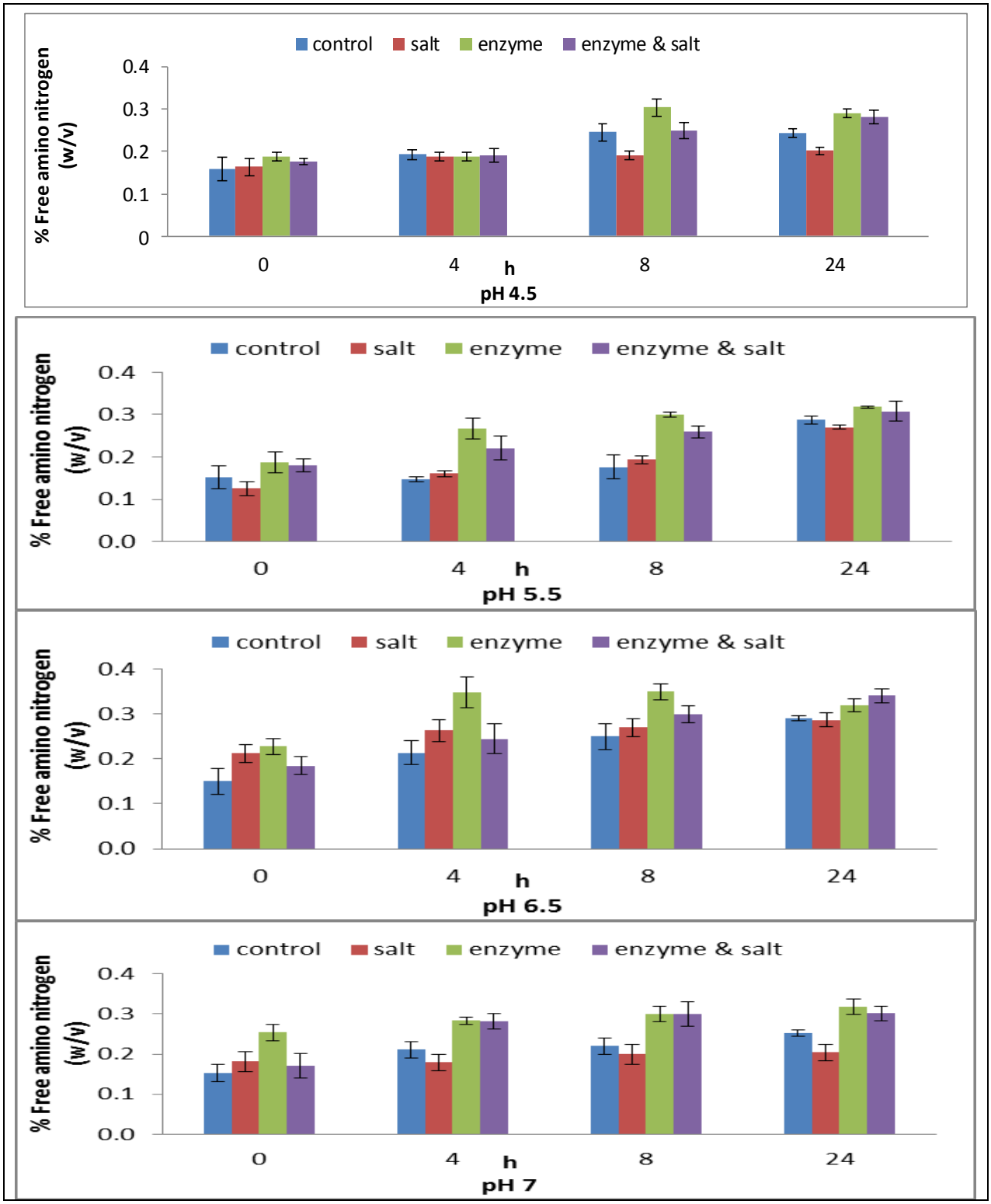


Figure 3.1 Free amino nitrogen released during autolysis of SDYK at various initial pH values, in the presence and absence of salt and enzyme papain. The values presented are the means of four replicate trials.

Table 3.2 Variance analysis of free amino nitrogen content of yeast extracts of SDYK.

Dependent Variable: % free amino nitrogen

Source of variation	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	1343.429	1	1343.429	5007.305	P < 0.001
Treatments	24.603	3	8.201	30.568	P < 0.001
pH	7.825	3	2.608	9.723	P < 0.001
Treatments * pH	7.328	9	.814	3.035	P < 0.05
Residual	64.390	240	.268		
Total	1447.576	256			

3.3.1.1a Enzyme papain addition

Multiple comparisons of the means also showed the effect of enzyme papain addition to be statistically significant with respect to improving the release of free amino nitrogen ($p < 0.05$).

Several studies (Chao et al., 1980; Conway et al., 2001) have shown that use of the protease papain is an effective method to aid in brewers yeast autolysis and in solubilisation of yeast biomass when compared with other enzymes. These enzymes included proteases such as rennin, pepsin, trypsin and pancreatin.

While the addition of exogenous enzymes such as papain resulted in high free amino nitrogen values, the exact mode of action of this enzyme during induced autolysis is not fully understood. It was observed that in the presence of papain during autolysis of SDYK, the cell pellet (yeast cell wall) gradually reduced as autolysis proceeded. This would indicate that the added papain in addition to hydrolysing cytoplasmic protein was also digesting the yeast cell wall and membrane, releasing other attached endogenous enzymes and cell wall components (Ryan and Ward, 1988).

One of the reasons why papain has proved to be most effective during autolysis is that this enzyme has a relatively small molecular weight of 23,400 Dalton and therefore can pass through the yeast cell wall and membrane resulting in hydrolysis of intracellular components including protease inhibitors. This ultimately leads to an acceleration of the autolysis process because of cellular structure disorganization and an increase in the availability of substrate for endogenous enzymes (Chao, 1980).

Proteolytic system of K. marxianus

Although the proteolytic system of *K. marxianus* is not as well studied as that of *S. cerevisiae*, there have been reports on the characterization of serine carboxypeptidase (CPY) and lysine aminopeptidase (APE) from this yeast (Ramirez-Zavala et al., 2004a,b). As shown in Table 3.3 both enzymes were reported to be stable under neutral or slightly alkaline pH conditions with the serine carboxypeptidase abruptly inactivated under acid pH conditions. It was therefore possible that endogenase peptidases of SDYK were working synergistically with the added enzyme papain in promoting the release of higher amounts of free amino nitrogen at pH 6.5 and the autolysis temperature of 50°C. It has been suggested that endogenous proteases originally present in an inactive form because of association with protease-inhibitor complexes, can be activated by environmental conditions which caused destruction of protease inhibitors and activation of protease activity (Halasz and Lasztity 1991b).

The use of exogenous enzymes, particularly plant proteases such as papain were also shown to work in a synergistic fashion with depleted yeast endogenous enzymes during autolysis of brewers yeast (Milic et al., 2007). The optimal pH of the proteolytic enzyme papain, which was added in order to accelerate proteolysis, is also in the neutral pH region while the enzyme has been shown to be very thermostable.

Table 3.3 Proteolytic system of *K. marxianus*

Enzyme	**Type / Features**	**Molecular weight**	**Substrate**	**Optimum pH & Temp.**	**Inhibitors**	**Organism & Reference**
Serine Carboxypeptidase (CPY)	Serine exopeptidase	67 kDa (by gel filtration)	N-benzoyl-L-tyrosine-p-nitro-anilide (NBTpNA)	pH 9.0 Stable in pH range 6.0-9.0, abruptly inactivated under acid pH conditions 45 °C & unstable at > 55 °C	strongly inhibited by PMSL & to lesser degree by *trans* epoxysuccinyl -L-leucylamido-4-guanidine-butane	*K. marxianus* (Ramírez-Zavala et al., 2004b)
Lysine amino-peptidase (APE)	Lysine exopeptidase	46 kDa (by gel filtration)	L-Lysine–p-nitroanilide	pH 7.0 Stable in pH range 4–8 45 °C & unstable at > 55 °C	strongly inhibited by bestatin & *o*-phen-anthroline& to lesser degree by EDTA	*K. marxianus* (Ramírez-Zavala et al., 2004a)

For the yeasts, *S. cerevisiae* and *K. marxianus*, the temperature applied for promoting autolysis has frequently been reported to be 50ºC (Amrane and Prigent, 1996; de Palma Revillion et al., 2003; Tanguler and Erten, 2007).

3.3.1.1b Sodium chloride addition

Further analysis of the results illustrated in Figure 3.1 indicated that after 24 h induced autolysis of SDYK the addition of salt alone did not result in an increase in free amino nitrogen with respect to the control. From these results it emerged that the primary role of sodium chloride salt was not in the release of free amino nitrogen by a direct

acceleration of endogenous proteinases. However, the beneficial effects of salt in reducing susceptibility to microbial contamination and as a flavouring agent cannot be underestimated (Reed and Nagodawithna, 1991). The use of sodium chloride as a plasmolyser is well documented (Liu et al., 2006; Moresi et al., 1995)

Sugimoto, (1974), did however show that although NaCl can inhibit most yeast proteinases, it can in the presence of certain activators or under the correct environmental conditions, activate proteinases at 5% (w/v) salt concentration. Proteolytic enzymes of yeasts have been characterised and referred to as proteinase A, B, C and D. In the presence of NaCl, yeast proteinase B was reported to form a strong enzyme inhibitor complex which resulted in yeast proteinase A not being activated and a reduction in total proteolytic activity (Lenney, 1975, Matern et al., 1974). At 50 °C, carboxypeptidase C which is known to form a weak enzyme inhibitor complex was considered responsible for overall proteolytic activity.

3.3.1.1c The effect of combining autolytic inductors on free amino nitrogen

For the benefit of the reader, and to clarify further the effect of autolytic inductors, Figure 3.2 illustrates the time course of the % free amino nitrogen released during the autolysis of SDYK at 50°C, using a combination of papain and salt at various initial pH values. After 24 h, the effect of pH in the presence of these inductors on free amino nitrogen released was shown to be significant ($p < 0.05$). As shown previously, this can be attributed to the enzyme papain**.** The release of free amino nitrogen during induced autolysis of SDYK (Figure 3.2) indicated that there was no abrupt release of free α-amino nitrogen, but rather a continuous release of this material was evident. The net increase in concentration of free amino nitrogen released for the control (no additives or pH adjustment) was similar to the 1.0 $g.L^{-1}$ obtained by Amrane and

Prigent (1996) where no inductor were used using primary grown as opposed to spent dairy yeast . However, the higher initial concentration of 0.15% - 0.2% (w/v) free α-amino nitrogen that was obtained during autolysis of SDYK can be attributed to the initial higher biomass utilized for autolysis and the variable age of SDYK and associated endogenous enzyme activity. Amino nitrogen from residual whey based material may also have been a contributory factor (Moresi et al., 1995).

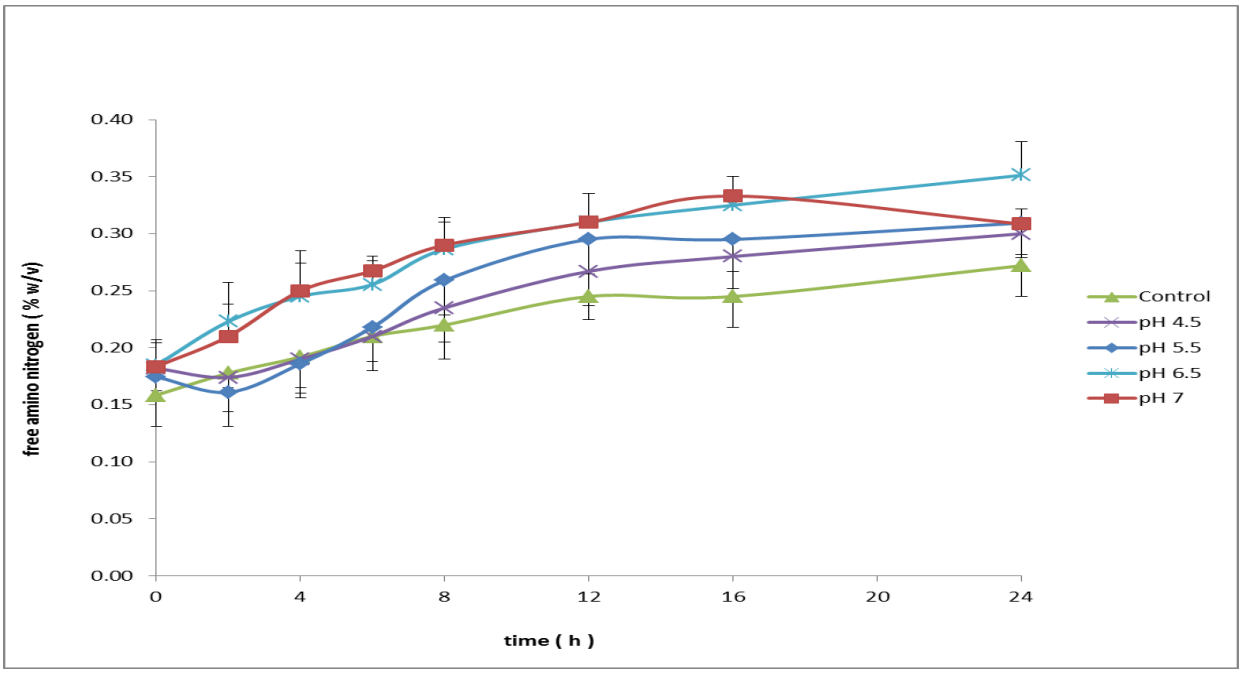


Figure 3.2 Time course of the % free amino nitrogen released into extracellular medium during the autolysis of SDYK at 50°C, at different initial pH values in the presence of salt and papain. The values presented are the means of four replicate trials.

3.3.1.2 Degree of hydrolysis (% DH) during autolysis of SDYK

% DH, a derived value of amino nitrogen and expressed as amino nitrogen / total nitrogen (AN/TN), was used to monitor protein hydrolysis as it is known to give a good estimate of the % of protein bonds enzymatically hydrolysed and has also been shown to be related to final flavor of yeast extracts **(**Godfrey, 1996**).** Figure 3.3 shows the % DH obtained in autolysates of SDYK after 24 h of induced autolysis under different conditions.

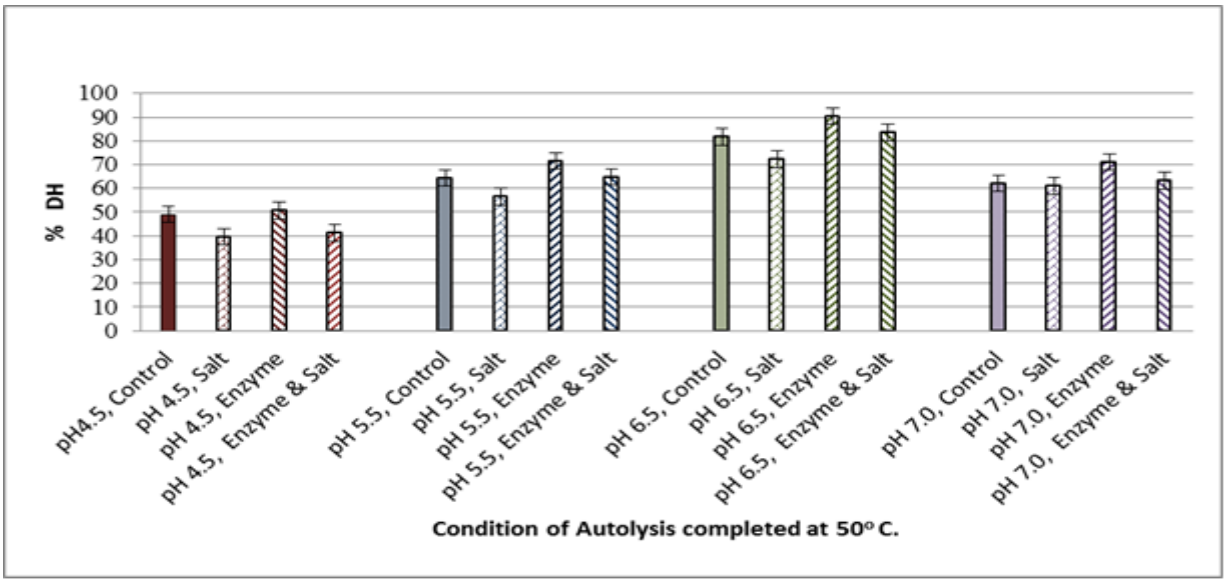


Figure 3.3 Overview of % degree of hydrolysis (% DH) of in extracellular medium after 24 h of induced autolysis of SDKY at 50°C, at different initial pH values in the presence and absence of salt and enzyme papain. The values presented are the means of three replicate trials.

When examining the % DH of all autolysis treatments (Figure 3.3), the % DH at pH 6.5 was significantly higher ($p < 0.05$) than the other autolytic treatments. It has been generally been accepted that when an exoprotease is used in conjunction with an endoprotease such as papain, a more acceptable taste and higher % DH result because it produces small non-bitter peptides (Chae et al., 2001). The characterization of

serine carboxypeptidase (CPY) and lysine aminopeptidase (APE) as outlined previously in Table 3.3 would concur with the lower values of % DH observed in this study at pH 4.5, which is outside both enzyme's optimal range. This would explain the high % DH obtained in autolysates of SDYK at pH 6.5 and the lower % DH values as conditions deviated from the optimal pH of the enzymes involved.

Although APE is reported to be stable at pH 4.0-8.0., it is quite possible that the temperature of 50°C used for autolysis of SDYK resulted in a significant loss of activity as this enzyme is quite thermolabile with 80% of activity reported to be lost after 30 min at 55°C (Ramirez-Zavala et al., 2004 a,b).

Most intact proteins will have extremely low AN/TN values typically below values of 8% DH (Godfrey, 1996). The increase in this ratio value is therefore an effective measurement of protein hydrolysis and today protein hydrolysates may be classified and described by reference to this ratio with values for multi-enzyme hydrolysis being quoted in the 60-70% range.

3.3.1.2a. Natural autolysis of SDYK

Natural autolysis of SDYK was responsible for the relatively high % DH prior to commencing induced autolysis trials. Measurement of the initial % DH for 10% (w/v) of SDYK suspensions were calculated to be 42.7% ± 0.9 when compared to intact proteins (7% ± 0.2) previously studied

However, too high a % DH poses its own problems. Undesirable bitter flavours can be produced when the % DH rises above levels required for satisfactory solubilization. It has been reported that amongst various methods, correct control of enzyme hydrolysis is an effective method for obtaining the desired % DH (Adler-Nissen, 1974; Wang et

al., 2010). Endoproteases used in conjunction with exopeptidases can remove the bitterness of high-DH hydrolysates or autolysates by degrading the bitter tasting peptide groups of proteins, while still obtaining a degree of hydrolysis of greater than 50% (Godfrey, 1996).

Although all yeast samples were adjusted to 10% (w/v) total solids prior to autolysis, there were differences ($p < 0.05$) in the concentration of free amino nitrogen studied and consequently % DH, at the initial time of autolysis. This was due to the nature of spent yeast, which can vary in composition according to age, method of cultivation and the growth phase at which the cells were separated from the primary fermentation. Manufacturing facilities also store spent yeast for varying lengths of time which results in variation in the quality of the raw material received from alcohol producers which can be attributed mainly to the onset of the natural yeast autolysis process.

3.3.1.3 The effect of induced autolysis parameters on total dissolved solids (TDS) content of yeast extract derived from SDYK

As measurement of TDS indicates the concentration of soluble products released into the extracellular medium during autolysis, this parameter was also monitored during the autolysis process using autolytic inductors salt and enzyme on their own and as a combination of both (Figure 3.4). The additional solids as a result of adding 5% (w/v) NaCl in various treatments, was excluded in the computation of the TDS results to give a better indication of the actual % dissolved solids released over time.

Analysis of variance indicated that autolytic treatments had a significant effect on release of TDS after 24h of induced autolysis ($p \leq 0.05$) while unlike free amino nitrogen release, the effect of pH was shown not to be significant ($p > 0.05$). The positive effect of salt in releasing dissolved solids was enhanced when used in

conjunction with the enzyme papain. Addition of salt and papain resulted in an increase of 22%, 11% and 6% total dissolved solids over respective controls when added at pH 6.5, pH 7 and pH 5.5, respectively.

Salt functions as a plasmolyser by facilitating the separation of the cytoplasm from the yeast cell wall and causing the yeast to lose water in an attempt to equilibrate its osmotic pressure with that of the surrounding medium. This results in a loss of cell turgor pressure and a rapid decrease in cytoplasmic water content and cell volume. (Reed et al., 1991; Walker, 1998b) with resultant leakage of intracellular material that can then be hydrolysed by both endogenous and added enzymes such as papain (Azarkan et al., 2003). From a commercial point of view, the fact that the yield of SDYK yeast extracts (increase in release of TDS into the extracellular matrix) was increased by the addition of salt and papain was noteworthy. Other advantages included viscosity reduction and improved clarification of the yeast extract (Sommer, 1998). With respect to induced autolysis of primary grown *K. marxianus* on whey, Moresi et al., (1995), undertook a study to investigate the effects of sodium chloride addition, temperature and cell disruption on the overall performance of the process concerned. Both whole and mechanically disrupted yeast cream was submitted to autolysis in the presence or absence of sodium chloride at the optimum concentration of 5% (w/v) sodium chloride. The highest release of soluble material (72%) was achieved when the yeast cream was preliminarily ground in the presence of NaCl. However, the autolysis of disrupted cells in pure water yielded just slightly lower results.

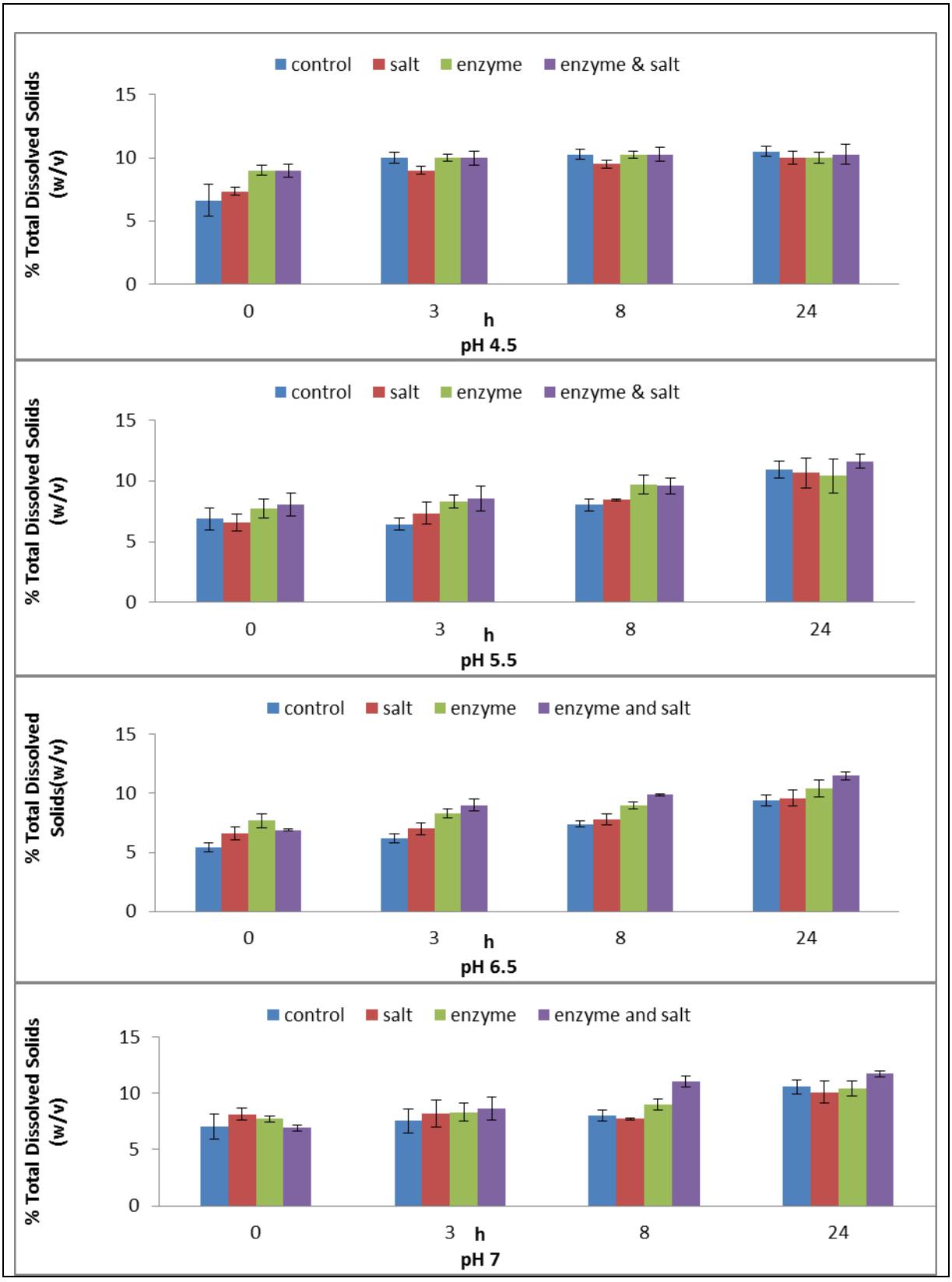


Figure 3.4 Total dissolved solids (%w/v) in extracellular medium during the induced autolysis of SDYK in the presence and absence of salt and enzyme papain, at 50°C and at different initial pH values. The values presented are the means of four replicate trials.

Moresi, 1994, concluded that for maximum protein extraction from *K. marxianus*, a preliminary mechanical breaking treatment followed by addition of NaCl would be necessary, but as the net increase when adding NaCl was not significant, a cost/benefit analysis was merited.

3.3.1.3a The effect of combining autolytic inductors on total dissolved solids

For the benefit of the reader and to further observe the effects of using a combination of salt and papain during autolysis, Figure 3.5 illustrates a time course of the % total dissolved solids released during the induced autolysis of SDKY at 50°C and various initial pH values. From this graph it is clear that most of the dissolved solids were released during the first 10h of autolysis.

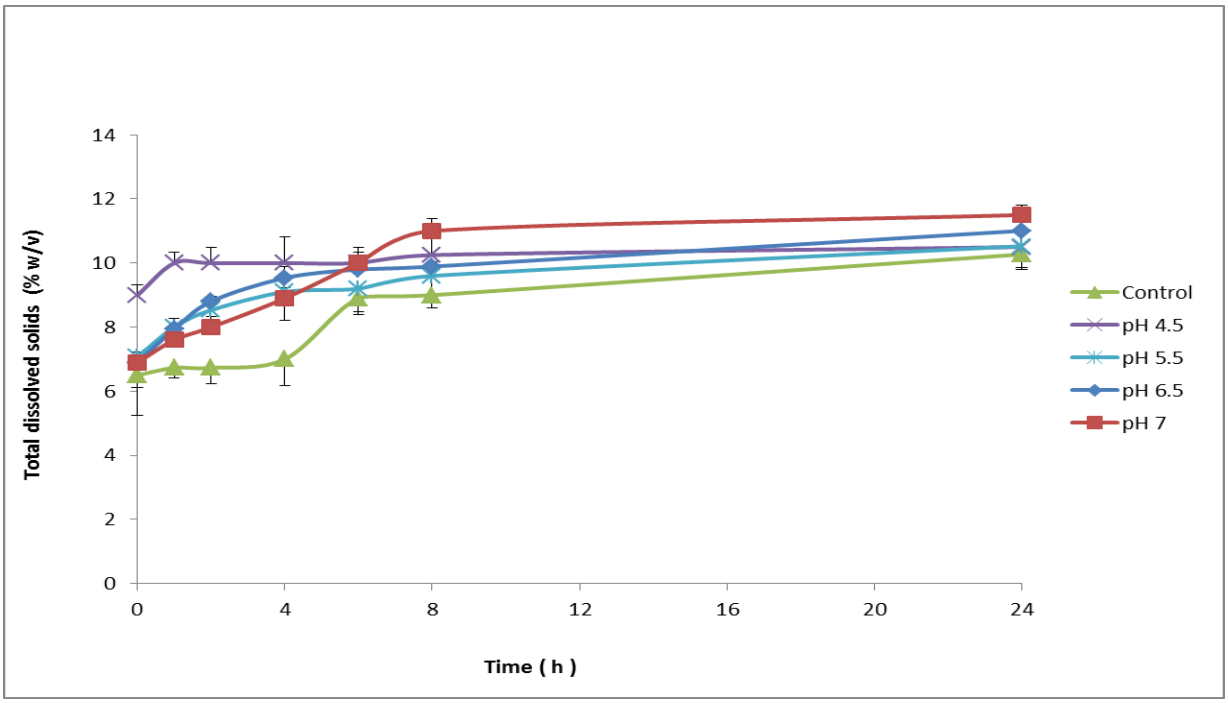


Figure 3.5 Time course of dissolved solids released into extracellular medium during the induced autolysis of SDYK at 50°C and at different initial pH values using a combination of salt and papain. The values presented are the means of three replicate trials.

While this study has shown that free amino nitrogen was released into the extracellular medium during induced autolysis of SDYK, it was also quite likely that other solubles were also being released. Yeasts are also known to produce β-glucanases, a cell wall bound endo-β-glucanase (Bath, 2000, Mrsa et al., Ryan and Ward, 1988) and extracellular exo-β-1,3-glucanases (Satyanarayana et al., 2009) which are localized in the periplasmic space. These enzymes have been reported to be released into the extracellular medium during autolysis (Hough and Maddox, 1970). Improved yields of total released solids has been reported when using viable yeast with active endogenous enzymes and the addition of the enzyme papain (Conway et al., 2001).

Reducing sugars which are products of β-glucanases were therefore measured in autolysates of SDYK at pH 5.5 and 6.5, pH values at which endogenous β-glucanases are active (Figure 3.6). It was found that reducing sugars increased from 0.15% (w/v) to 0.6% (w/v) during autolysis when using salt and papain at pH 6.5 and was significantly higher than the control ($p < 0.05$). As the control with no additives also released reducing sugars, endogenous enzymes capable of hydrolysing polysaccharides were active in SDYK during autolysis. The presence of additional reaction products such as reducing sugars is of relevance to flavoured yeast extracts as reducing sugars are known to interact with amino acids of protein in the flavour forming Maillard reaction.

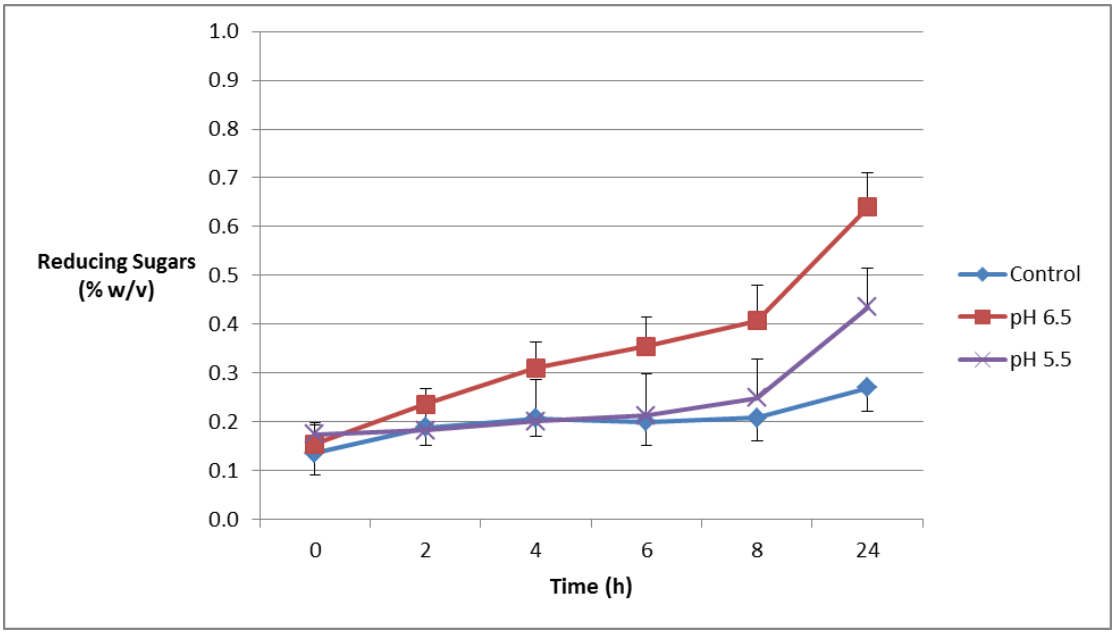


Figure 3.6 Time course of reducing sugars released into extracellular medium during the induced autolysis of SDYK at 50°C and at different initial pH values using a combination of salt and papain. The values presented are the means of three replicate trials.

3.3.2 Microscopy studies

Differential interference contrast microscopy established that the cell wall of SDYK retained its integrity during autolysis. Presumably, the core β-1-3 glucans responsible for wall rigidity were not degraded. However by observing micrographs before and after induced autolysis,it was observed that the cell walls of SDYK were elongated after induced autolysis indicating that salt and papain were assisting in alteration of cell wall structure (Figures 3.7 - 3.8). While autolytic loss of mannoprotein would not affect cell wall integrity, it would be expected to alter cell wall porosity which increases in order to allow external passage of intracellular macromolecules (De Nobel and Barnett 1991).

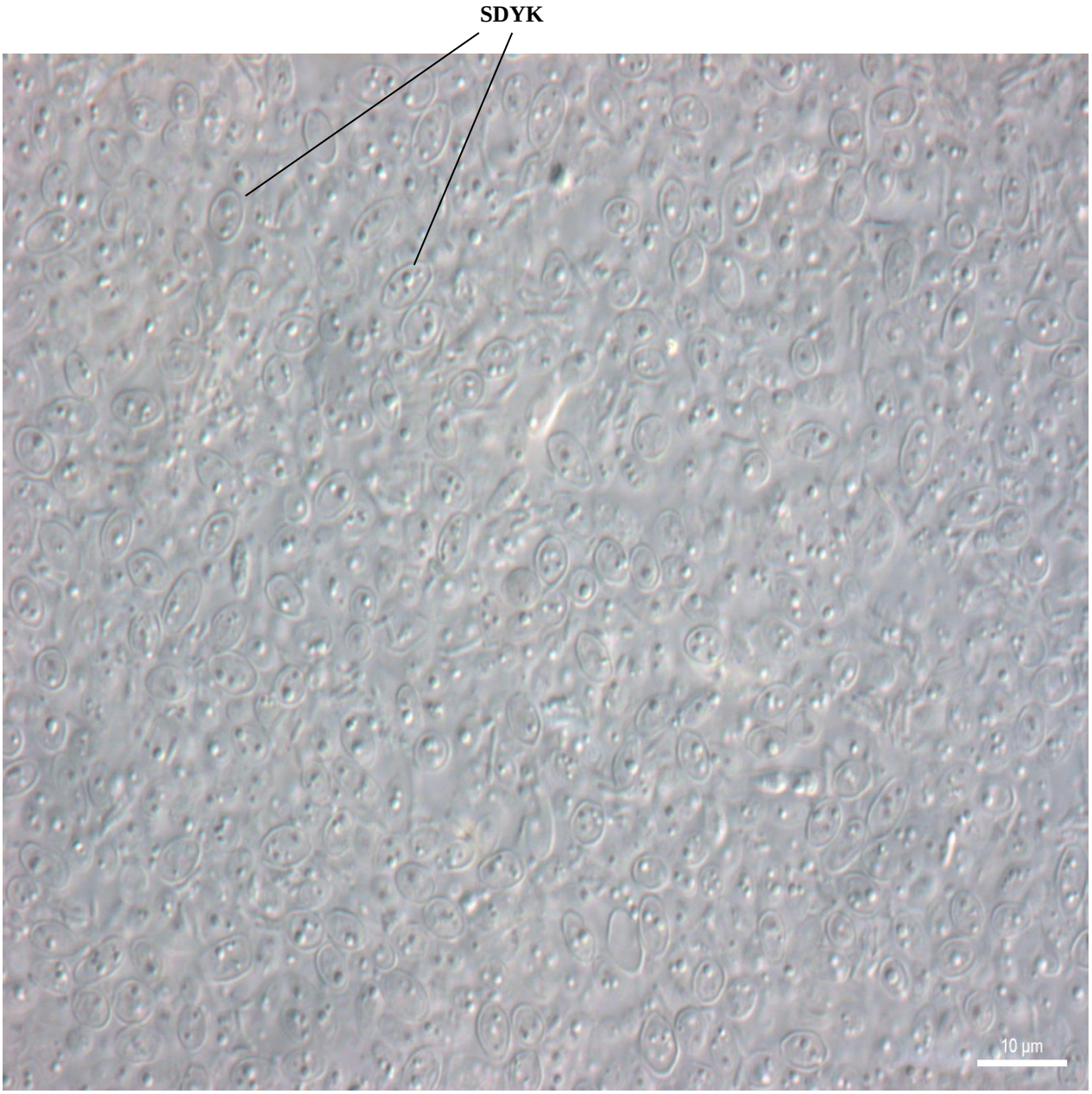


Figure 3.7 Differential interference contrast microscopy of spent yeast SDYK with no additives (control).

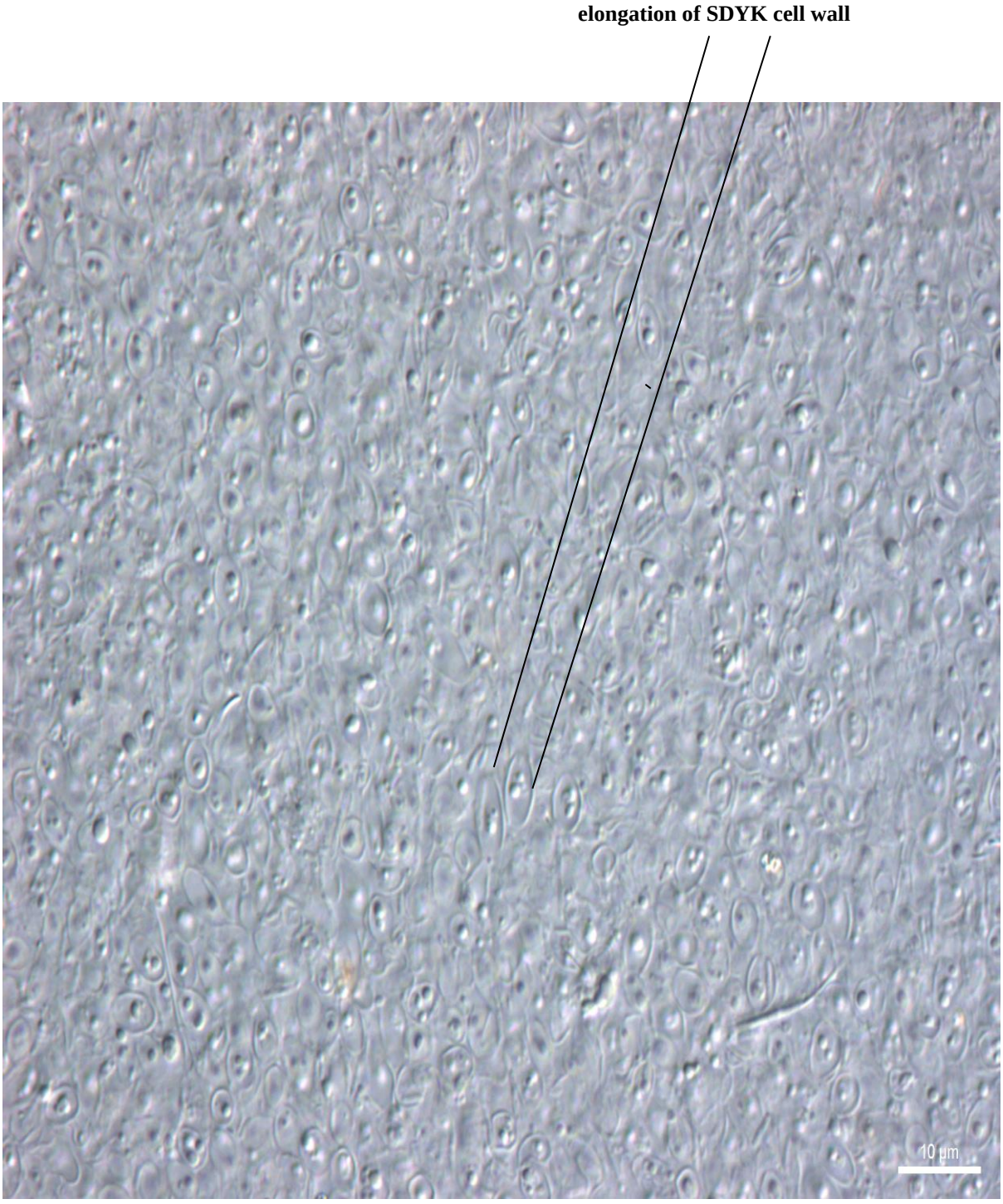


Figure 3.8 Differential interference contrast microscopy of SDYK with additives salt and papain.

3.3.3 Induced autolysis of SDYK with yeast cell wall as a by-product

After induced autolysis of yeast, the autolysate is normally centrifuged to separate the yeast cell wall from the yeast extract. As yeast cell wall is considered in many cases to be a waste product of yeast extract production, a modified enzymatic extraction method to that of Sedmark, (2006) was completed on SDYK in order to isolate yeast cell wall polysaccharides. A flow diagram of this proces is illustrated in Figure 3.9. Autolysis of SDYK was carried out at pH 5.5 using the inductors salt and papain as previously described in section 3.2.1. Cell wall material (CWM) which typically had a dry solids content of 25% w/v ± 1.4 was diluted to 10% w/v and pH adjusted to pH 8.0. As insoluble β-glucan is the main yeast cell wall polysaccharide (Manners et al., 1973), it was enzymatically extracted under alkaline conditions using an alkaline protease (section 3.2.1.1)

Similar to Thammakiti et al., (2004) who isolated β-glucan from spent brewers yeast, the polysaccharides β-glucan and α-mannan were found to be the main components of CWM of spent dairy yeast *K. marxianus* (refer to Table 2.3). Using the process as outlined in Figure 3.9, the β-glucan concentration in the isolated SDYK preparation increased to 55.0% (w/w) ± 0.3. β-glucan is known to improve the functional properties of food which includes its ability to function as a noncaloric food thickener, emulsifier, and fat replacer as well as being an excellent dietary fibre (Thammakiti et al., 2004). Induced autolysis at pH 5.5 and pH 6.5 has been reported to be an effective initial isolation method of a glucan-mannan preparation (yeast cell wall) by Liu et al., (2006) and Sedmak (2006) using spent brewers yeast.

The composition and functionality of cell wall β-glucan enzymatically extracted from SDYK has also been reported to be affected by conditions of induced autolysis which led to improved oil-binding and water-binding capacities (Mc Carra and Luz Prieto, 2008)). On evaluation of primary grown *K. marxianus* as a source of a natural bioemulsifier, Lukondeh, (2003b) observed that the emulsion phase decreased when NaCl was added, an indication that the addition of this inductor during autolysis may result in a lowering of the emulsion stabilizing capacity. Improved functionality of cell wall preparations from SDYK could also be attributed to the fact that induced autolysis conditions followed by enzymatic treatment of yeast cell wall does not destroy the natural properties of polysaccharides when compared to the harsher alkali and acid methods of extraction (Thammakiti et al., 2004). This would agree with studies by Jamas et al., (1989) who observed that yeast cell wall material isolated enzymatically contained small amounts of chitin cross-linked to β-glucans and proteins. Sedmak (2006) has also reported that enzymatic treatment of spent brewers yeast cell wall did not achieve complete separation of mannan and β-glucan. The concentration of β-glucan found in enzymatically extracted preparations of SDYK also indicated that complete separation of mannan and β-glucan had not occured and that further process optimization was required.

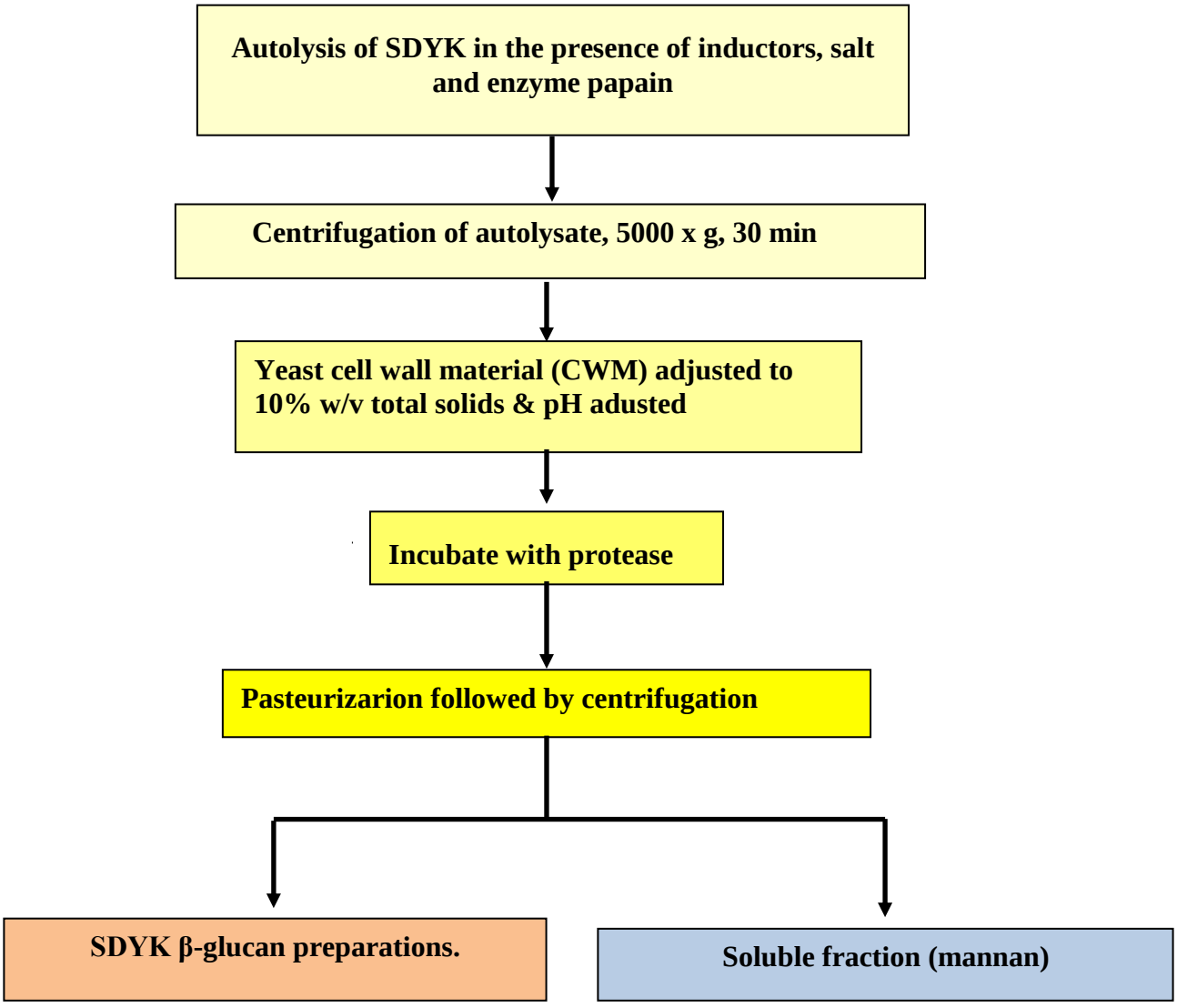


Figure 3.9 Flow diagram of a natural enzymatic process for isolation of yeast cell wall polysaccharides from SDYK.

3.4 Conclusion

The effects of the autolysis inductors pH, salt and the proteolytic enzyme papain on the composition of yeast extracts derived from SDYK was determined by measurement of the hydrolysis products, free amino nitrogen content and total dissolved solids released in the autolysate. Both pH and autolytic treatment applied were found to have a statistically significant effect on release of free amino nitrogen ($p < 0.001$). Autolysis at pH 6.5 yielded the highest free amino nitrogen content and was found to be significantly different to pH 4.5 and pH 5.5 ($p < 0.05$) which indicated optimal enzyme activity in the range pH 6.5 – pH 7.0. The effect of enzyme papain addition was also found to be statistically significant with respect to improving the release of free amino nitrogen ($p < 0.05$). Yeast extracts with a high degree of protein hydrolysis are desirable in extracts destined as food ingredients and the optimal pH of induced autolysis was found to be pH 6.5. Analysis of variance also indicated that autolytic treatments had a significant effect on release of TDS after 24h of induced autolysis ($p \leq 0.05$) while unlike free amino nitrogen release, the effect of pH was shown not to be significant ($p > 0.05$). This highlighted the important role of inductors such as salt in releasing soluble solids and improving yeast extract yield. These conditions also had the advantage of a short autolysis time and the prevention of bacterial contamination. An induced autolysis procedure was therefore developed for production of dairy yeast extract using SDYK. Cell wall material, a by-product of this process, was also shown to be a good source of β-glucan and mannan, polysaccharides with the potential to be used more widely as functional food ingredients.

Chapter 4

Effect of induced autolysis on the concentration of natural flavour enhancers in yeast extracts produced from spent dairy yeast *Kluyveromyces marxianus*

4.1 Introduction

It is now known that free amino acids and RNA derived nucleotides in yeast extracts can act synergistically to improve and enhance flavour (Nandakumar et al., 2003). Yeasts such as *S. cerevisiae* are the preferred source of flavour enhancing ribonucleotides and amino acids due to their high nucleic acid and protein content respectively (Andreu et al., 1988; Charpentier et al., 2005; Zhao and Fleet, 2005). The composition of free amino acids in yeast extracts can also directly or indirectly exert a major influence on food flavour. The Maillard reaction between amino acids and reducing sugars leads to aroma (Varavinet et al., 2000) while the products of these reactions have distinctive colouring properties and cover a wide spectrum of flavour intensive compounds (Nagodawithana, 1992; Sommer, 1998).

To date, no detailed data is available on the effect of autolysis inductors on the flavour enhancer levels of yeast extracts derived from spent dairy yeast SDYK. The objective of this study was to investigate if controlled autolysis of SDYK in cooperation with established inductors, could influence development of alternative flavoured yeast extracts.

4.2 Materials and Methods

4.2.1 Induced autolysis conditions using SDYK

Autolysis trials with inductors were carried out at Carbery Group R&D laboratories in order to observe any scale-up effects and to provide larger sample volumes (0.5-0.75L) for a detailed sensory analysis of yeast extracts.

The conditions used for of autolysis of SDYK are described in Table 4.1. One litre stainless steel beakers with automated stirring (Heidolph) in a temperature controlled

water-bath, were used as the reaction vessels for yeast autolysis. pH adjustments and additions were performed as described in section 3.2.1. An additional treatment was included (T5) to investigate the effect on resultant flavour of increasing initial yeast solids at pH 6.5. The pH of 6.5 was selected on the basis that this pH provided a good degree of hydrolysis using the standard 10% yeast solids for autolysis trials, while there is some evidence to suggest that increasing the yeast solids has a desirable impact on flavour (Spellman et al., 2005).

Table 4.1 Induced yeast autolysis conditions using SDYK at 50 ºC and 10% (w/v) yeast solids, with varying initial pH values.

	Autolysis Conditions- Initial pH and additives used @ 10 % yeast solids
T 1	Control – no pH adjustment, no additives; pH 4.5
T 2	pH 4.5, salt and papain enzyme[a]
T 3	pH 5.5, salt and papain enzyme[a]
T 4	pH 6.5, salt and papain enzyme[a]
T 5	pH 6.5, salt and papain enzyme[b]
T 6	pH 7 salt and papain enzyme[a]

[a]5% (w/v) NaCl and 0.2 % (w/v) papain enzyme added.

[b]5% (w/v) NaCl and 0.2 % (w/v) papain enzyme added, with yeast solids increased to 17.5% w/v.

After induced autolysis of SDYK, the yeast autolysates were centrifuged at 5000 x g for 30 min in order to remove yeast cell walls and produce SDYK yeast extracts, T1 to T6

4.2.2 Measurement of free amino acids in yeast extracts of SDYK

0.75 ml aliquots of the SDYK yeast extracts, T1 to T6 were deproteinised by mixing equal volumes of 24% (w/v) tri-chloroacetic acid (TCA) and sample. These were allowed to stand for 10 min before centrifuging at 14400 x g (Microcentaur, MSE, UK) for 10 min. Supernatants were removed and diluted with 0.2 M sodium citrate buffer, pH 2.2 to give approximately 250 nmol of each amino acid residue. Samples were then diluted with the internal standard, norleucine, to give a final concentration of 125 nm/ml. Amino acids were quantified using a Jeol JLC-500/V amino acid analyser (Jeol (UK) Ltd., Garden city, Herts, UK) fitted with a Jeol Na^+ high performance cation exchange column using known concentrations of amino acid standards (Fenelon et al., 2000). Colourimetric detection of amino acids was achieved by postcolumn ninhydrin derivatisation. Data analysis was carried out using a Minichrom data handling system (VG Data Systems, Altrincham, Cheshire, UK).

4.2.3 Measurement of total amino acids in yeast extracts of SDYK

0.75ml of SDYK yeast extracts T1 to T6 were hydrolysed in 6 M HCl at 110°C for 23 h (Hill, 1965) and the resulting hydrolysates were analysed as per measurement of free amino acids (section 4.2.2).

4.2.4 Measurement of free nucleotides in yeast extracts of SDYK

HPLC analysis of 5'-mononucleotides in SDYK yeast extracts was completed using reversed phase HPLC chromatography on a Shimadzu Prominence Liquid Chromatograph (LC-20AT) with Shimadzu LC Solutions software.

The chromatography was carried out in isocratic mode at a flow rate of 1 ml/min with UV detection at 254 nm. The mobile phase was prepared (Table 4.2) and then degassed in a sonicator bath (Cole Parmer 8893) for 20 min.

Table 4.2 Reversed phase HPLC conditions for the separation of SDYK derived 5'-ribonucleotides in isocratic mode

Column	Waters Atlantis dC_{18}, 4.6 x 150 mm, 5 μm
Mobile phase	1% acetonitrile, 0.063% ammonium formate and 0.05% tetrabutylammonium chloride (adjusted to pH 3.20 using formic acid)
Flow rate	1 ml/min
Injection volume	20 μL
Temperature	Ambient
Detection	UV @ 254 nm

4.2.4.1 Preparation of ribonucleotide standards

The following standards were prepared: 5'- IMP disodium salt, 5'- GMP disodium salt, 5'-AMP disodium salt, 5'-UMP disodium salt and 5'-CMP disodium salt (Sigma Aldrich). A stock solution (200 μg/ml) of each standard was prepared using distilled water which was then used to prepare a concentration range of 50-200 μg/ml. Each standard was injected in triplicate. Standard curves were plotted (Figure 4.1) and the equation of the line was used to calculate the concentration of 5'-ribonucleotides in the samples, correcting the values with the appropriate dilution factor.

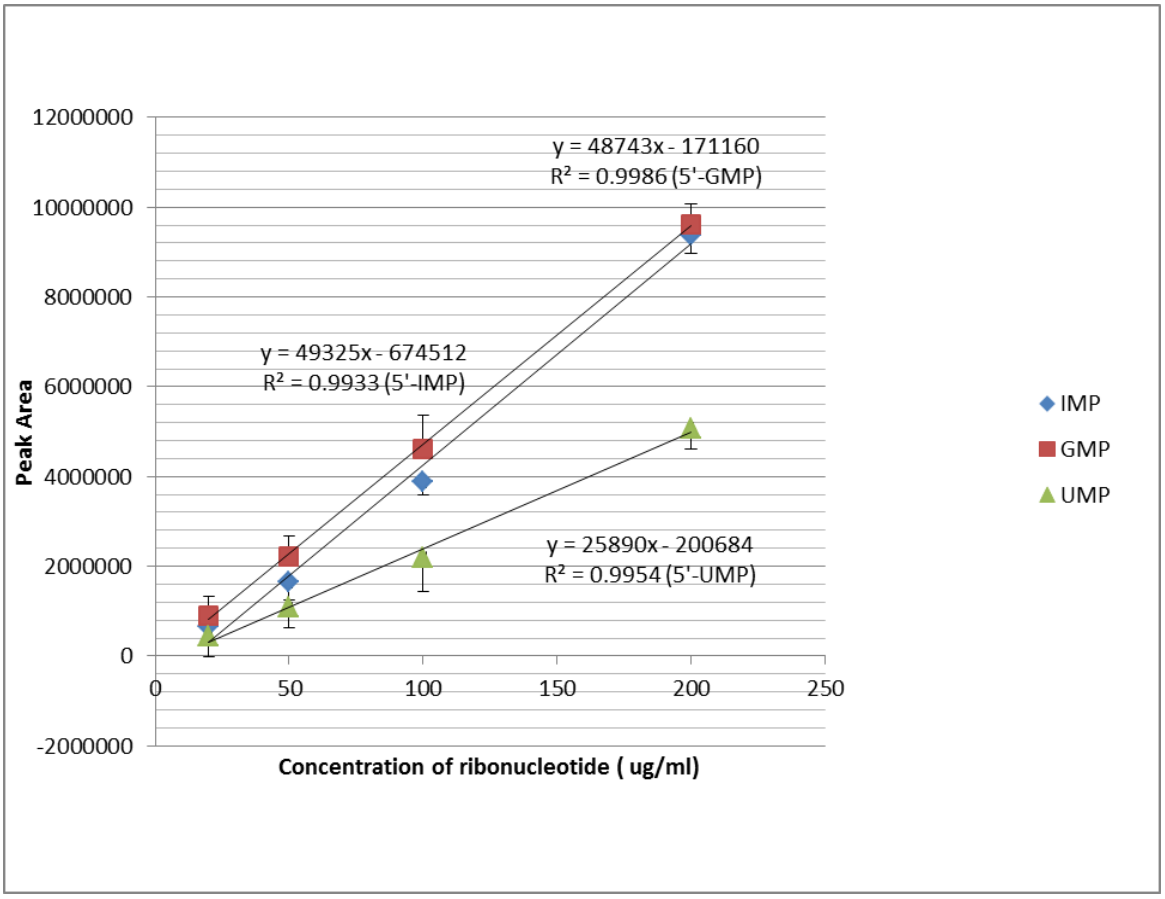


Figure 4.1 Standard curves for measurement of ribonucleotides using HPLC reversed phase chromatography.

4.2.5 Size-exclusion chromatography (SEC)

SDYK yeast extracts were fractionated in isocratic mode using a Waters size exclusion column (Protein-Pak SEC Column 300Å, 10 µm, 7.5mm X 300 mm) which was operated at room temperature. This was completed for the purpose of obtaining molecular weight data and as a means of sample preparation for LC/MS characterisation. The mobile phase consisted of 0.1 M K_2HPO_4, pH 7.0. Molecular weight standards between the range of 1000 Da and 440,000 Da were supplied as dry preparations from Sigma. The chromatography was carried out at a flow rate of 0.5 ml/min with UV detection at 280nm using a Shimadzu Prominence Liquid

Chromatography System (LC 20 AT). The mobile phase was degassed in a sonicator bath (Cole Parmer 8893) for 20 min prior to use.

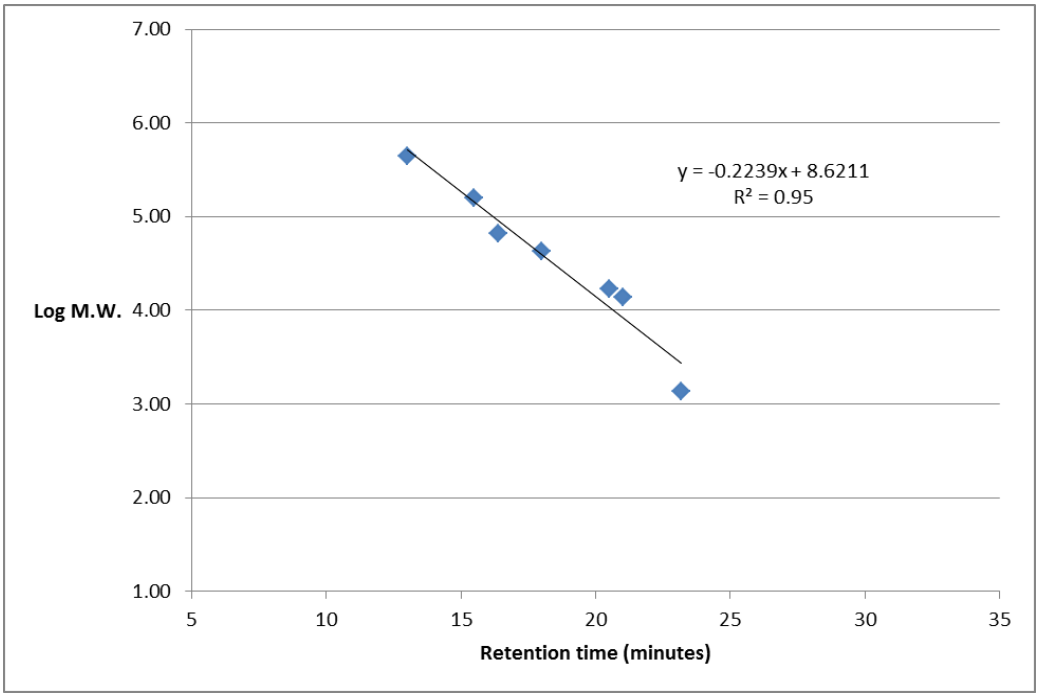


Figure 4.2 Calibration curve showing relationship between molecular weight of protein standards (Da) and retention time on a Waters Protein-Pak HPLC size exclusion column.

4.2.6 Liquid chromatography/mass spectrometry (LC/MS) characterisation of SDYK yeast extract

The yeast extract T4 (non-fractionated) was analysed using online LCMS consisting of a 75µ diameter C18 microcapillary HPLC column linked to a Waters Q-TOF mass-spectrometer equipped with a nanoelectrospray ion source (LC/MS/MS).

An initial desalting trap column was installed to protect the C_{18} reversed phase LC analytical column. After an initial centrifugation to remove cellular debris followed by sterile filtration, the yeast extract was applied to the reversed phase C_{18} LC column whereby the fractionated eluting components were detected on a Q-TOF mass-spectrometer to confirm signal strengths. The Q-TOF mass spectrometer was a hybrid

quadrupole time of flight mass spectrometer with MS/MS capability. The quadrupole was operated as an ion guide in MS mode and as a mass selection device in MS/MS mode. A reflectron time-of-flight (TOF) analyzer served as a mass resolving device for both MS and MS/MS modes. The final detector was a microchannel plate with high sensitivity. The ESI interface consisted of an electrospray probe and a Z-spray source. The instrument was controlled from a PC using MassLynx software.

4.2.6.1 Liquid chromatography/mass spectrometry (LC/MS) characterisation of SDYK yeast extract fractions derived from size exclusion chromatography (SEC)

SDYK yeast extract fractions from size exclusion chromatography were centrifuged at 3000 x g for 15 min followed by filtration through 0.2µm disposable filters to remove any particulate matter present. The SEC fractions of T1-T6 yeast extracts were analysed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS) with the fractions pretreated and desalted using C18 solid-phase-extraction on Millipore ZipTips. Analysis took place on a Waters, MALDI –TOF-MS in positive-ion reflectron mode and using alpha-cyano-4-hydroxycinnamic acid (α-CHC) matrix.

4.2.7 Statistical methods

Results were analysed for mean, standard error and statistical significance with respect to the different autolytic treatments (in triplicate) which were evaluated using one way analysis of variance (ANOVA). The autolytic treatments are summarised in Table 4.1. A value of $p < 0.05$ was considered statistically different. All statistical analysis was performed using the program SPSS for Windows, Version 20, SPSS Inc., Chicago, IL.

4.3 Results and Discussion

4.3.1 Comparison of free amino acid concentration in yeast extracts derived from induced autolysis of SDYK under different autolysis conditions.

Biochemical analyses on compounds that have an impact on flavour were completed on SDYK yeast extracts. Figure 4.3 illustrates the free amino acid (FAA) profile of all the yeast extracts produced in this study by induced autolysis of a suspension of 10% spent yeast solids under different conditions of pH. An evaluation of the free amino acid profile of SDYK yeast extracts using analysis of variance, indicated a statistically significant difference ($p < 0.001$) between the concentration of free amino acids in the extracts. The amino acid alanine was the highest individual amino acid concentration at 0.345% (w/v) – 0.482% (w/v) with the lowest concentrations of free amino acids being proline, tyrosine, cysteine and methionine, ranging from 0.008% (w/v) to 0.087% (w/v**)**. The amino acid alanine has previously been shown in Chapter 2 to be the prevalent amino acid in protein of SDYK. and has also been reported to be characterized by a sweet taste (Nandakumar, 2003) With respect to the induced autolysis treatments, there was no significant difference in the amino acid profile at pH 5.5 and pH 6.5, and similarly between pH 4.5 and pH 7.0.

4.3.1.1 Comparison of free amino acids associated with flavour

The release of certain amino acids, particularly aromatic amino acids, branched chain acidic amino acids and sulphur amino acids are known to coincide with various types of flavour development (Hannon, 2005; Sommer, 1998; West, 1996). In addition to this, both glutamic acid and aspartic acid are well-known amino acids contributing to

the “umami” or savoury taste in food. It is clear from Figure 4.3 that these free amino acids are all present in all the yeast extracts tested but with important concentration differences. In most cases the addition of autolytic inductors resulted in an increase in free amino acid concentration in the yeast extracts produced, and ANOVA followed by multiple comparisons of the means indicated a significant difference between the control yeast extract and all other treatments ($p < 0.05$). This is particularly important with respect to free amino acids that have an impact on flavour. Figure 4.4 graphically represents the % differences in free amino acid concentration for autolysis trials with inductors when compared to the control (no inductors added).

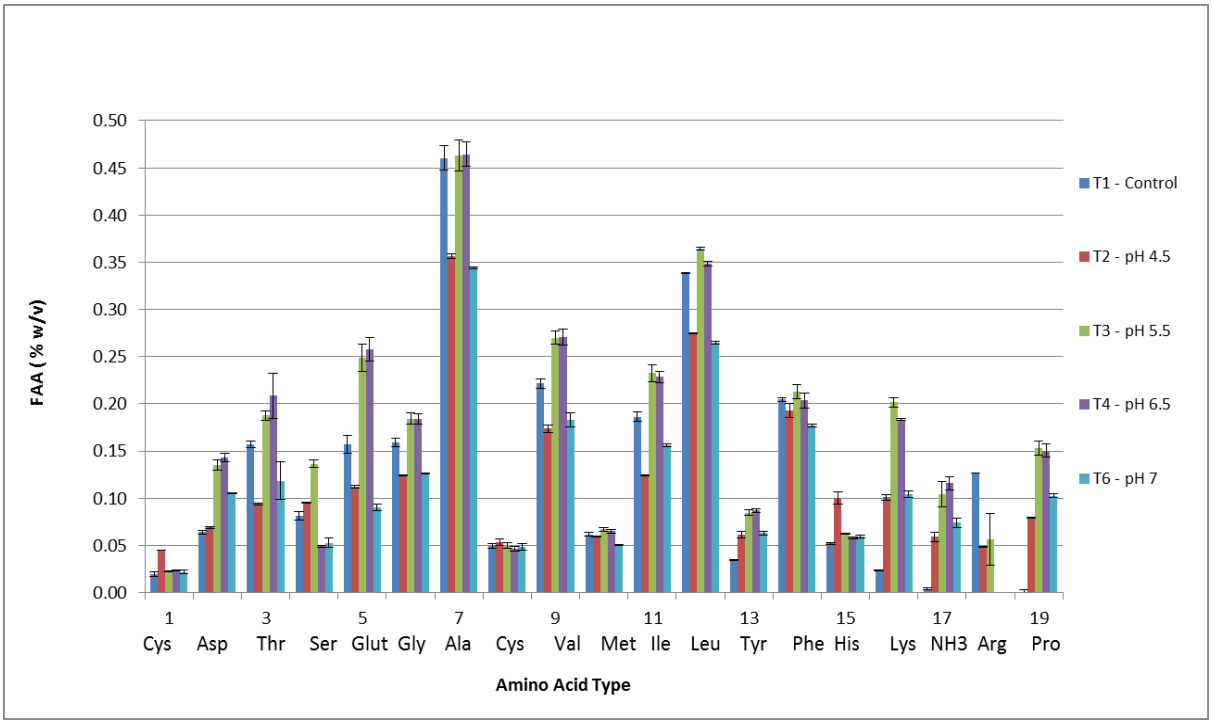


Figure 4.3 Free amino acid profile of SDYK yeast exreacts developed in this study.

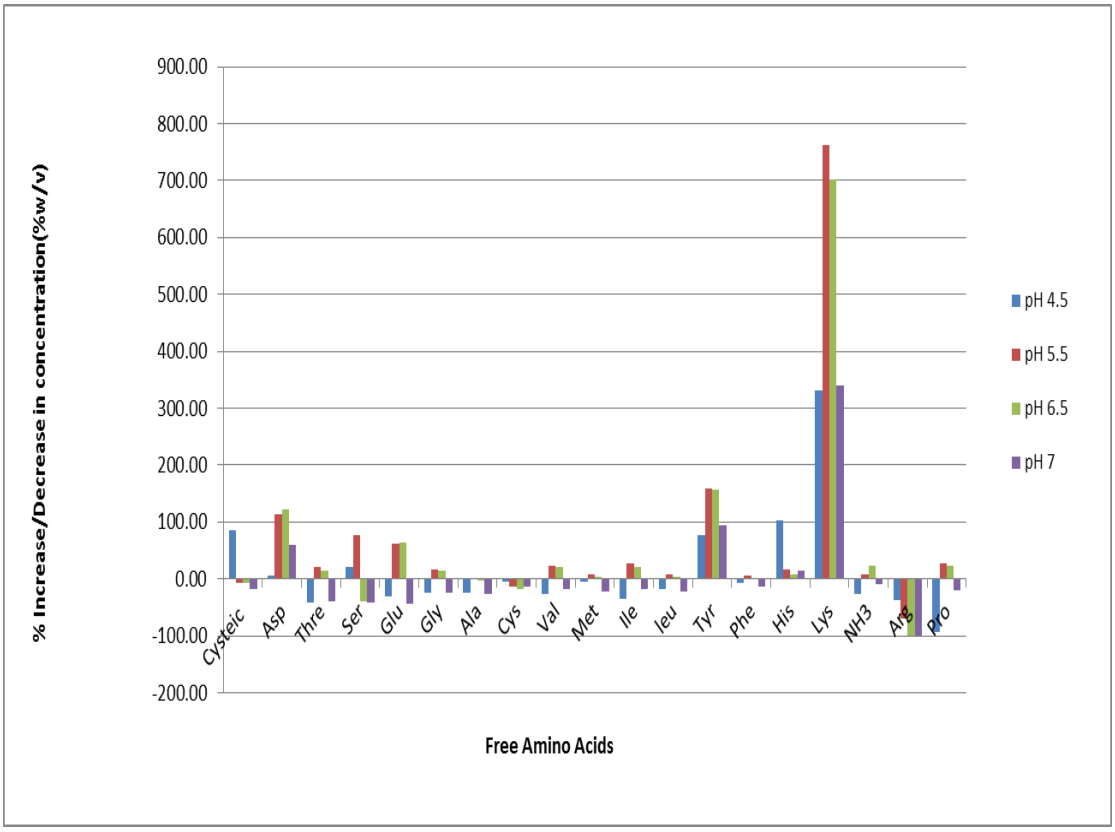


Figure 4.4 Difference in concentration of free amino acids in yeast extracts derived from induced autolysis of SDYK and that of the control yeast extract (no inductors added).

From Figure 4.4, it can be observed that aspartic acid levels released during induced autolysis of SDYK have increased when compared to the control. When inductors were used at pH 5.5 and pH 6.5 the % increase was 114% and 122%, respectively. Similarly, glutamic acid levels increased by 62% and 65%, respectively, under the same conditions but decreased at pH 4.5 and pH 7.0. Both these enzymes are products of enzyme action - aspartic acid is a product of aspariginase-glutaminase catalysed reaction and is converted to alanine, an important amino acid component of soy sauce (Nandakumar, 2003) while the yeast enzyme L-glutaminase, catalyses the hydrolysis of L-glutamine to L-glutamic acid, a very important yeast derived flavour enhancer (Figure 4.5).

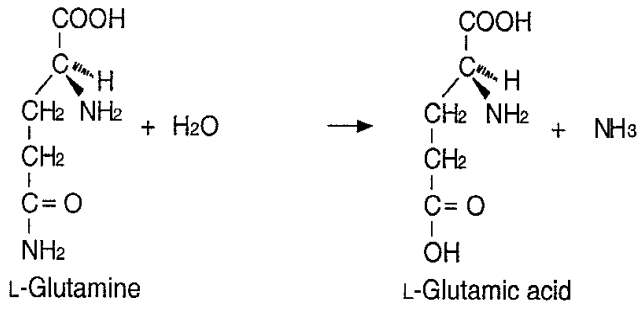


Figure 4.5 The enzymatic hydrolysis of glutamine to the flavour enhancer, glutamic acid, catalysed by glutaminase

The flavour enhancing properties of glutamic acid can only be achieved within a pH range of 5.0 – 8.0, as only dissociated glutamate has any flavour enhancing properties (Sommer, 1998). It has also been reported that flavour enhancers, like glutamic acid and 5'-nucleotides, taste acidic in their free form but if they are converted to salts with Na or some other salt, the umami taste becomes more pronounced (Yamaguchi, 1991). These findings emphasised the important effects of autolysis pH in the presence of inductors such as salt, in optimising the autolysis process of SDYK towards particular taste requirements.

4.3.1.2 Flavour inducing action of papain hydrolysis

The flavour inducing action of papain hydrolysis has also been attributed to its high preference for bonds containing glutamine and glutamic acid (Kelly, 1983). Papain is also known to hydrolyse bonds adjacent to lysine and this was highlighted by the results shown in Figure 4.4 where a large increase in the essential amino acid lysine was observed in all yeast extracts in which papain had been added to accelerate autolysis. Unpleasant bitter flavour in yeast extracts and other protein hydrolysates can be a major hindrance to their use in food formulations. Intact food proteins do not

display bitterness as their molecular size suggests their inability to interact with receptors in the oral cavity (Fitzgerald and O'Cuinn, 2006).

Bitterness of food protein hydrolysates is associated with the exposure of interior hydrophobic amino acid chains of proteins and peptides as a results of enzyme action and includes the amino acids, leucine, isoleucine, proline, tyrosine and tryptophan (Pedersen, 1994, Transfiguracion et al., 1998). Peptides containing arginine and phenylalanine also generally have a bitter taste (Halasz and Lasztity, 1991c). While the concentration of arginine was quite low in all yeast extracts, the concentration of phenylalanine was relatively high, with the control yeast extract (T1) having a concentration of 0.207% (w/v). Methods employed to reduce bitterness include the use of activated carbon (Cogan et al., 1981), use of specific exopeptidases which cleave hydrophobic amino acids from the terminal end of the peptide chain (Raksakulthai and Haard, 2003) and more recently alteration of the total solids content in the hydrolysis reaction (Spellman et al., 2005).

4.3.1.3 Effect of increasing yeast solids on release of FAA during autolysis of SDYK.

From Figure 4.6 it was clear that increasing the yeast solids in autolysis led to an increase in the release of free amino acids. This resulted from the higher amount of intracellular solids being enzymatically hydrolysed during autolysis. However, it was interesting to note that while many of the flavour contributing amino acids increased, the flavour enhancing amino acid, glutamic acid did not increase in concentration.

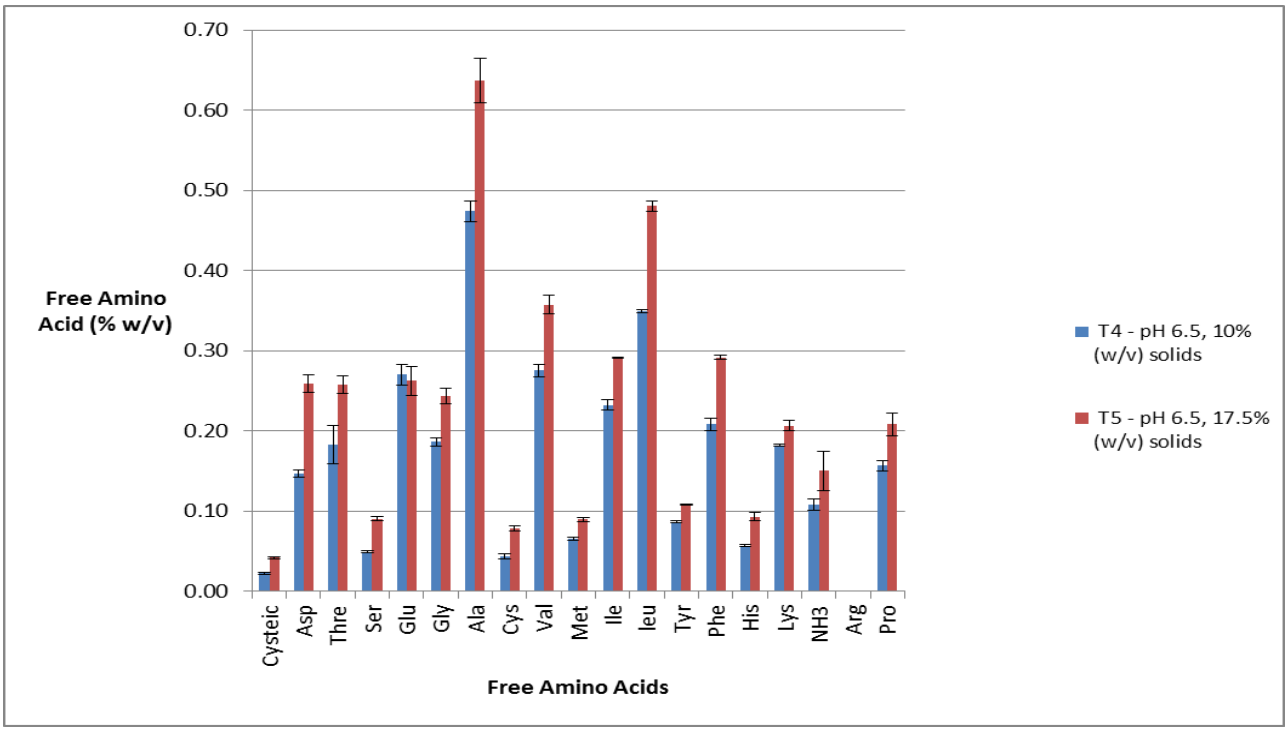


Figure 4.6 Difference in concentration of free amino acids in SDYK yeast extracts derived from induced autolysis at pH 6.5 with 10% (w/v) yeast solids (T4) compared to that using 17.5% (w/v) yeast solids (T4)

In a scenario where large volumes of SDYK are available, increasing the initial yeast solids used for autolysis is therefore one solution to producing yeast extracts having higher concentrations of free amino acids. However, it has been reported that too high a concentration could lead to feedback inhibition of the yeast's autolytic enzymes (Champagne et al., 2003). It is quite possible therefore, that partial inhibition of enzymatic hydrolysis of glutamine to glutamic acid has occurred as a result of the increased yeast solids used during the induced autolysis SDYK.

4.3.2 Free ribonucleotides in yeast extracts of SDYK

4.3.2.1 Development of improved reversed phase HPLC method of ribonucleotide detection

As the autolytic fate of yeast nucleic acids is not as well understood as that of protein degradation, it was considered important to quantify the 5'-ribonucleotide products that contribute to flavour enhancement. To date, the techniques utilised to quantify nucleotides are mainly chromatographic, with many of these techniques requiring quaternary gradient systems to separate the free nucleotides effectively (Belem and Lee, 1998; Charpentier et al., 2005; Hernawan and Fleet, 1995; Zhao and Fleet, 2005). In order to provide a more rapid and user friendly method of detecting free 5'-ribonucleotides produced during autolysis of SDYK, a reversed phase isocratic method separation was developed. This involved the use of a Waters Atlantis HPLC column in association with modification of mobile phase by the addition of ammonium formate and the ion-pair reagent tetrabutylammonium chloride. The charged ribonucleotides present (Figure 4.7) were therfore capable of forming an ion-pair with the ion-pair reagent present in the mobile phase to become electrically neutral. The resulting increase in hydrophobic character of the ion-pair resulted in a greater affinity for the reversed stationary phase and led to improved sample resolution (Figure 4.8). The separation of free ribonucleotides, particularly 5'-GMP (10.17 min) and 5'-IMP (11.24 min) was now possible using the improved mobile phase which were previously very difficult to separate and quantify. This mobile phase also resulted in reproducible standard curves for the individual ribonucleotide standards with correlation cooefficients $R^2 = 0.993 – 0.990$.

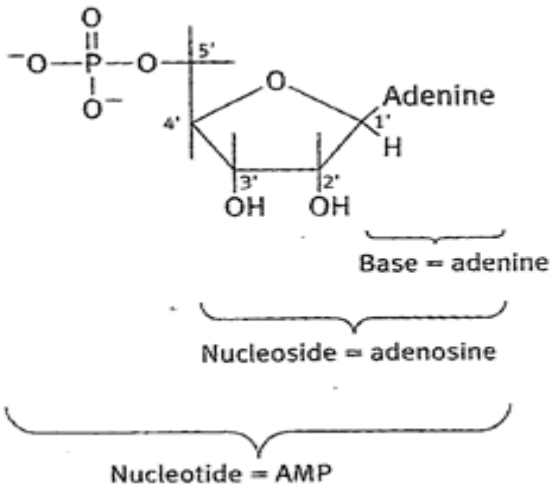


Figure 4.7 The structure of ribonucleotide AMP and the corresponding charged phosphate group available for ion-pairing.

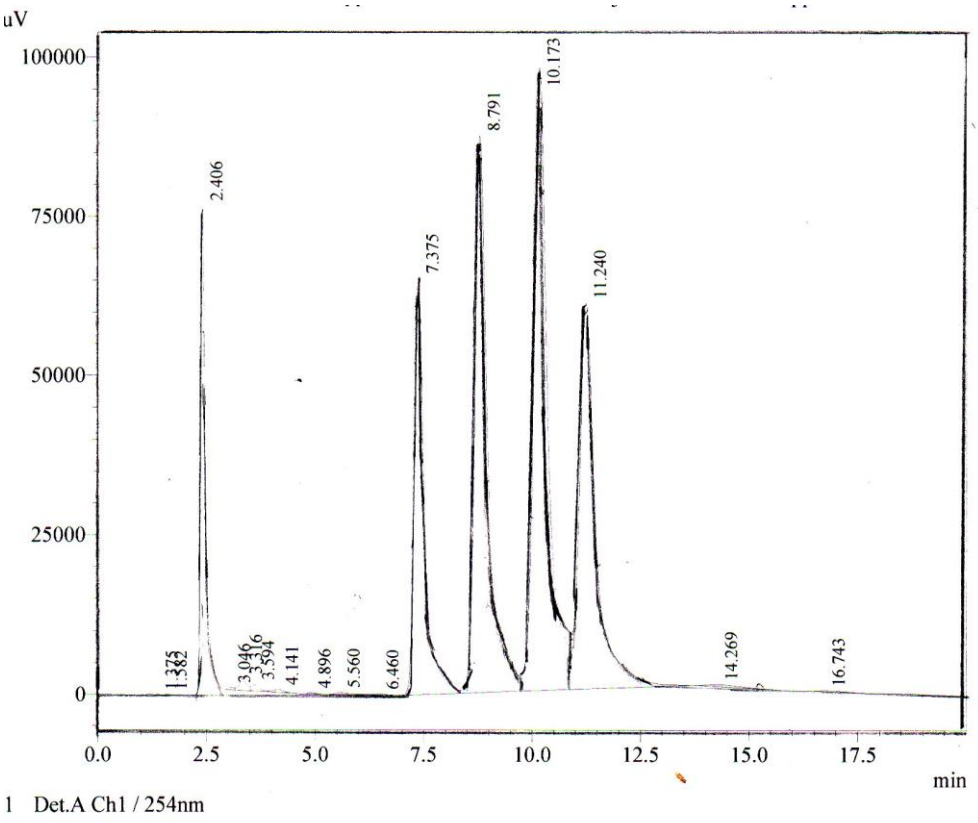


Figure 4.8 Effective reversed phase HPLC separation of 5'-CMP (2.406 min), 5'-UMP (7.375 min), 5'-AMP (8.79min), 5'-GMP (10.17 min) and 5'-IMP (11.24 min) in this study, using ion pair reagent interaction with 5'- ribonucleotide standards

4.3.2.2 Quantification of free ribonucleotides in yeast extracts of SDYK

Reversed phase HPLC analysis of yeast extracts (T1 to T6) from all autolysis trials of SDYK was carried out to quantify the concentrations of the flavour enhancing 5'-ribonucleotides, 5'-GMP and 5'-IMP present.

4.3.2.2a 5'-IMP

The concentrations of 5'ribonucleotides (%w/v) obtained in yeast extracts produced from induced autolyis of SDYK are shown in Figure 4.9. The existence of 5'-nucleotide-forming exonucleases in *S. cerevisiae* has been reported which resulted in flavour enhancing 5'-ribonucleotides been released during autolysis (Charpentier et al., 2005). HPLC analyses of SDYK yeast extracts revealed that there were levels of 5'-IMP present in all yeast extracts with the exception of T2. The highest levels of 5'-IMP obtained using 10% (w/v) initial yeast solids was T6 with 0.085 g 5'-IMP/100 g dry yeast. Belem et al., (1997), carried out autolysis of primary grown *K. marxianus* yeast at different pH's (6.5-7.5) and different temperatures (45-55^{o}C) and obtained values in the range of 0.0096g to 0.04 g of 5'IMP/100g yeast using temperature and pH as the autolytic inductors. Optimal yields were obtained at pH 6.5 -7.0 and 50^{o}C (Belem et al., 1997).

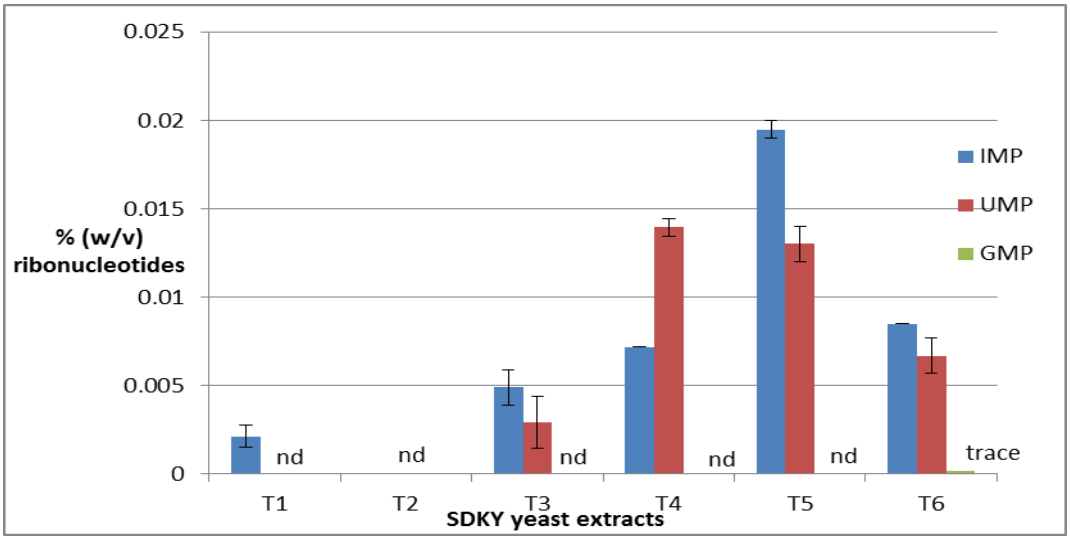


Figure 4.9 Concentration of 5'ribonucleotides (%w/v) obtained in yeast extracts produced from induced autolyis of SDYK (nd = not detected).

Analysis of variance indicated a significant difference in concentration ($p < 0.05$) of 5'ribonucleotides between the six SDYK dervived yeast extracts T1-T6. The yeast extract T6 (autolysis at pH 7.0 in the presence of inductors) yielded 0.0085 g/100 mls or 0.085g/100g yeast solids while yeast autolysis carried out at higher initial yeast solids of 17.5 % (w/v) at pH 6.5 yielded 0.0195 g/100mls 5'-IMP or 0.1114 g/ 100g yeast solids. This result indicated that the increase of initial yeast solids provided one approach to increasing the release of the flavour enhancing 5'-IMP from intracellular RNA of SDYK. Intracellular RNA was calculated to be 7.5% ± 0.75 (w/w) for SDYK in this study. Alteration of the pH of induced autolysis of SDYK at 10% (w/v) yeast solids also had a significant effect on 5'-IMP in the yeast extract. The addition of autolytic inductors resulted in an increase in 5'-IMP concentration in SDYK yeast extracts with the exception of yeast extract T2. Interestingly, all yeast extracts with the exception of yeast extracts T1 and T2 also had higher levels of 5'IMP than that obtained by Belem et al., 1997 using primary grown *K. marxianus* on whey. Figure 4.10 shows a typical RP HPLC chromatogram of the yeast extract T4 in this study.

The concentration of ribonucleotides present suggested limited endo-nucleases and exo-nuclease activity. Similiar to Belem et al, 1997, the first group of compounds eluted were nitrogen bases followed by 5'-ribonucleotides which were found to be products of phosphodiesterase activity and included 5' CMP, 5'UMP and 5' IMP. These results suggested that activities of different RNA degrading enzymes were influenced by environmental factors such as pH and autolytic inductors. Manipulation of these factors could allow autolysates with desired amounts of particular ribonucleotides to be produced. There have also been reports on the addition of commercial preparations of phosphodiesterase and adenylic deaminase to complement yeast endogenous enzymes and target release of higher concentrations of 5 -ribonucleotides particularly the flavour enhancing 5'-ribonucleotides 5' IMP and 5' GMP (Chae et al., 2001).

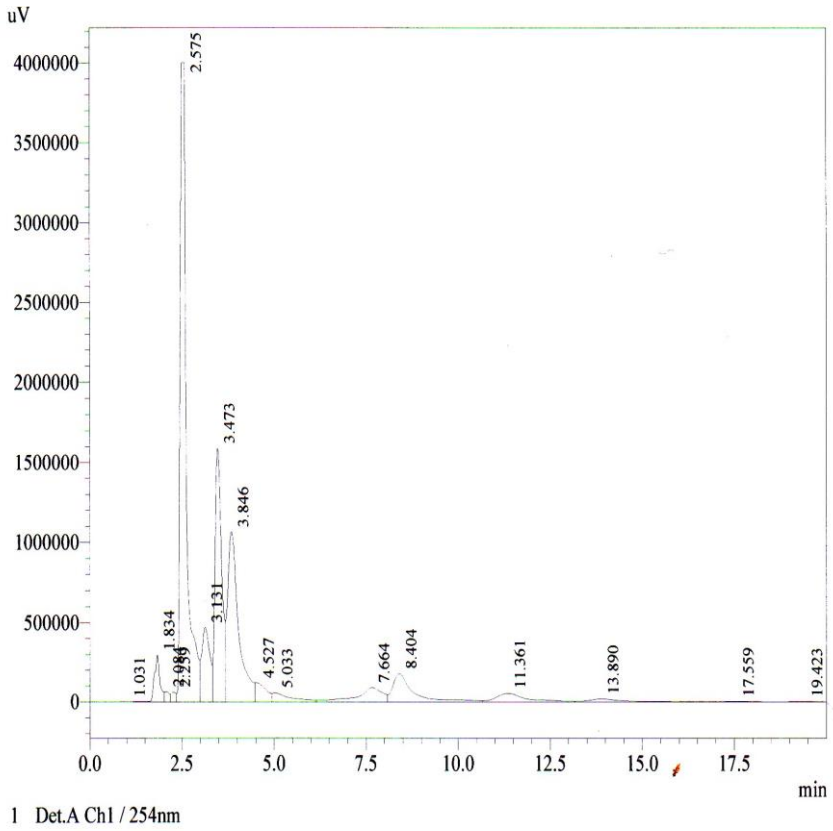


Figure 4.10 Reversed phase HPLC chromatogram of yeast extract T4 indicating the presence of 5' IMP with a retention time of 11.36 min.

4.3.2.2b 5'-GMP

The explanation why non-detectable and trace levels of 5-GMP were obtained in SDYK yeast extracts of this study can be attributed to a number of reasons. Firstly, 3'-ribonucleotides are by far the most predominant compounds for many autolysis conditions studied, accounting for 50–94% of total ribonucleotides (Zhao and Fleet, 2005). The levels of 5'- ribonucleotide and 2'- ribonucleotide isomers are much lower in comparison, representing only 3–21% and 3–29% of total ribonucleotides, respectively. Secondly, 5'-GMP can be hydrolysed in autolysates, indicating the presence of an extracellular RNase activity. Yeast organelles contain an array of nucleic acid hydrolysing enzymes (RNAases), including endo-ribonucleases,

exoribonucleases, nucleotidases and nucleosidases all of which could be involved in the autolytic degradation of RNA components (Zhao and Fleet, 2005).

With respect to nuclease activities, endo-nucleases hydrolyse the phosphodiester bond between adjacent ribose units within the polymer to produce poly- and oligo-ribonucleotides. Exo-nucleases hydrolyse this bond in a step-wise manner from one end of the polymer to produce mono ribonucleotides. For both types of enzymes, there are sub-classes that hydrolyse at eitherthe 5'or 3' side of the phosphate, generating ribonucleotides with either a 3'- or a 5'-phosphate group respectively. These enzymes have a diverse range of properties with regard to their optimal pH and temperature and substrate specificity which dictates a particular range of products of RNA degradation.

4.3.2.2c 5'-AMP and 5'-UMP

Although it is only 5'-GMP and 5'-IMP that provide flavour enhancement it is important to note that although 5'-AMP provides no flavour enhancement properties, it can be converted to 5'-IMP by the enzyme adenylic deaminase (Figure 4.11). The absence of the ribonucleotide 5'-AMP may be due therefore to the fact it can serve as a precursor for the flavour-enhancing 5'-IMP, which was present in most of the SDYK yeast extracts. Previous reports have also indicated that there was an increase in concentration of 5'-IMP produced at the expense of 5'-GMP in the presence of this enzyme, indicating that 5'-GMP can be converted to 5'-IMP by adenylic deaminase (Kim, Lee and Lee, 2002). It has been suggested that the formation of 5'-IMP in *K. marxianus* autolysates may depend exclusively on prior conversion of RNA into 5'-GMP, but not on conversion of these molecules into 5'-AMP (Belem et al., 1997).

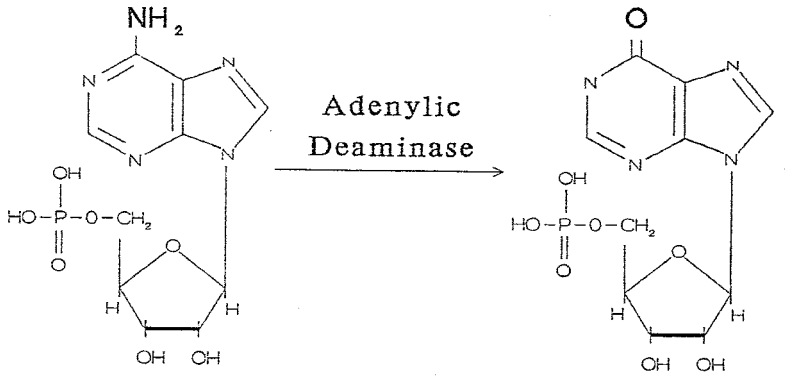


Figure 4.11 Formation of the flavour enhancing 5'-IMP ribonucleotide from the 5'-AMP ribonucleotide using the enzyme adenylic deaminase (Reed and Nagodawithna, 1991)

4.3.2.3 Synergistic interactions

Yeast RNA degradation is a characteristic feature of yeast autolysis**.** Many of the flavour improving properties of commercial yeast extracts today are the result of the synergistic interaction of various amino acids in combination with the RNA degradation products consisting of the 5' ribonucleotides, 5'-IMP and 5'-GMP. For this reason, 5'- ribonucleotides are now considered as high added-value bioingredients, used as natural flavour enhancers in foods.

One study attempted to quantify the synergism among flavour enhancers, by measuring resultant taste thresholds of individual and mixed solutions of these substances (Nagodawithana, 1992). These test evaluations have demonstrated dramatic decreases in taste thresholds. 5'-GMP and 5'-IMP are able to enhance flavour when present in concentrations ranging from 0.012% (5'-GMP) to 0.02% (5'-IMP) on a weight per volume basis. Through a synergistic effect (when present in a 1:1 ratio), this taste threshold can be lowered even further to 0.0063%. Moreover, used in combination with monosodium glutamate, the resulting taste threshold for

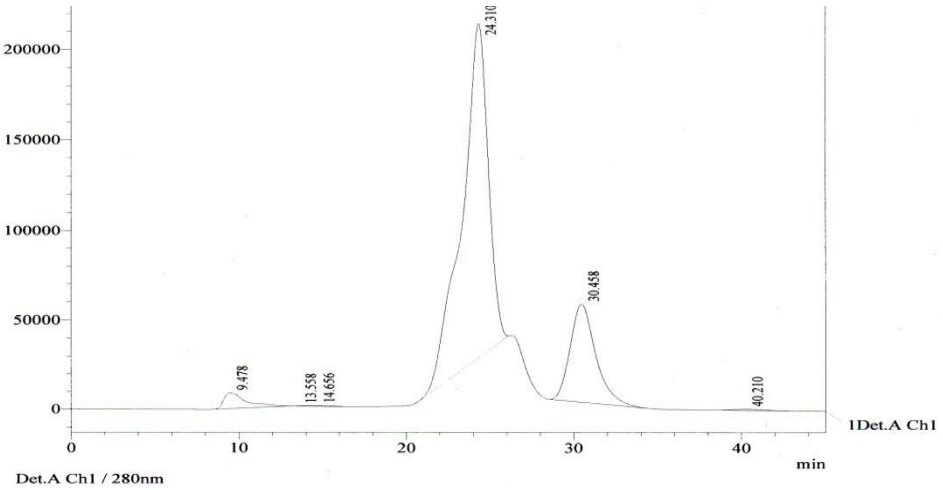
combined 5'-GMP and 5'-IMP was decreased to 0.000031%, representing a dramatic reduction of the taste threshold by a powerful taste synergism. As the concentration of 5'-IMP in yeast extract T5 is very close to 0.02% (w/v), it is possible for a taste synergism to occur with the concentration of glutamic acid in this yeast extract at 0.262% (w/v). The high concentration of glutamic acid in T4 could also result in a similar synergistic effect in this yeast extract.

It has also been reported that when 5'-IMP was added to several sweet amino acids, i.e. alanine, serine and glycine, the flavour enhancement was shown to be synergistic rather than additive by measuring mixtures of these amino acids with 5'-IMP using magnitude estimation (Okiyama and Ueda, 2002). The results showed that the flavour enhancement ratios were larger than 1.0 in the cases of the amino acids, alanine, serine and glycine. The amino acid alanine happened to be the amino acid with the highest concentration in the yeast extracts T1-T6 of this study using SDYK.

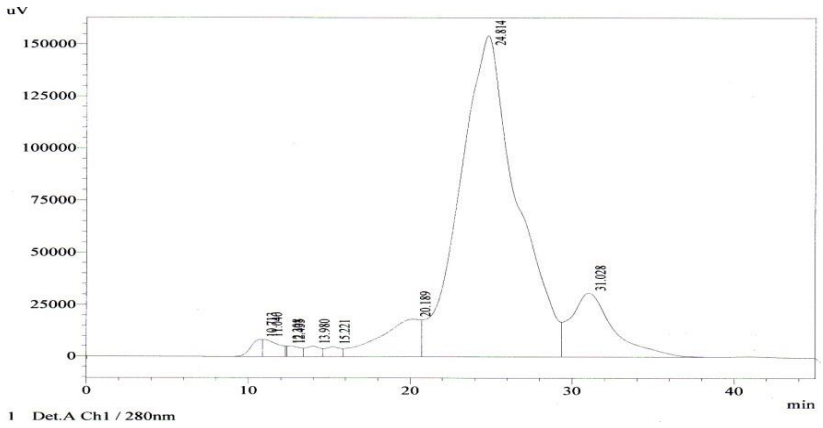
4.3.3 Size-exclusion chromatography (SEC) of yeast extracts derived from induced autolysis of SDYK under different autolysis conditions

The HPLC-SEC chromatograms of the six SDYK yeast extracts T1 –T6 produced under different autolysis conditions are shown in Figures 4.12 and 4.13. All chromatograms exhibited a characteristic profile or fingerprint with the main peak observed at a retention time of 24.8 min corresponding to an average molecular weight of 1182 Da. The next most prominent peak with a retention time 30.9 min corresponded to an average molecular weight of 100 – 200 Da. Small quantities of high molecular weight components as indicated by initial elution of small peaks in these chromatograms, indicated a high level of enzymatic hydrolysis, which is

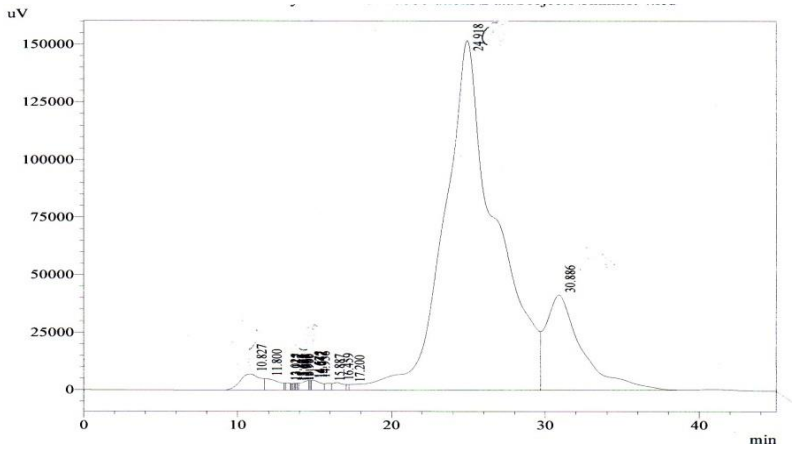
consistent with the high % DH previously obtained for these yeast extracts. Sommer (1998) described a typical baker's yeast extract protein content as consisting primarily of oligopeptides, di-, tri-, and tetrapeptides ranging from 500 – 3000 Da. The next largest fraction were free amino acids and the smallest fraction were oligopeptides with molecular weights of 3000 - 100000 Da. This distribution of components was also apparent in the yeast extracts of SDYK (Figures 4.12 and 4.13) where the peaks of 24.8 min and 30.9 min had molecular weights within the range reported of oligopeptides and amino acids respectively. Variation in yeast extract composition can be attributed to yeast extract processing conditions, particularly yeast autolysis conditions. It has been reported that by modification of enzymatic processes during autolysis, the ratio of the free amino acids to the di-, tri-, tetra-, and oligopeptides can be altered significantly, thus providing new yeast extract applications (Sommer, 1998). When observing the SEC fractions of yeast extracts derived from SDYK, the variation in the size of free amino acid fraction (30.9 min) to oligopeptide fraction (24.8 min) was evident. This application of size-exclusion chromatography in high performance mode (HPLC-SEC) has also proved useful in the study of chromatographic profiles of protein hydrolysates (Lemieux et al., 1989; Silvestre, 1997; Van der Ven et al., 2001). By comparing molecular weight profiles or "fingerprints" of yeast extracts derived from various autolysis processes or indeed from different yeasts, valuable information can therefore be obtained regarding the effect of process conditions on the final product while helping to maintain quality and reproducibility of new and existing processes.



Yeast Extract T1: (Control) Autolysis at pH 4.5 with no added inductors



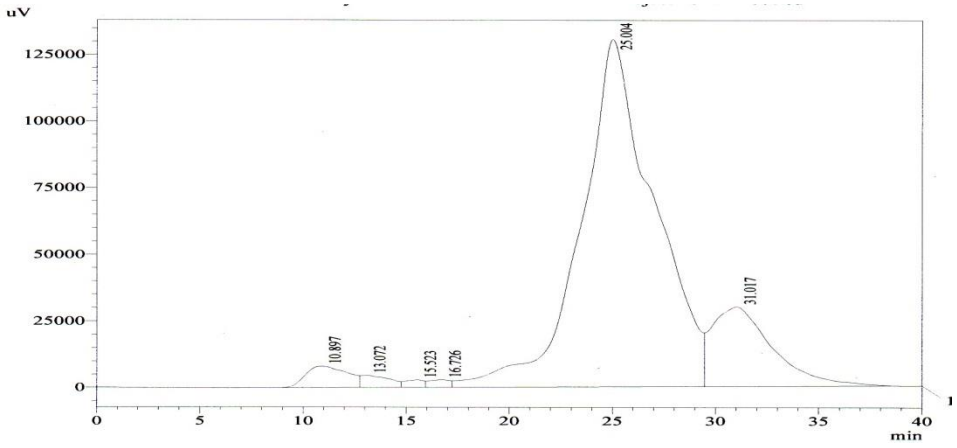
Yeast Extract T2: Autolysis at pH 4.5 with added inductors.



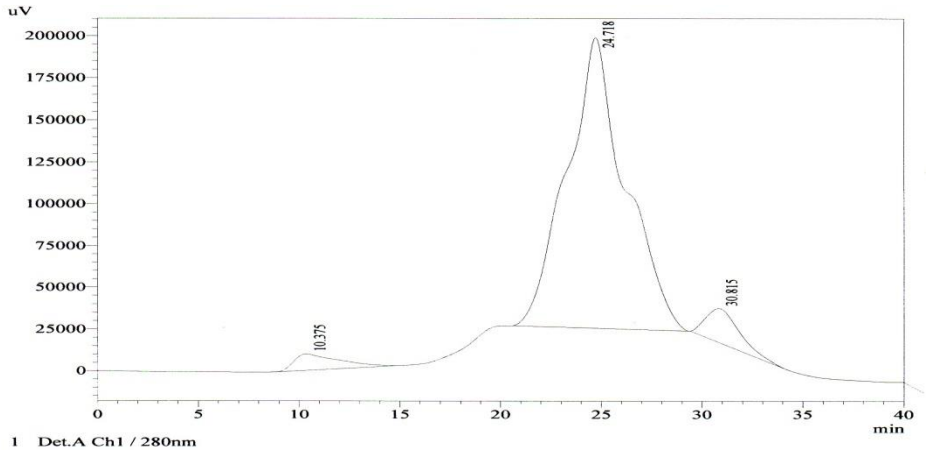
4.5

Yeast Extract T3: Autolysis at pH 5.5 with inductors

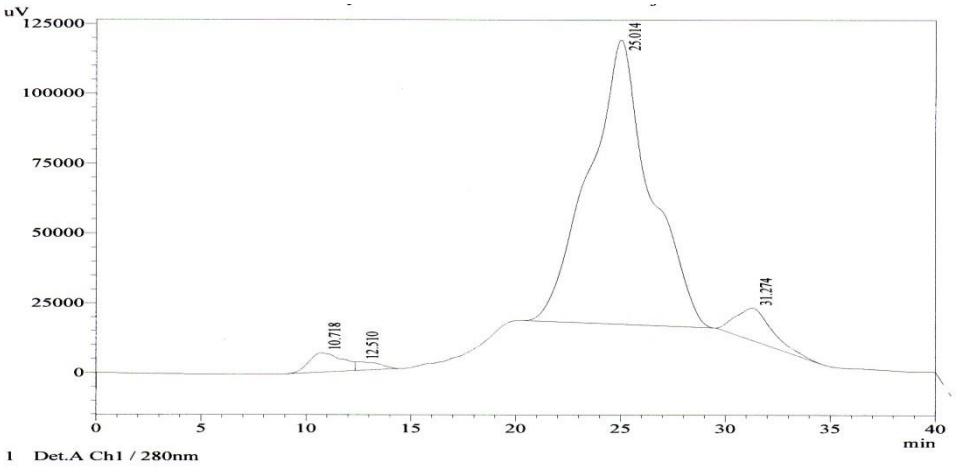
Figure 4.12 Size-exclusion chromatography (SEC) of yeast extracts (T1–T3) derived from induced autolysis of SDYK using inductors NaCl and proteolytic enzyme papain.



Yeast Extract T4: Autolysis at pH 6.5 with inductors.



Yeast Extract T5: Autolysis at pH 6.5 with inductors and higher yeast solids (17.5% w/v).



Yeast Extract T6: Autolysis at pH 7.0 with inductors

Figure 4.13 Size-exclusion chromatography (SEC) of yeast extracts (T4–T6) derived from induced autolysis of SDYK using inductors NaCl and proteolytic enzyme papain.

4.3.4 Liquid chromatography/mass spectrometry (LC/MS) characterisation of SDYK yeast extract

In order to obtain more information regarding the nature of molecular components in yeast extracts of SDYK, it was decided to carry out LC/MS analysis on a selected yeast extract (T4). This yeast extract was chosen on the basis of results obtained regarding its biochemical composition. The eluting components were detected on a Q-TOF mass-spectrometer to confirm signal strengths and mass spectra from all ions that were eluted from the liquid chromatography run ('intact masses spectrum') are shown in Figure 4.14. A fragment ion spectrum from the intact ion which was seen at 2209 mass units on the intact spectrum is shown in Figure 4.15. This shows the fragment ion series from a doubly charged parent ion originally observed at 1105.96 mass units. This daughter ion spectrum was achieved from fragmenting the doubly charged parent ion at 1105 mass units (as it was doubly charged the actual singly charged parent was 2209.83 mass units). Initial results indicated that there were multiple charged species being identified and triggering fragmentation.

While these masses do not correspond directly to amino acids, they were believed to be complex sugars as many fragment ion 'gaps' in the series are 162 mass units (i.e. the difference in mass between one daughter ion and another was uniform at 162 mass units) which is correct for a number of hexose sugars. Theoretically sugars alone do not protonate sufficiently on their own to give large multiple charged parent ions, as normally there should be amino groups present to protonate. One explanation is that fragments of glycoproteins were being observed due to the additional presence of peptides.

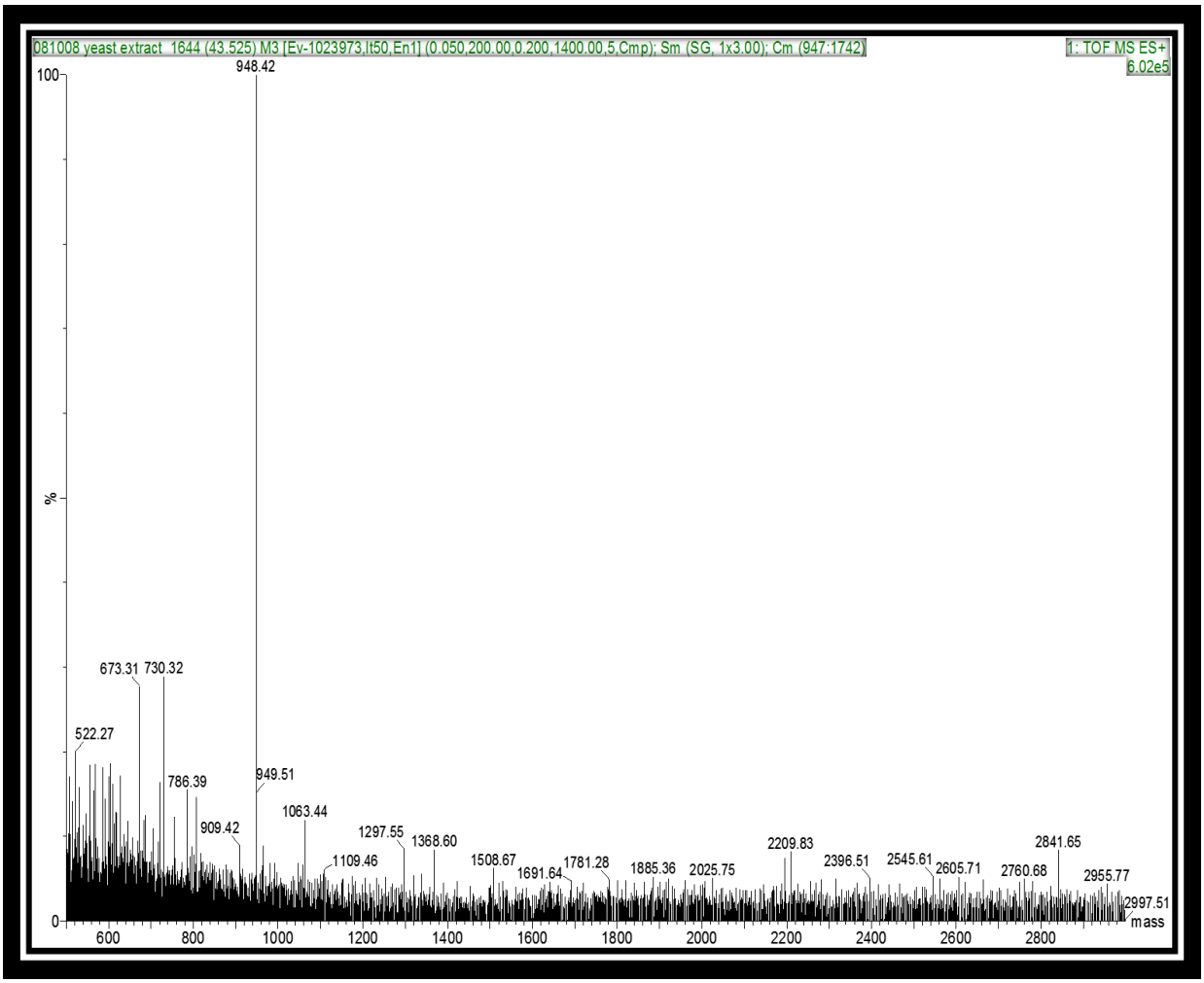


Figure 4.14 Mass-spectrum of eluted singly charged ions (intact masses) from the multiply protonated ions detected during online LCMS analysis of SDYK T4 yeast extract

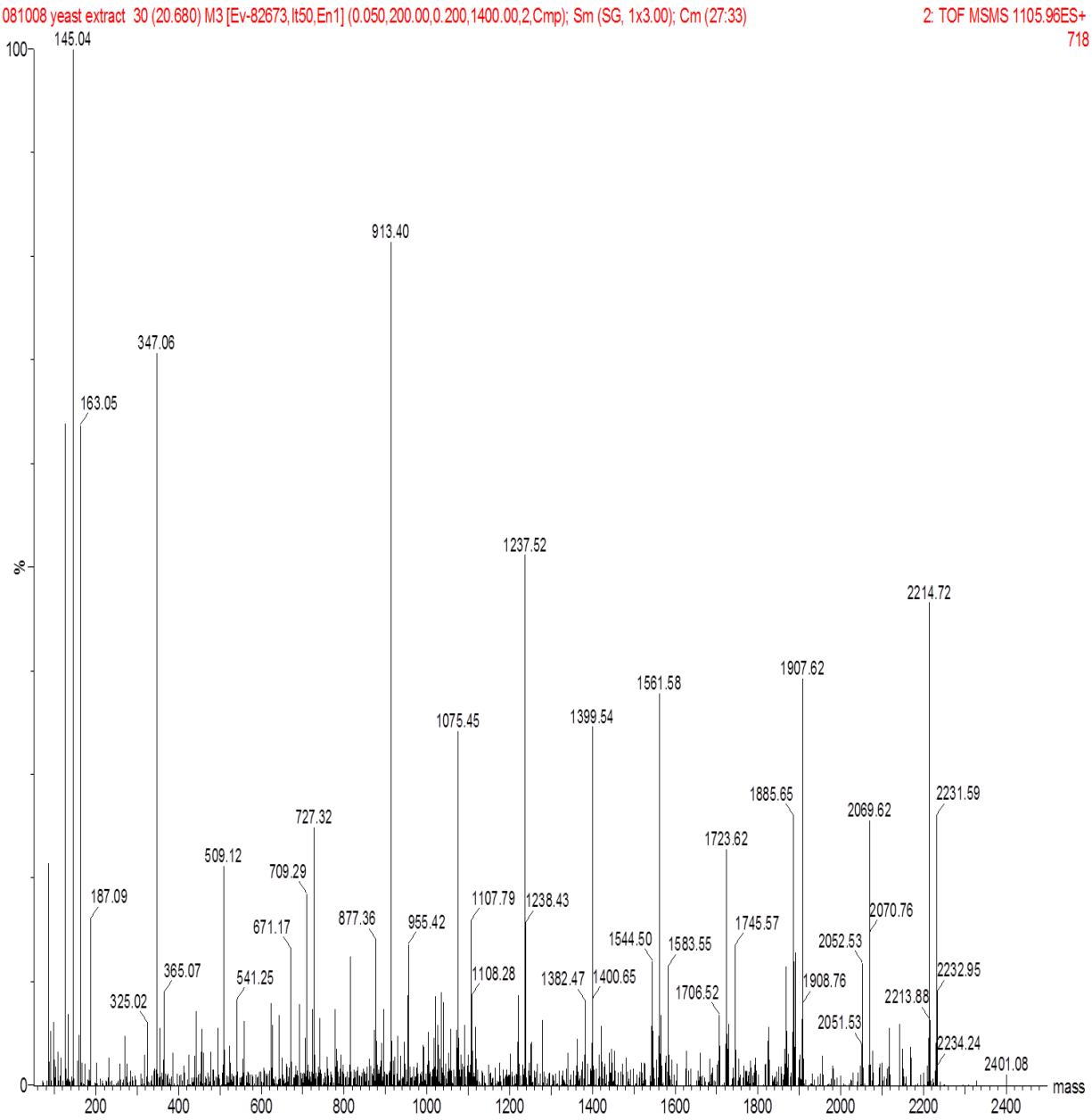


Figure 4.15 Fragment ion spectrum (product ion spectrum) from the intact ion which was seen at mass 2209 (1105++) on the intact spectrum in Figure 4.14

Figure 4.14 and Figure 4.15 have therefore indicated the compositional complexity of this yeast extract. Software Interpretation of the product ion spectrum for mass 1105++ (Table 4.3) suggested that it corresponded to peptide GNPISIK (Gly-Asn-Pro-Ile-Ser-Ile-Lys) linked to a glycan composed of N-acetylhexosamine (HexNAc) and 8 hexose sugars. It was also noted that both Asn and Ser were in this region,

indicating that it was a candidate for either N- or O-linked glycosylation. The amino acid composition of this peptide was characterized by having two essential amino acids (Ile and Lys) and amino acids associated with sweet and bitter flavours. Additional heterogeneity associated with the glycan was also evident which may be explicable by the additional presence of deoxy-hexose sugars.

Table 4.3 LC/MS output with respect to the product ion spectrum for mass 1105++ in SDYK yeast extract (T4)

Type of search	: MS/MS Ion Search
Enzyme	: Protease
Mass values	: Monoisotopic
Protein Mass	: Unrestricted
Peptide Mass Tolerance	: ± 1.2 Da
Fragment Mass Tolerance	: ± 1.2 Da
Max Missed Cleavages	: 1
Instrument type	: ESI-QUAD-TOF
Number of queries	: 1
Peptide.....	:.GNPISIK
Protein matching this peptide	Yeast oleate activated transcription factor 3 (OAF3)
Significance threshold	: $p < 0.05$

In order to obtain further information on the nature of peptides present in the yeast extracts from induced autolysis of spent SDYK, all yeast extracts were passed through a Protein-Pak SEC column and fractions in the region of 1000-2000 Da were run on the MALDI mass spectrometer to attempt characterisation of peptides present (Figure 4.16). Using this system, peptides in the mass range of 1000 - 3000 Da would be most likely to give interpretablet mass spectrum fragmentation data, while peptides above this size rarely give acceptable fragmentation data. Below 1000 mass units they are likely to be present as singly charged species and would not normally be selected for fragmentation with the present system. Low level signals in the 1000 - 1500 mass range were detected in the yeast extracts, T4, T5 and T6. The mass of 1222.74 in

yeast extract T4 was considered to be a probable peptide as were the masses of 1174 in extracts T5 and T6. There were again some indications of the presence of various forms of carmelised/heat modified sugars such as those related to lactose and mannose. Masses in very close proximity to masses of these compounds were listed on the National Institute for Science and Technology (NIST) database for molecular masses. As induced yeast autolysis was always completed by heating the yeast solution to 85°C for 30 min to inactivate enzymes present, the sugars present may have been modified by this heat treatment.

The Expasy database was utilized to obtain information relating to the peptide molecular masses from yeast sources. While there were many hits with masses showing up as potential peptide fragments, these potential peptides were not considered to be originating from typical yeast sources. It is possible that there may have been residual whey peptides present, as SDYK is a by-product of whey fermentations. Additional purification of the yeast extract samples combining SEC and reversed phase HPLC would be required in order to enrich and further characterise the peptides present.

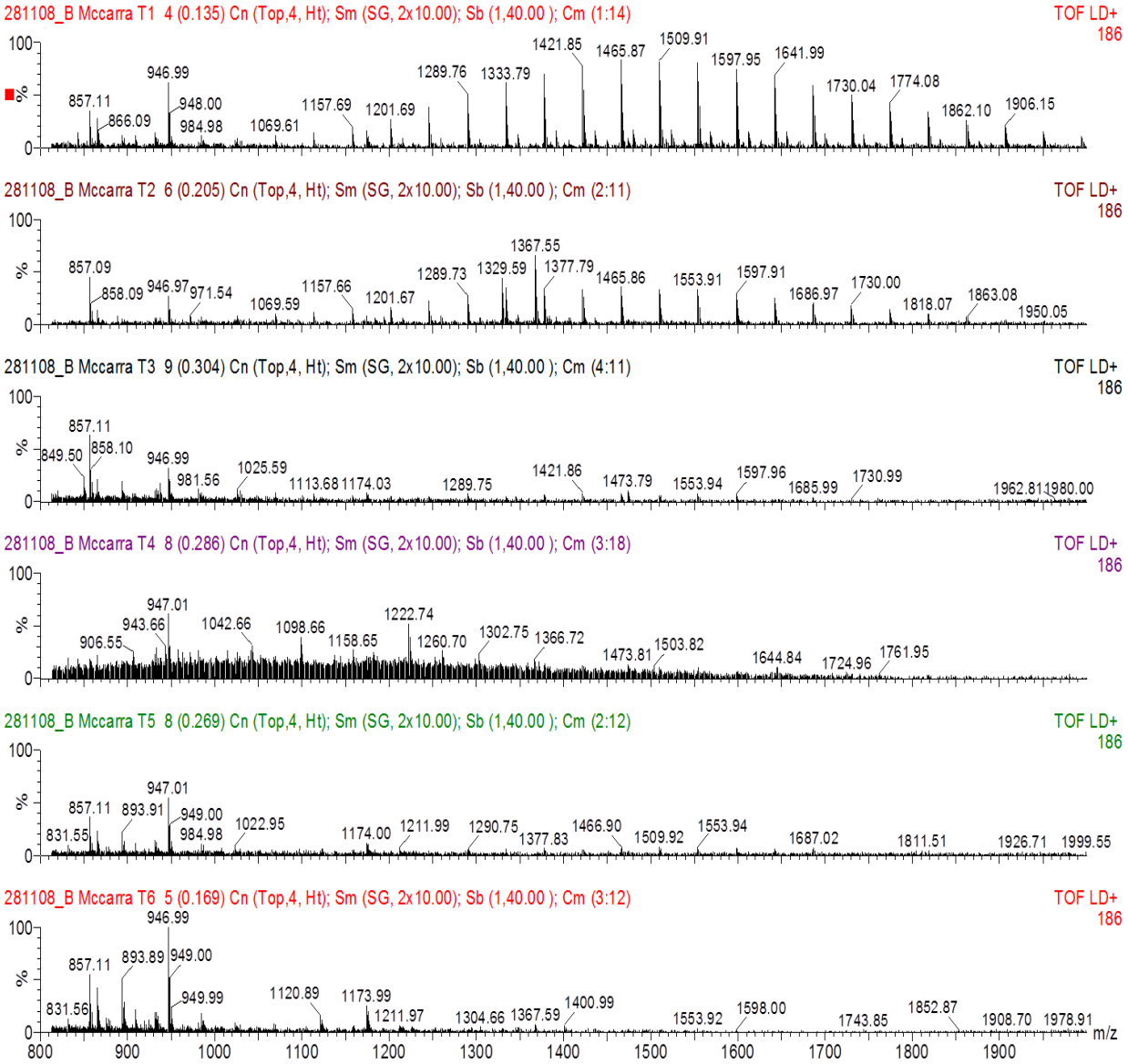


Figure 4.16 LC Mass-Spec of SEC fractions (1000 Da) of T1 – T6 yeast extracts (SDYK) derived from different autolysis conditions.

4.4 Conclusion

The results regarding the flavour enhancing components of SDYK yeast extracts emphasised the important effects of autolysis pH in the presence of inductors and salt in optimising the autolysis process of SDYK towards particular flavour requirements. The yeast extract corresponding to induced autolysis carried out at pH 5.5 - 6.5 had increased concentrations of flavour enhancers, glutamic and aspartic acid, while increasing the initial yeast solids improved significantly the concentration of the flavour enhancing ribonucleotide 5' IMP. SEC and LC/MS characterisation of yeast extract also indicated the presence of glycopeptides and further highlighted the complex biochemical nature of SDYK autolysate composition. This was believed to be due to the induced autolysis conditions utilised which promoted enzyme hydrolysis by the yeasts own proteases, peptidases, carbohydrases and nucleases and that of the exogenous enzyme, papain.

The application of descriptive sensory analysis in conjunction with data on flavour enhancing components of SDYK yeast extracts was therefore applied to assess the effect of induced autolysis pH on flavour development of yeast extracts derived from SDYK.

Chapter 5

Application of descriptive sensory analysis in assessing the effect of induced autolysis and pH on flavour development of yeast extracts derived from spent dairy yeast *Kluyveromyces marxianus.*

Introduction

5.1 Introduction

The taste of yeast extracts can be characterized by qualitative descriptors such as sweet, sour, salty, bitter and umami, a term to describe the savoury or meaty taste quality of protein hydrolysates, amino acids, and 5-ribonucleotides combinations (Mion-Du Crest, 1998; Nagodawithana, 1992; West, 1996; Varavinit et al., 2000). Yeast extracts derived from brewers or baker yeast have resulted in established flavours associated with commercial yeast extracts and tend to be distinctly strong, meaty or broth-like. Although spent brewers yeast is available in large quantities it contains undesirable flavour characteristics as a result of the carryover of hop resins and beer solids. The yeast therefore requires debittering before usage where hop resins are dissolved by increasing the pH level and then removed by further washing.

Methods to control and accelerate desirable and alternative flavour quality are sought by the food industry to reduce costs, improve consistency and produce products with distinctive characteristics that consumers find attractive (Hannon et al., 2005). The availability of large volumes of SDYK following the whey-to-ethanol fermentation process has the potential to meet these demands. The aim of this work was to investigate the impact of induced autolysis conditions on the flavour attributes of yeast extracts from SDYK and to highlight any flavour differences arising from the different autolysis treatments. This was achieved using modern sensory analysis techniques, combined with the multivariate statistical method, Principal Component Analysis. Table 5.1 summarises reported yeast associated flavour compounds and their attributes.

Table 5.1 Summary of yeast associated flavour compounds and attributes.

Flavour Compound(s)	Flavour attributes	Sources	Reference
5'GMP, 5' IMP, & glutamic acid	Enhancement of savoury and meaty flavours	Primary bakers yeast, Spent brewers yeast (*S. cerevisiae)*	Mion-Du Crest, 1998;; West, 1996; Sommer, 1996; Hay, 1993; Walker, 1998b.
5'GMP, 5' IMP	Enhancement of salty taste	*Candida utilis*	Reed and Nagodawithna, (1991)
Glutamic acid in the presence of salt and 5' IMP	Enhancement of salty and umami taste	*S. cerevisiae*	Nandakuma et al., 2003; Yamaguchi, 1991;
i)Yeast β-glucan ii)Yeast mannoproteins	i)slightly sweet taste and a fat-like mouthfeel. ii) increase in mouthfeel	*S. cerevisiae*	Alexandre and Guilloux-Benatier, 2011; Palomero et al., 2007
L-aspartic acid / L-aspartate	Acidic/	*S. cerevisiae*	Nagodawithana, 1992; Sommer, 1996
L- alanine	Sweet taste	*S. cerevisiae*	Sommer, 1996
i)Free amino acids . ii)Free amino acids with lactic acid and succinic acid	i)Characteristic " broth" flavour. ii) Enhanced meaty and savoury attributesr	Primary bakers yeast (*S. cerevisiae)*	Hay, 1993, Peppler, . 1979
2-phenylethanol ; 2-phenylacetic acid	flowery and sweet aroma.	Primary grown *K. marxianus*	Dragone et al., 2009; Fabre et al., 1997; Fabre et al., 1998)
Methyl-Ketones	Mushroom odour	*S. cerevisiae*	Varavinit,2000.
1-Octen-3-ol	Flavor of edible mushrooms		Pan et al., 2007.
Short peptides containing aspartic acid	Savoury /glutamate like taste qualities	*S. cerevisiae*	Matsushita and Ozaki, 1994
Peptides with hydrophobic amino acids	bitter	*S. cerevisiae*	Nishimura and Kato, 1988
Acidic oligopeptides	Savoury taste	Fish protein hydrolysate	Noguchi et al., 1975)
organic acids - propionic, succinic acid and acetic acid	sourness and pungency	*S. cerevisiae*.	Hernawan and Fleet, 199)
Free fatty acids	pungency,	*K. marxianus*	(Deive et al., 2003,)
organic acids, alcohols and aldehydes	Savoury volatile	*K. marxianus*	Vitova et al., 2006; Dragone et al., 2009
organic acids, alcohols , ketones & aldehydes	dairy products/sweet taste	*K. marxianus*	Vitova et al., 2006; Dragone et al., 2009)
3-mercapto-2-pentanone	sulphur aroma	*Yeast extracts*	Cerny and Guntz-Dubini, 2008
N-lactoyl-L glutamate,	Savoury volatile	*K. marxianus*	Frerot and Escher, 1997

5.2 Materials and Methods

5.2.1 Descriptive sensory analysis

A panel of eight highly trained assessors participated in the sensory evaluation of the yeast extract products. Prior to descriptive sensory analysis testing, the panel members were required to attend two focus group sessions in order to develop a consensus vocabulary for odour, flavour and mouthfeel attributes of the SDYK yeast extracts. During each discussion, the panel evaluated the odour and flavour of each of the yeast extracts and added new descriptors to the previously existing vocabulary where necessary (Hannon et al., 2001). Using the six yeast extracts involved in the study (Table 5.2), a list of initial attributes was developed. These focus group sessions were designed to familiarise panellists with the test products and acquaint panel members with terms used to describe yeast extracts through the use of reference standards (Murray and Delahunty, 2001). In this respect, a sample of SDYK yeast cream and a 0.2% (w/v) solution of monosodium glutamate were provided.

Panellists were trained during two separate sessions on different days, to ensure a uniform understanding of the developed sensory attributes. Some of the initial attributes were discarded. After completing their training, the panel rated the intensity of the six products with respect to the remaining 21 sensory attributes (8 odour terms, 12 flavour terms and 1 mouthfeel term).

Table 5.2 Composition (% w/v) of liquid yeast extracts provided for sensory analysis. The yeast extracts were produced using induced autolysis[a] at different initial pH values at 50 ºC and 10% (w/v) yeast solids.

Yeast Extract Code	Autolysis Initial pH	Total Solids	Total Free Amino acid	Protein	Final pH
T 1	pH 4.5±0.25	7.9 ±0.03	2.67±0.04	4.68 ±0.18	5.19±0.20
T 2 [a]	pH 4.5	9.2 ±0.22	2.33±0.01	4.57 ±0.19	4.47±0.05
T 3 [a]	pH 5.5	9.4 ±0.59	3.37±0.05	4.40 ±0.25	5.33±0.13
T 4 [a]	pH 6.5	8.8 ±0.71	3.15±0.07	4.50 ±0.26	5.80±0.29
T 5 [b]	pH 6.5	12.7 ±0.06	4.41±0.13	6.88 ±0.20	6.32±0.20
T 6 [a]	pH 7.0	7.7 ±0.44	2.19±0.02	4.12 ±0.30	6.83±0.15

[a]5% w/v NaCl and 0.2 % w/v papain enzyme added.

[b]5% w/v NaCl and 0.2 % w/v papain enzyme added, with yeast solids increased to 17.5% w/v.

5.2.1.1 SDYK yeast extract preparation

In cooperation with Carbery R&D laboratories it was agreed that the yeast extract products for evaluation would be in liquid form as this would give a more representative profile of flavours produced by SDYK. Induced autolysis of SDYK was carried out as described in 4.2.1. The yeast extracts were obtained by centrifugation of SDYK autolysates at 5000 x g for 30 min followed by pasteurisation of the supernatant (yeast extract) for 45 min. Table 5.2 summarises the codes and composition (% w/v) of the liquid yeast extracts provided for sensory analysis.

On the morning of analysis, samples were removed from refrigeration at 4 ºC, (having being defrosted from -20 ºC) and made up to the required concentration of 1% total solids with water and equilibrated at room temperature (~ 20 ºC). Approximately 100 ml of each sample was presented for evaluation in a clear-glass tumbler covered with a clock glass and coded with a random three-digit code (Figure 5.1).

Figure 5.1 SDYK yeast extract samples (T1 – T6) as presented for sensory evaluation.

A warm-up sample, the results of which were discarded, was given to panellists prior to assessment of the full set of samples. Each panellist was provided with spring water and sliced carrots to cleanse their palate between samples. A list of definitions for each of the attributes included in the final vocabulary was also available.

The panel rated the intensity of the attributes on 100 mm unstructured line scales. Descriptive terms were anchored on both ends (at 5% and 95%) with extremes of each descriptive term. The samples were evaluated in duplicate over a two-day period (Figure 5.2) and order of presentation was balanced to account for first order and carry-over effects (MacFie et al., 1989). Data were collected using Compusense Five, Version 4.0 (Compusense Inc. Ontario, Canada).

Figure 5.2 Sensory evaluation of SDYK yeast extract samples T1-T6 by panel of trained assessors.

5.2.2 Statistical analysis of the data

The mean panel scores from descriptive sensory analysis were subjected to one-way ANOVA to determine whether significant differences ($p \leq 0.05$) existed between the products. The ANOVA was performed using SPSS version 20 (SPSS Inc., Chicago, IL, USA). Duplicate scores were then averaged, standardized (1/standard deviation), cross validated and analysed using Principal Components Analysis (PCA) using Guideline® v 7.5 (CAMO ASA, Box 1405 Vika, N-0115 OSLO, Norway).

5.3 Results and Discussion

5.3.1 Development of yeast extract descriptors and results of descriptive sensory analysis of yeast extracts derived from SDYK

After completion of focus group sessions, the sensory panel rated the intensity of the six SDYK yeast extracts products T1- T6 with respect to 21 sensory attributes (8 odour terms, 12 flavour terms and 1 mouthfeel term). These yeast extract descriptors with definitions are clearly described in Table 5.3. Using ANOVA it was found that 10 of the 21 attributes significantly ($P < 0.05$) discriminated between the yeast extracts and the attributes that did not significantly discriminate between the yeast extracts ($P > 0.05$) were not included in PCA. Results of descriptive sensory analysis are presented in Table 5.4 which shows the averaged attribute scores (1- 100) for the 6 yeast extracts (T1-T6) assessed by the sensory panel and also includes the results of ANOVA analyses.

Figure 5.3(a), Figure 5.3(b) and Figure 5.3(c) illustrate PCA plots showing the first two principle components (PCs) of descriptive sensory analysis of the six yeast extracts T1 –T6, derived from induced autolysis of SDYK at different pH values. It was found that principle component 1 (PC 1), significantly discriminated ($P < 0.05$) between the yeast extracts and accounted for most of the variation (85%) between the sensory characteristics of yeast extracts. PC , separated these products on the basis of addition of autolytic inductors, salt and protease (papain) enzyme.

Table 5.3 SDYK yeast extract descriptors

Odour Attributes	***Definition***
Pungent	Physically penetrating sensation in the nasal cavity. Sharp smelling irritant. At the high end of this scale, a stinging sensation.
Sulphur	The smell of sulphur - in particular that associated with hardboiled eggs or a struck match.
Chemical	Odour associated with non specific solvents.
Rancid	Odour associated with sour milk and oxidised fats. Having the rank aroma or taste characteristics of fats when no longer fresh.
Yeast	Reminiscent of the odour associated with farms/ cow dung/ manure/ slurry. Reference - Yeast sample.
Dirty dog/Damp wool/Stale water/Damp cardboard	Aromatics associated with dirty dogs, stale water or damp wool & cardboard.
Mushroom	Odour associated with raw mushrooms.
Savoury	Odour associated with foods containing salt and/or MSG.
Flavour Attributes	
Pungent Sensation	Physically penetrating sensation in the nasal cavity. Sharp smelling irritant. At the high end of this scale, a stinging sensation.
Rancid	Flavour associated with sour milk and oxidised dairy fats. Having the rank aroma or taste characteristics of diary fats when no longer fresh.
Acetone/Blue Cheese	Flavour associated with acetone or blue cheese.
Sweaty	The flavour reminiscent of perspiration generated foot odour which are are sour, stale and slightly cheesy.
Savoury	Chemical feeling factor elicited by certain peptides and nucleotides. Reference - 0.2% MSG.
Earthy/ Mushroom/ Mouldy	Flavour characteristics associated with earth, raw mushrooms or moulds.
Dirty Farm/ Slurry/ Manure	The flavour characteristics associated with the smell of dirty farms, slurry or manure.
Basic Tastes	
Sweet	The fundamental taste sensation of which sucrose is typical.
Salty	The fundamental taste sensation of which sodium chloride is typical.
Sour/Acidic	A sour, tangy, sharp, citrus-like taste. The fundamental taste sensations of which lactic acid and citric acids are typical.
Bitter	The fundamental taste sensations of which caffeine and quinine are typical.
Astringent	A mouthdrying and harsh sensation. The complex of drying, puckering and shrinking sensations in the lower cavity causing contraction of the body tissues.
Mouthfeel Attribute	
Fatty	The range of sensations typically associated with fat content.

Table 5.4 Results of quantitative descriptive sensory analysis of liquid yeast extracts derived from different induced autolysis conditions of SDYK showing the
(i) Averaged attribute scores (1-100), (ii) Analysis of indices[1]and (iii) ANOVA[2].

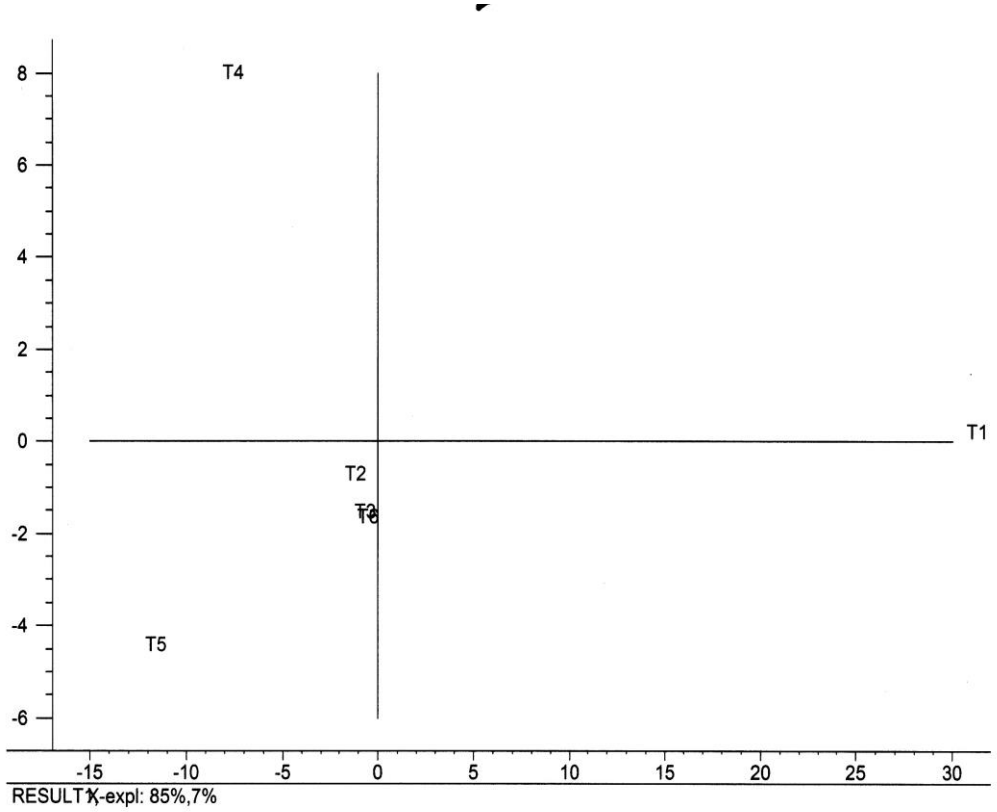
Attributes	pH	4.8	4.5	5.5	6.5	6.5	7.0	
	Sample	T_1	T_2	T_3	T_4	T_5	T_6	P_{value}
Odour								
Pungent		29.3	22.5	26.9	25	17.3	24.3	0.072
Sulphur		5.2	6.5	7.2	4.1	4.8	1.6	0.203
Chemical		8.4	9	8.4	7.8	7.8	8.5	0.979
Rancid		15.3	9.4	11	8.9	6.4	9.3	0.188
Yeast		39.8^{a}	27.1^{bc}	28.2^{b}	29.7^{b}	20.6^{c}	27.3^{bc}	**0.008**
Cardboardy		11.9	14.6	17.8	15.6	15.4	22.1	0.206
Mushroom		0.1^{a}	0.2^{a}	0.1^{a}	2.2^{ab}	4.6^{b}	0.1^{a}	**0.05**
Savoury		12.9	23.5	14.2	19	14	13.4	0.088
Flavour								
Pungent Sensation		35.8^{a}	29.5^{ab}	23.1^{bc}	23.8^{bc}	19.3^{c}	27.7^{b}	**0.02**
Rancid		20.5	16	12.1	12.1	12.1	14.2	0.065
Acetone/Blue Cheese		26.7^{a}	18.7^{b}	15.7^{b}	18.8^{b}	15.6^{b}	18.2^{b}	**0.002**
Sweaty		10.8	9.5	8	7	9	9.9	0.073
Savoury		22.9^{a}	35.6^{bc}	34^{bc}	42.1^{c}	39.5^{bc}	31.6^{b}	**0.012**
Mouldy		9	5	2.7	4.7	4.5	3.8	0.681
Dirty Farm		42.6^{a}	21.3^{b}	26.4^{b}	23.4^{b}	19.9^{b}	23.6^{b}	**0.003**
Taste								
Sweet		2.7^{a}	5.9^{c}	4.2^{abc}	4.4^{abc}	3.3^{ab}	4.8^{bc}	**0.027**
Salty		11.9^{a}	23.9^{b}	24.2^{b}	32^{c}	25.3^{b}	25.6^{b}	**0.000**
Acidic		17.9	10.9	12.8	11.9	7.8	12.3	0.076
Bitter		21.4^{a}	14.1^{b}	12.9^{b}	11.4^{b}	10.8^{b}	10.8^{b}	**0.019**
Astringent		13.3^{a}	9.6^{bc}	8.6^{bc}	10.5^{abc}	11.1^{ab}	7.8^{c}	**0.026**
Fatty Mouthfeel		0.7	3.2	6.3	4.2	4.8	4.5	0.08

[1]Values within a row not followed by the same letter, differ (P < 0.05).

[2]Descriptors that significantly discriminated between the yeast extracts (T1 –T6) from different autolytic conditions have P value highlighted (P < 0.05).

Comparison of the relative positions of the individual yeast extracts (T1 – T6) on the PCA bi-plot to the position of the control yeast extract was a useful index of the impact of the applied treatment and also how the flavour of the yeast extracts differed from the control (Hannon et al., 2005). From Figure 5.3(a), the position of the control yeast extract T1 (no added autolytic inductors) was positioned quite separately from the remaining yeast extracts indicating that autolytic treatments had an impact on flavour. It was also interesting to note that positions of yeast extracts T4 and T5 were positioned quite distinctly and furthest from the control yeast extract. The relevant flavours associated with these yeast extracts were also shown on the associated PCA bi-plot Figure 5.3(b).

Principal Component 2 (7%)



Principal Component 1 (85%)

Figure 5.3(a) Principle Component Analysis (PCA) of descriptive sensory analysis of SDYK yeast extracts T1 – T6 derived from different autolysis conditions of pH in the presence of autolytic inductors.

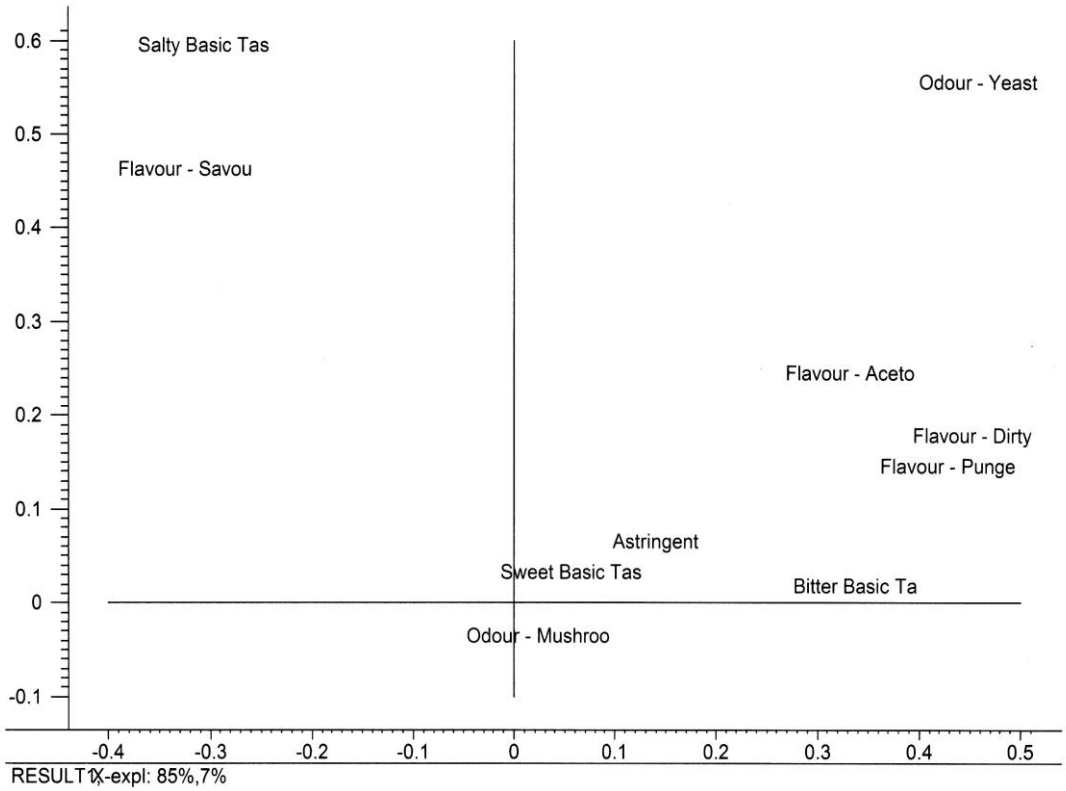


Figure 5.3 (b) Principle Component Analysis (PCA) of descriptive sensory analysis of yeast extracts T1 – T6 flavour and taste attributes

It was clear from the relevant positions of the flavour attributes on the PCA biplot in Figure 5.3(c) that the more undesirable flavours in terms of yeast extract requirements were positioned quite separately to the right from the more interesting flavours of savoury and salty. This indicated that the addition of autolytic inductors was having an important effect on the sensory characteristics of SDYK yeast extracts.

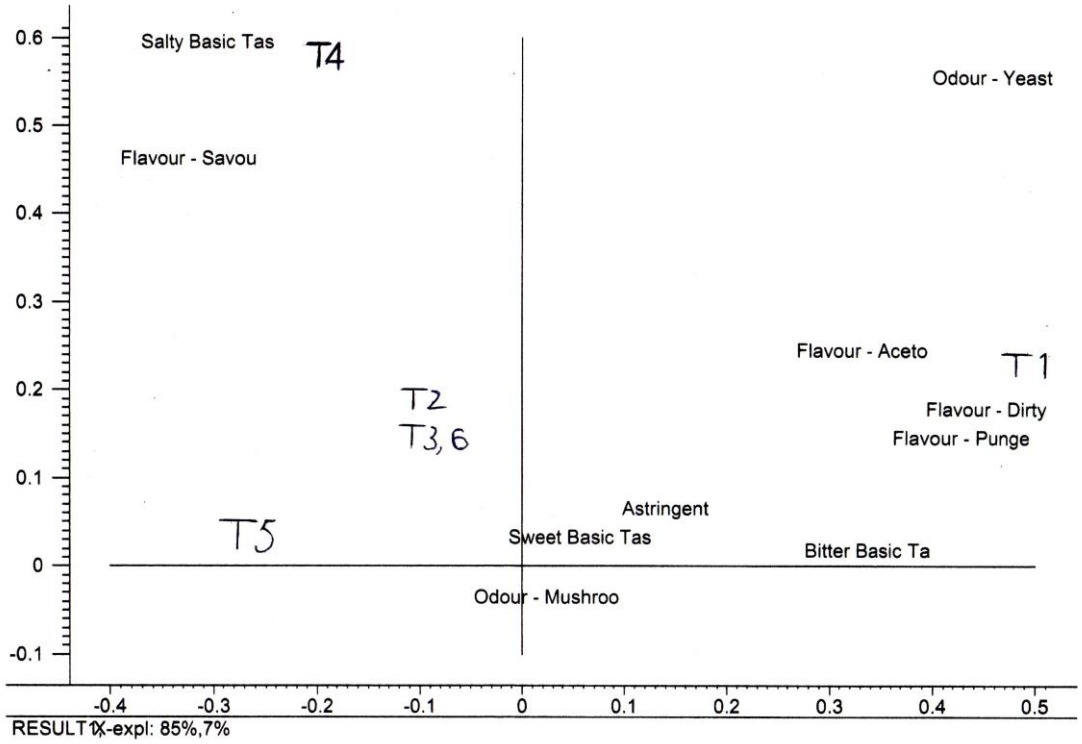


Figure 5.3(c) Principle Component Analysis (PCA) of descriptive sensory analysis of SDYK yeast extracts T1 – T6 with flavor and taste attributes

5.3.1.1 Use of analysis of indices to evaluate the effect of autolysis conditions on the sensory descriptors

A better understanding of the effect of induced autolysis conditions on the sensory descriptors associated with the developed yeast extracts from SDYK was obtained from the analysis of indices included in Table 5.4. Using this statistical technique, the indices values (superscript) above a given attribute score within a row which are not followed by the same letter indicated a significant difference ($P < 0.05$). The important findings were summarised as follows:

Salty Basic Taste:

Yeast extract T4 (autolysis treatment carried out at pH 6.5) was significantly higher in salty basic taste ($P < 0.05$) when compared to the other yeast extracts (Figure 5.4.).

This was relevant as the same salt concentration was used to promote autolysis in all induced treatments, indicating that modification of autolysis conditions could provide a method for salt reduction in yeast extracts and other processed food. High intake of salt is known to lead to hypertension and heart disease. The food industry must therefore find innovative methods to reduce the sodium content in food without a detrimental impact on taste. The results of this study indicated that the salty taste could be enhanced in yeast extracts by controlled autolysis of SDYK and could therefore allow sodium concentrations to be reduced in food products.

Flavour enhancers like glutamic acid and 5'-nucleotides which taste acidic in their free form are converted to salts with sodium chloride and the umami or savoury taste becomes more pronounced while greatly enhancing salt perception (Yamaguchi, 1991). The synergistic action of 5' ribonucleotides, glutamic acid and salt in yeast extracts also contribute a background flavour that helps in reducing harsh and undesirable flavours. However, this effect can only be achieved within a pH range of pH 5.0 - 8.0, as only dissociated glutamate has any flavour enhancing properties (Sommer, 1998). As no salt was added to the control, it was not surprising that this yielded the lowest salt score ($p < 0.05$).

Savoury Flavour:

All autolysis treatments had a significantly higher savoury flavour rating ($p < 0.05$) than the control and this is illustrated in Figure 5.5. The highest savoury rating was achieved when autolysis was carried out at pH 6.5 with inductors present which indicated that autolysis inductors had a positive effect on savoury flavour at this pH value. Again, the flavour enhancing properties of dissociated glutamic acid in the

presence of salt and the ribinucleotide IMP ensured that the savoury and salt tastes were more pronounced. Indeed savoury scores would indicate that the presence of salt and papain during autolysis resulted in similar savoury flavour profiles at most pH values with the exception of autolysis treatment carried out at pH 7.0 (T6) which had a significantly lower savoury rating ($p < 0.05$) than at pH 6.5. Autolysis induced at pH 7.0 with salt addition appeared not to be optimal for endogenous enzyme activity responsible for the required free amino acid liberation in the extract. Similar concentrations of the flavour enhancing amino acids were found in the control yeast extract (T1) and yeast extract T2 (autolysis at pH 4.5 with salt and enzyme). However the addition of salt and the enzyme papain appeared to be counteracting pungent, astringent and bitter flavour effects at pH 4.5 and other pH values of autolysis.

It was also quite likely that in addition to free amino acids, low molecular weight peptides were present in SDYK yeast extracts, the taste of which is related to their amino acid composition. Similar to amino acids, peptides can be classified as savoury and bitter tasting peptides. Short peptides consisting of aspartic acid and glutamic which are present as oligopeptides, tetrapeptides and tripeptides, have been reported (Saha and Hayashi, 2001; Lemieux and Simard, 1992; Nishimura and Kato, 1988). Similar peptides have been reported to have glutamate like taste qualities (Matsushita and Ozaki, 1994; Noguchi et al., 1975). LC-Mass-Spec findings in the previous chapter have already indicated the presence of peptides in yeast extract T4.

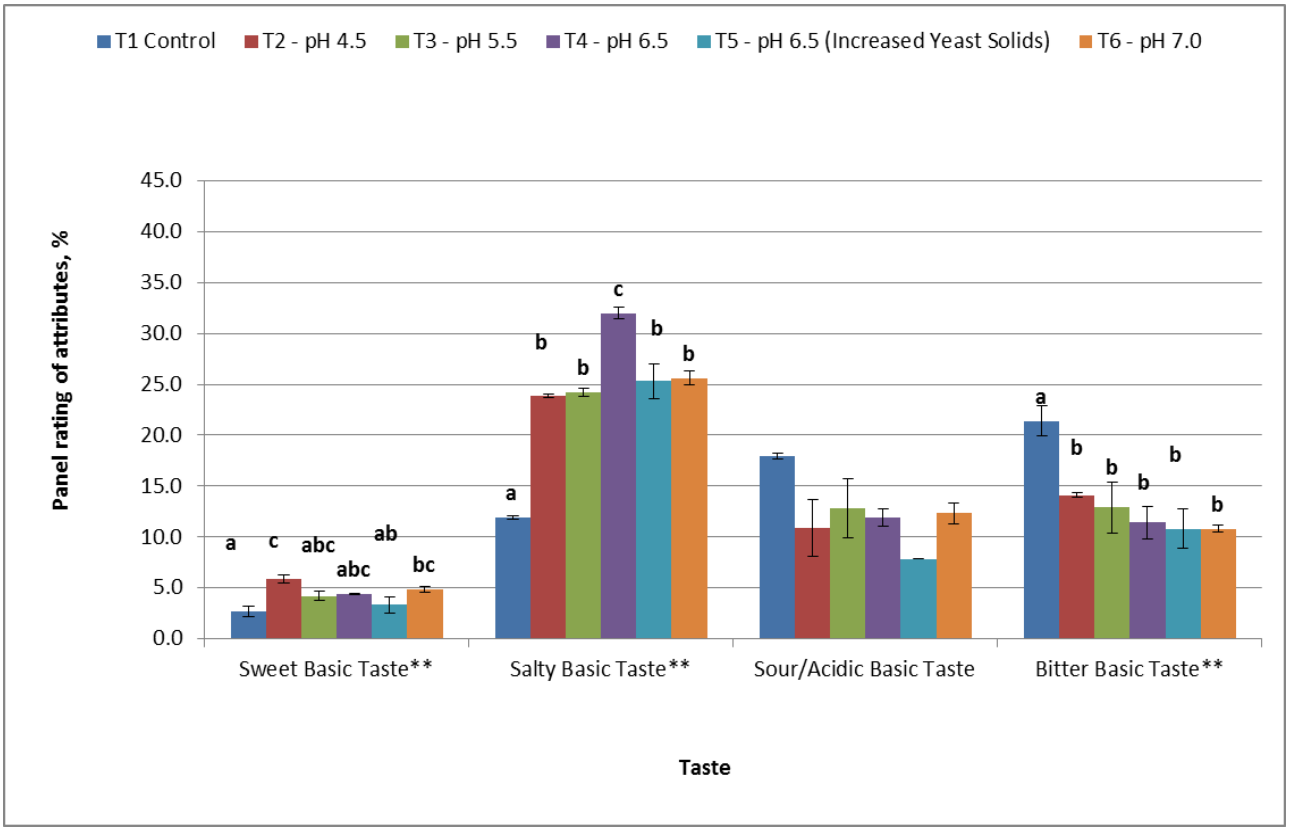


Figure 5.4 Bar chart showing results of six yeast extracts assessed by sensory panel for the four basic tastes of sweet, salty, sour and acidic.

**** indicates attributes in which a significant difference in taste score existed with respect to six yeast extracts T1-T6. Bars with different letters are significantly different ($p < 0.05$)**

Bitter Taste:

Yeast extract derived from autolysis with no inducers or pH adjustment (control) was characterised by a significantly higher bitter taste rating ($P < 0.05$). The characteristics of the control were interesting in that it yielded characteristics that are to be avoided with respect to production of flavoured yeast extracts. In addition to the higher bitter values, the control yeast extract showed a significantly higher pungent sensation ($p < 0.05$) in all the induced autolysis samples with the exception of induced autolysis carried out at the lowest pH of 4.5 (Figure 5.5). Carrying out yeast autolysis in this pH region therefore resulted in a higher level of undesirable flavour attributes

even with inductors present. However, at the higher pH values, it was likely that papain acted more effectively by hydrolysing the interior of the yeast protein, exposing hydrophobic amino acids, while the endogenous exopeptidases cleaved these amino acids from the terminal end of the peptide chain, a process known as debittering of proteins (Saha and Hayashi, 2001; Pawlet and Bruce, 1996; Fitzgerald and O' Cuinn, 2006). Bitterness is associated with peptides having a high content of bitter (hydrophobic) amino acids and dipeptides with hydrophobic amino acids have been found to be more bitter than free amino acids (Matoba and Hata, 1972). While a high % DH was achieved in the control yeast extract, indicating good endogenous enzyme activity, the products of hydrolysis were most probably composed of bitter peptides (Nishimura and Kato, 1988).

Sourness and Acidity

It is believed that the presence of organic acids can also contribute to sourness and pungency of yeast extracts. Organic acids such as propionic, succinic acid and acetic acid have been detected during autolysis of *S. cervisiae*. (Hernawan and Fleet, 1995) and are believed to be linked to the metabolism of sugars and lipids in yeasts. In relation to lipids, free fatty acids which can also contribute to pungency, have been shown to be present as a result of lipolysis by the action of extracellular lipase present in *K. marxianus* (Deive et al., 2003, Molimard and Spinnler, 1996).

While this attribute was present to some degree in all SDYK yeast extracts tested , the scores obtained were significantly lower in the extracts derived from autolysis in the presence of salt and papain at pH 5.5 – pH 7.0. Another noticeable feature with respect to the control yeast extract was significantly higher acetone/blue cheese and dirty farm flavours when compared to yeast extracts derived from autolysis with

inductors salt and papain. Again, compounds generated by lipid metabolism in the yeast *K. marxianus* are known to produce aldehydes and ketones, while catabolism of whey lactose also produces organic acids, alcohols and aldehydes, compounds that are common constituents of many dairy products (Vitova et al., 2006; Dragone et al., 2009). It was also likely that different concentrations of these compounds in the yeast extracts were also responsible for the different levels of sweet taste in the SDYK yeast extracts.

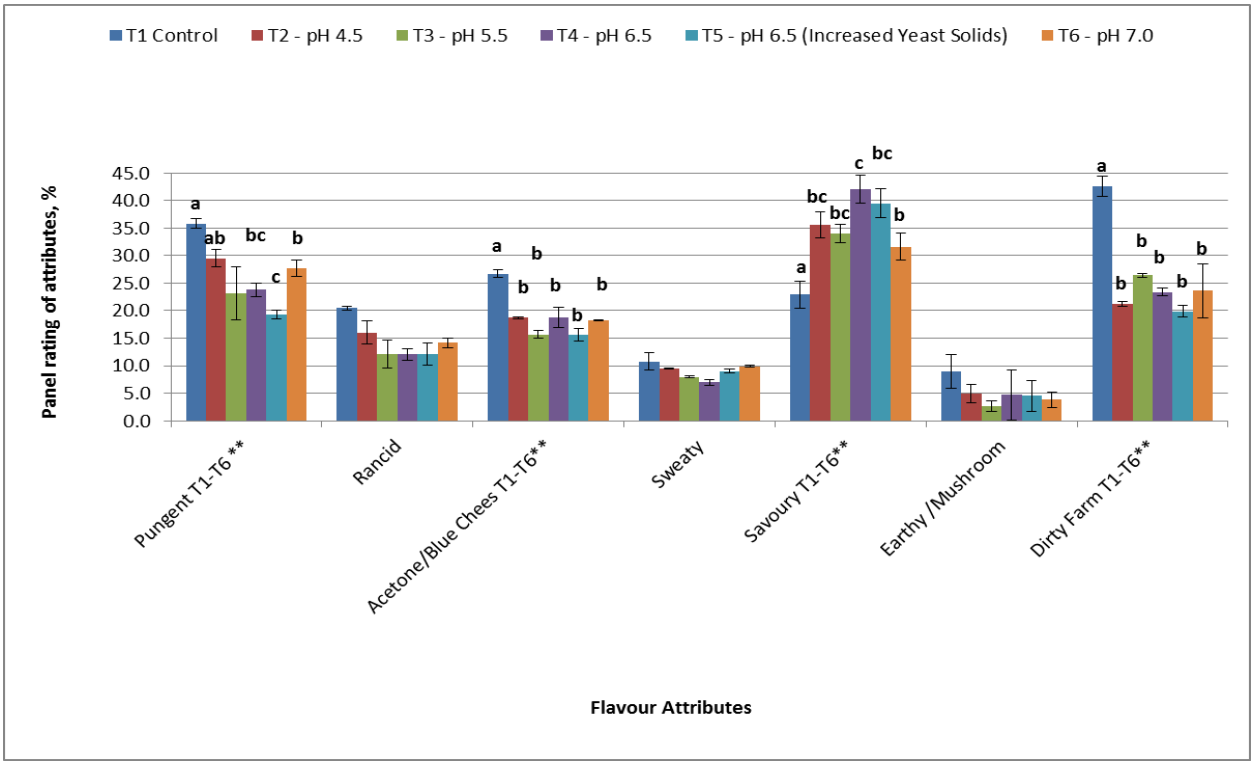


Figure 5.5 Bar chart showing results of six SDYK yeast extracts derived from autolysis trials carried out at different initial pH values and assessed by sensory panel for the selected flavour attributes.

**** indicates attributes in which a significant difference in flavour score existed with respect to six yeast extracts T1-T6. Bars with different letters are significantly different ($p < 0.05$).**

Odour

With respect to odour, the control yeast extract had a significantly higher yeast odour than all the yeast extract samples derived from induced autolysis of SDYK (Figure 5.6), indicating that the addition of autolytic inductors assisted in reducing this odour. Yeast odour of *K. marxianus* can be attributed to 2-phenylethanol and 2-phenylacetic acid which are also characterised by a flowery and sweet aroma respectively (Dragone et al., 2009; Fabre et al., 1997; Fabre et al., 1998). The other odour of interest, mushroom odour, was a distinguishing characteristic of yeast extract T5, a yeast extract derived from induced autolysis using a higher concentration of yeast biomass. Methyl ketones from yeast are derived from fatty acids by β-oxidation or from β-ketoacids, and are known for their mushroom odour (Vitova et al., 2006). Linoleic acid is also enzymatically converted to 1-Octen-3-ol which is known to contribute significantly to the flavor of edible mushrooms (Pan et al., 2007).

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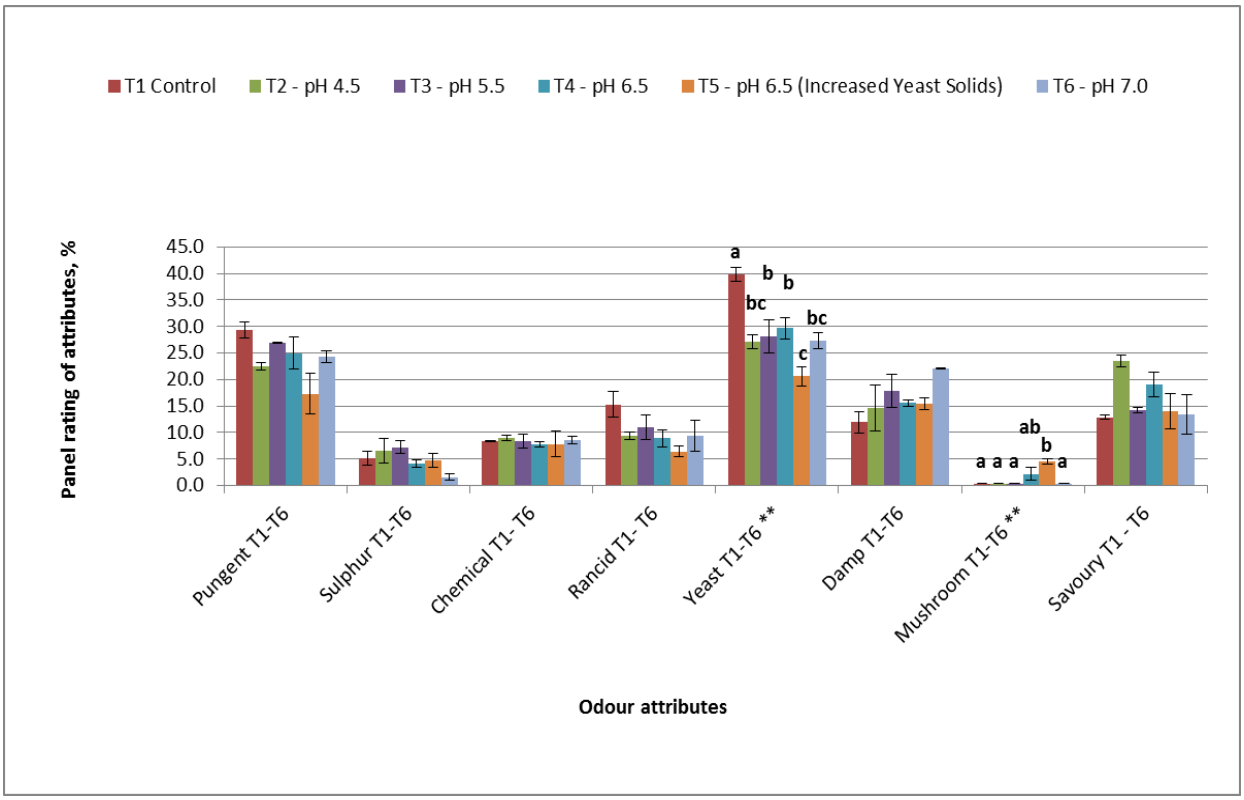

Figure 5.6 Bar chart showing results of six SDYK yeast xtracts derived from autolysis trials carried out at different initial pH values and assessed by sensory panel for the selected odour attributes.

**** indicates attributes in which a significant difference in odour score existed with respect to six yeast extracts T1-T6. Bars with different letters are significantly different ($p < 0.05$).**

The Maillard reaction also has an important role in flavour development of yeast extracts (Nagodawithana, 1992). The Maillard or browning reaction, involves a series of temperature controlled reactions usually beginning with the condensation of a reducing sugar with an amino acid. This study has shown the diverse range of reaction products in SDYK yeast extracts which included free amino acids, peptides, reducing sugars and ribonucleotides. Consequently, taste and odour of yeast extracts are developed during the heat processing step following autolysis which promotes a complex series of reactions involving precursors formed during autolysis

(Nagodawithana, 1992; Yaylayan and Wnorowski, 2001; Varavinet et al.,2000). Of relevance to SDYK is the reaction of lactic acid, commonly produced in whey fermentations, and glutamic acid in the formation of a savoury volatile such as N-lactoyl-L glutamate, which also adds to the flavour enhancing properties of yeast extracts (Frerot and Escher, 1997). Other reactions that occur in the final heat processing of yeast extracts include a combination of fat oxidation products and thermal degradation products of sugars, amino acids, nucleotides and other components. The differences in flavour attributes of SDYK yeast extracts can therefore be related to both the presence of volatiles and non-volatiles which function together in the construction of flavour.

5.4 Conclusion

Descriptive sensory analysis of SDYK yeast extracts developed in this study have indicated that when autolytic inductors were utilised to accelerate autolysis, the yeast extracts were characterised by quite different flavour profiles to autolysis carried out without these inductors. Induced autolysis at pH 6.5 resulted in yeast extracts with a high savoury score, good flavour enhancement and the option of providing lower salt levels in yeast extracts. Alteration of the yeast solids level of SDYK at this pH resulted in a yeast extract with a distinctive mushroom odour. In addition to higher bitter values, the control yeast extract showed a significantly higher pungent sensation ($p < 0.05$) to the other yeast extracts with the exception of yeast extract from induced autolysis at the lowest pH of 4.5 This study has therefore highlighted the importance of autolysis pH and inductors salt and papain in the development of flavoured SDYK yeast extracts.

Induced autolysis of SDYK performed under different conditions of pH should therefore be considered as distinct enzymatic bioprocesses which can be controlled or directed towards the production of yeast extract products with the required flavor attributes. Using this approach, yeast extracts derived from SDYK were shown to be capable of providing additional benefits to conventional yeast extracts which included a novel method of salt reduction and the availability of lighter coloured yeast extracts, which could provide natural flavour enhancement of dairy based and savoury products.

General Conclusions

6.1 General Conclusions

This study has shown that spent dairy yeast *K. marxianus* (SDYK), a by-product of whey to ethanol fermentations, can be utilized effectively to produce natural and alternative yeast extracts with a range of flavour attributes. This was achieved by the improved understanding and consequent development of an induced yeast autolysis process using the autolytic inductors sodium chloride and proteolytic enzyme papain at different pH values.

The overall conclusions based on the objectives of this thesis were:

1) The composition of SDYK biomass indicated this yeast's suitability as a source of alternative flavoured yeast extracts. While the concentration of flavor enhancing amino acids in SDYK was similar to spent brewers yeast, important differences included a significantly higher content of the sweet tasting amino acid alanine and the presence of lactic acid bacteria which are known flavour contributors.

2) Autolysis pH in association with autolytic inductors papain and salt was shown to be a major factor in the development of an induced autolysis process for the production of flavoured yeast extracts using SDYK.

The adjustment of initial pH of autolysis upwards to pH 6.5 yielded the highest free amino nitrogen content and % DH respectively and was attributed to endogenous peptidases of SDYK working synergistically with the added enzyme papain in promoting the release of higher amounts of free amino nitrogen. The role of salt as a plasmolyser during the autolysis process was evidenced by the increased release of solubilised material through the cell wall of SDYK.

3) Measurement of flavour enhancer levels in SDYK yeast extracts indicated that induced autolysis carried out at initial pH values of 5.5 and 6.5 resulted in significantly higher values of the flavour enhancing amino acids glutamic acid and aspartic acid. 5'-IMP was found to be the main flavour enhancing 5'ribonucleotide present in SDYK yeast extracts with the highest value obtained at pH 7.0. These results suggested that similar to yeast proteases, the activities of different RNA degrading enzymes were also influenced by factors such as induced autolysis pH and initial yeast concentration. Optimisation of these factors together with addition of exogenous enzymes such as papain have therefore the potential to allow autolysates with the desired amounts of ribonucleotides, free amino acids and peptides to be produced.

4) Descriptive sensory analysis together combined with the statistical technique, PCA, separated the developed yeast extracts on the basis of addition of autolytic inductors, salt and papain. Autolysis carried out without inductors resulted in yeast extracts characterised by a a significantly higher bitter taste, pungent flavour and acetone/blue cheese flavor. ($p < 0.05$). Further analysis of the significant sensory descriptors showed that induced autolysis at pH 6.5 resulted in yeast extracts with a high savoury flavour score and a significantly higher salty basic taste when compared to all yeast extracts ($P < 0.05$). This was attributed to the synergistic action of 5'IMP, glutamic acid and salt which in addition to enhancing savoury flavour and salt perception were also contributing to a reduction in harsh and undesirable flavours. This induced autolysis process could therefore be considered as a novel approach to reduce the sodium content in dairy yeast extracts without a detrimental impact on taste.

This study has shown that desirable flavour profiles of SDYK yeast extracts can be achieved the addition of autolytic inductors and by alteration of pH of the induced yeast autolysis process. The results from both biochemical and descriptive sensory analysis would support the conclusion that autolysis of SDYK performed under different process conditions could be considered as different enzymatic processes capable of producing yeast extracts with particular flavor attributes .An interesting by-product of the autolysis process was yeast cell wall material, a good source of the functional polysaccharides β-glucan and mannan.

The whey-to-ethanol fermentation process is a good example of the efficient use of a natural waste stream such as whey permeate. However, the quantities of SDYK biomass produced as a by-product of this process are likely to increase as a result of increased whey production, coupled with the high demand for both potable and fuel alcohol from renewable sources (Ling, 2008). The production of flavoured yeast extracts from SDYK is therefore relevant in terms of the efficient utilization of a fermentation by-product and the provision of alternative food ingredients. In addition to this, the increasing demand by food manufacturers and consumers for clean label and natural food ingredients have led to growing interest in this area.

The composition and sensory attributes of SDYK yeast extracts therefore offer an alternative to conventional yeast extracts which are mainly derived from bakers and spent brewers yeast (Alexandre, 2011; Tanguler and Erten, 2007; Champagne et al., 1999; Chae et al., 2001; Conway et al., 2001; Liu et al., 2006).

In particular, it is believed that SDYK yeast extracts can deliver important benefits to dairy based and savoury food products ranging from salt reduction to the provision of

light coloured yeast extracts which can provide both natural flavour enhancement and desirable flavour attributes

Further improvements in the development of SDYK yeast extracts will be accelerated by optimization of the induced autolysis process and further assessment of the sensory and ingredient acceptability of SDYK yeast extracts included in suitable model food systems.

Bibliography

Alder-Nissen, J. (1991). Determination of the degree of hydrolysis of food proteins hydrolysates by trinitrobenzene sulfonic acid. *American Chemical Society,* 1256-1262.

Adler-Nissen, J. (1996). Enzymic Hydrolysis of Food Proteins. Applied Science, London.

Aguilar-Uscanga, B. and François, J.M. (2003). A study of the yeast cell wall composition and structure in response to growth conditions and mode of cultivation. *Letters in Applied Microbiology*,37, 268-274.

Akin, C. and Murphy, R.M. (1981). Method for accelerating autolysis of yeast. United States Patent # 4, 285,976.

Alcaide-Hidalgo, J.M., Puego, E., Polo,M.C., and Martinez-Rodriguez (2007). Bioactive Peptides Released under Accelerated Autolysis in a wine model system. *Journal of Food Science,* 72, (7), 276-279.

Aldsworth, T., Dodd, C.E.R., and Waites,W. (2009). Food microbiology, in *Food Science and Technology,* 1st edition, edited by Campbell-Platt, Geofrey. Wiley Blackwell, UK and IUFoST, Chapter 6, p 115-173, ISBN 978-0-632-06421-2.

Alexandre, H. (2011). Autolysis in Yeasts, in *Comprehensive Biotechnology (2nd edition) volume 2, Engineering Fundamentals of Biotechnology,* edited by Murray Moo-Young, Elsevier B.V. Chapter 2.45, p. 641-649. ISBN: 978-0-08-088504-9.

Amrane, A. and Prigent, Y. (1996). Behaviour of the yeast *Kluyveromyces marxianus* var. *marxianus* during its autolysis. *Antonie van Leewenhoek*,69, 262-272.

Amrane, A. and Prigent, Y. (1998). Effect of culture conditions of *Kluyveromyces marxianus* on its autolysis, and process optimization. *Bioprocess Engineering,* 18, 383-388.

Andreu, G., Benaiges, M.D., Lopez, J. and Sola, C. (1988) A simple method for RNA extraction from yeasts. *Biotechnology and Bioengineering,* 32, 927-929.

AOAC (1980). *Official methods of analysis*, 15th edn. Arlington VA: Association of Official Agricultural Chemists. Washington, DC, pp.1422.

Azarkan, M., El Moussaoui, A., van Wuytswinkel, D., Dehon, G and Looze, Y. (2003). Fractionation and purification of the enzymes stored in the latex of *Carica papaya*. *Journal of Chromatography*, 790, 229-238.

Babayan, T.L., Bezrukov, M.G., Latov, V.K., Belikov, V.M., Belavtseva, E.M. and Titova, E.F. (1981). Induced autolysis of *Saccharomyces cerevisiae*: morphological effects, rheological effects and dynamics of accumulation of extracellular hydrolysis products. *Current Microbiology,* 5, 163-168.

Babayan, T.L.and Berzukov, M.G. (1984). About intermediate products from induced autolysis of intracellular proteins of yeasts. *Acta Biotechnologica*, 4, 129-136.

Babayan, T.L, Bezrukov, M.G. (1985). Autolysis in yeasts. *Acta Biotechnologica,* 5, 129,136.

Babayan, T. L. and Latov, V. K. (2003). Kinetic characteristics of protein hydrolysis during the process of induced utolysis of the biomass of *Saccharomyces cerevisiae*. *Applied Biochemistry and Microbiology,* 39(6), 559-563.

Banat, I.M., Singh, D. and Marchant, R. (1996). The use of a thermotolerant fermentative *Kluyveromyces marxianus* IMB3 yeast strain for ethanol production. *Acta Biotechnologica,* 16, 215-223.

Banchathanakij, R. and Suphantharika, M. (2009). Effect of different β-glucans on the gelatinisation and retrogradation of rice starch. *Food chemistry,* 114, 5-14.

Bath, M.K. (2000). Cellulases and related enzymes in biotechnology. *Biotechnology Advances,* 18(5), 355-383.

Becerra, M., Cerden, E. and Siso, M.I.G. (1998). Microscale purification of beta-galactosidase from *Kluyveromyces lactis* reveals that dimeric and tetrameric forms are active. *Biotechnology Techniques,* 12, 253-256.

Bekatorou, A., Psarianos, C., and Koutinas, A. (2006). Production of Food Grade Yeasts. *Food Technol. Biotechnol*., 44 (3), 407-415.

Belem, M.A.F., Gibbs, B.F. and Lee, B.H. (1997). Enzymatic production of ribonucleotides from autolysates of *Kluyveromyces marxianus* grown on whey. *Journal of Food Science,* 62, 851-857.

Belem, M.A.F. (1999). Application of biotechnology in the product development of nutraceuticals in Canada. *Trends in Food Science & Technology,* 10, 101-106.

Belem, M.A.F. and Lee, B.H. (1999). Fed-batch fermentation to produce oligonucleotides from *Kluyveromyces marxianus* grown on whey. *Process Biotechnology* 34, 501-509.

Belem, M.A.F. and Lee, B.H. (1998a). Production of Bioingredients from *Kluyveromyces marxianus* grown on whey: an alternative. *Critical reviews in Food Science and Nutrition,* 38 (7), 565-598.

Belem, M.A.F. and Lee, B.H. (1998b). Oligosaccharides extracted from cell walls of *Kluyveromyces marxianus* grown on whey. *Biotechnology Techniques,* 12(3), 229-233.

Belem, M.A.F., Gibbs, B.F. and Lee, B.H. (1998). Proposing sequences for peptides derived from whey fermentation with potential bioactive sites. *Journal of Dairy Science,* 82, 486-493.

Bellisle, F. (1999). Glutamate and UMAMI taste: sensory, metabolic, nutritional and behavioural considerations. A review of the literature published in the last 10 years. *Neuroscience and Biobehavioural Reviews*, 23, 423-438.

Ben-Hassan RM, Ghaly AE, Ben-Abdullah N. (1992). Metabolism of cheese whey lactose by *Kluyveromyces fragilis* for energy and growth under batch conditions. *Applied Biochem Biotechnol*, 33: 97–116.

Benton-Jones Jr, J. (1991). Kjeldhal method for nitrogen determination. Georgia, USA: *Micro-Macro Publishing.*

Bisswanger, H. (2004). *Practical Enzymology*, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, 255

Boyce, C.O.L. (1986). Methods for monitoring protease reactions. In *Novo's Handbook of Practical Biotechnology*, pp. 104-108. Novo Industri A/S Bagsvaerd, Denmark.

Breslin, P.A.S. (2001). Human Gustation and Flavour. *In Food Flavours and Chemistry, Advances of the New Millenium*, Edited by Spanier, A.H., Royal Society of Chemistry, pp. 33-64. ISBN: 0-85404-875-8.

Cameron, D.R., Cooper, D.G. and Neufeld, R.J. (1988). The mannoprotein of *Saccharomyces cerevisiae* as an effective bioemulsifier. *Applied and Environmenal Microbiolgy*, 54 (6), 1420-1425.

Carbery. (2012). http://www.carbery.com/index.asp?PageID=42.(Acessed 27/5/2012).

Cardona, A.C. and Sanchez, O.J. (2007). Fuel ethanol production: process design, trends and integration opportunities. *Bioresource Technology*, 98, 2415-2457.

Carvalho-Silva, M. and Spencer-Martins, I. (1990). Modes of lactose uptake in the yeast species *Kluyveromyces marxianus*. Antonie van Leeuwenhoek, 57, 77-81.

Castillo, F.J. (1990). Lactose metabolism by yeasts. *Bioprocess Technology*, 5, 297-320.

Cerny, C. and Guntz-Dubini, R. E. (2008). Identification of 5-Hydroxy-3-Mercapto-2- pentanone in the Maillard Reaction of Thiamine, Cysteine and Xylene. *Journal of Agriculture and Food Chemistry*, 56, 10679-10682.

Chae, H.J., Joo, H and In, M-J. (2001). Utilization of brewer's yeast cells for the production of food-grade yeast extract. Part 1: effects of different enzymatic treatments on solid, protein recovery and flavour characteristics. *Bioresource Technology*, 76, 253-258

Champagne, C.P., Barrette, J. and Goulet, J. (1999). Interaction between pH, autolysis promoters and bacterial contamination on the production of yeast extracts. *Food Research International*, 32, 575-583.

Champagne, C.P., Gaudreau, H., Conway, J. (2003). Effect of the production or use of bakers' or brewers' yeast extracts on their ability to promote growth of *lactobacilli* and *pediococci. Electronic Journal of Biotechnology*, 6, (3) 1-8.

Chao, K.C., McCarthy, E.F. and McConaghy, G.A. (1980). Yeast autolysis process. Unites Stated Patent Application 4,218,481.

Charpentier, Claudine.; Aussenac, Jerome.; Charpentier, Monique., Prome, Jean-Claude.; Duteurtre, Bruno., and Feuillat Michel (2005). Release of nucleotides and nucleosides during yeast autolysis: Kinetics and potential impact on flavour. *J. Agric Food Chem*., 53, (8) 3000-3007.

Chiang, W.D., Shin, C.J and Chu, Y.H. (1999). Functional properties of soy protein hydrolysate produced from a continuous membrane reactor system. *Food Chemistry*, 65, 189-194.

Chobert, J.M., Bertrand-Harb, C. and Nicolas, M.G. (1988). Solubility and emulsifying properties of casein and whey proteins modified enzymatically by trypsin. *Journal of Agricultural and Food Chemistry*, 36, 883-892.

Christensen, A.D., Kadar, Z., Oleskowicz-Popiel, P. and Thomsen, M.H. (2011). Production of bioethanol from organic whey using *K. marxianus*. *J. Ind. Microbiol Biotechnol*., 38, (2) 283-289.

Cogan, U., Moshe, M. and Mokady, S (1981). Debittering and nutrional upgrading of enzymic casein hydrolystes. *Journal of Science and Food Agriculture*, 32, 459-466.

Conway**,** J., Gaudreau, H. and Champagne, C.P. (2001). The effect of the addition of proteases and glucanases during yeast autolysis on the production and properties of yeast extracts. *Canadian Journal of Microbiology*. 47, 18-24.

Cortes, G, Trujillo-Roldan, M.A., Ram, O.T. and Galindo, E. (2005). Production of β-galactosidase by *Kluyveromyces marxianus* under oscillating dissolved oxygen tension. *Process Biochemistry* 40, 773-778.

Cristiani-Urbina, E., Netzahuatl-Munoz, A.R., Manriquez-Rojas, F.J., Juarez-Ramirez, C., Ruiz-Ordaz, N. and Galindez-Mayer, J. (2000). Batch and fed-batch cultures for the treatment of whey with mixed yeast cultures. *Process Biochemistry* 35, 649-657.

Cunningham, S.D., Cater, C.M. and Matil, K.F. (1975). Rupture and protein extraction of petroleum-grown yeast. *Journal of Food Science* 40, 732-735.

Dallies, N., François, J. and Paquet V. (1998). A new method for quantitative determination of polysaccharides in the yeast cell wall. Application to the cell wall defective mutants of *Saccharomyces cerevisiae*. *Yeast* 14, 1297-1306.

Darwicz, M., Dziuba, J. and Caessens, P.W. Jr. (2000). Effect of enzymatic hydrolysis on emulsifying and foaming properties of milk proteins- a review. *Polish Journal of Food Nutrition Science*, 9 (50), 3-8.

Davis, M. (2001). Market and Innovation: The development of the market in the US, in *Proceedings of the 3rd International Whey Conference. Munich 2001- The importance of whey and whey components in food and nutrition*. Chapter 1.2, pp 29 – 45.Behrs-Verlag GmbH. ISBN 3-86022-890-0.

Deive, F. J., Costas, M. and Longo, M. A. (2003). Production of a thermostable extracellular lipase by *Kluyveromyces marxianus. Biotechnology Letters*, 25, 1403-1406.

Deoda, A.J. and Singhal R.B. (2003). 5'-Phosphodiesterase (5'-PDE) from germinated barley for hydrolysis of RNA to produce flavour nucleotides. *Bioresource Technology*, 88, 245-250.

De Palma Revillion, J.P., Brandelli, A.and Záchia Ayub, M.A. (2003). Production of yeast extract from whey using *Kluyveromyces marxianus. Brazilian Archives of Biology and Technology*, 46 (1), 121-128.

Drago, S.R. and Gonzalez, R.J. (2001). Foaming properties of enzymatically hydrolysed wheat gluten. *Innovative Food Science and Emerging Technologies*, 1, 268-273.

Dragone, G., Mussatto, S.I., Oliveira, J.M. and Teixeira, J.A. (2009). Characterisation of volatile compounds in an alcoholic beverage produced by whey fermentation. *Food Chemistry*, 112, 929-935.

Dubois, M., Giles, K.A., Hamilton, J.K., Rebers, P.A. and Smith F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical chemistry* 28, 350-352.

Dziezak, J.D., (1987). Yeasts and Yeast Derivatives: Applications. *Food Technology* (February) 122-125.

Elliott, W.H. and Elliott, D.C. (2001). Nucleotide Metabolism in *Biochemistry and Molecular Biology*, 2nd Edn, Oxford University Press, UK. p. 287-298.

Enfors, Sven-Olof. (2001). Bakers Yeast. In *Basic Biotechnology*, edited by Ratelidge and Kristiansen, Cambridge University press, Chapter 17, 377-389.

EPA (USA), Environmental Protection Agency (2012). Yeast Extract Hydrolysates from*Saccharomycescerevisiae*.
http://www.epa.gov/oppbppd1/biopesticides/ingredients (Last accessed: 27/5/2012).
EPA, Environmental Protection Agency (2012). National Strategy on Biodegradable Waste(April2006).
http://www.epa.ie/downloads/advice/waste/municipalwaste/name,27246,en.html.

Eurasyp (2012). European association for speciality yeast products. available: http://www.eurasyp.org/public.eurasyp.home.screen (Last accessed: 20/05/2012)

EBC -European Brewing Convention (1975). 'Free alpha amino nitrogen in worts and beers.'. *Analytica*: 61. Third edition. Schweizer-Brauerei-Rundschau, Zürich.Fabre,

Fabre, C.E., Blanc, P.J. and Goma,G. (1997). Screening of yeasts producing 2-phenylethylalcohol. *Biotechnology techniques, (11) No.7, 523-525.*

Fabre, C.E., Blanc, P.J. and Goma, G. (1998). Production of 2-phenylethylalcohol by *Kluyveromyces marxianus. Biotechnol Prog*, 14, 270-274.

Fenelon, M.A., O'Connor, P. and Guinee, T.P. (2000). The effect of fat content on the microbiology and proteolysis in cheddar cheese during ripening. *Journal of Dairy Science*, 83, 2173-2183.

Fersht, A. (1999). *Structure and mechanism in protein science: A guide to enzyme catalysis and protein folding*. W.H. Freeman and Company, New York, p. 631.

Festring, D and Hofmann, T. (2010). Discovery of N2-(1-Carboxyethyl guanosine 5'-monophosphate as an umami-enhancing Maillard-modified nucleotide in yeast extracts. *Journal of Agricultural and Food Chemistry*, 58, 10614-10622.

Fitzgerald, R.J. and O' Cuinn, G. (2006). Enzymatic debittering of food protein hydrolysates. *Biotechnology Advances*, 24 (2), 234-237.

Fonseca, G.G., Heinzle, E., Wittmann, C. and Gorbert, A.K. (2008). The yeast *Kluyveromyces marxianus* and its biotechnological potential. *Applied. Microbiology and Biotechnology*, 79, 339–354.

Freimund, S., Sauter, M., Käppeli, O. and Dutler, H. (2003). A new non-degrading isolation process for 1,3-β-D-glucan of high purity from baker's yeast *Saccharomyces cerevisiae*. *Carbohydrate Polymers*, 54, 159-171.

Friedman, M. (1996). Nutritional value of proteins from different food sources. A review. *Journal of Agricultural and Food Chemistry*, 44, 6-29.

Geciova, J.,Bury, D., and Jelen, P. (2002). Methods for disruption of microbial cells for potential use in the dairy industry – a review. *International Dairy Journal*, (12) 6, 541-553.

Ghaly, A.E., Ben-Hassan, R.M., Mansour, M.H. and Nassar, M.A. (1993). Modeling batch production of single cell protein from cheese whey, II: Lactose metabolism. *Applied Biochemistry and Biotechnology*, 43, 15-24.

Ghaly, A.E. and Kamal, M.A. (2004). Submerged yeast fermentation of acid cheese whey for protein production and pollution potential reduction. *Water Research*, 38, (3) 631-644.

Giec, A. and Kosikowski, F.V. (1982). Activity of lactose fermenting yeasts in producing biomass from concentrated whey permeates. *Journal of Food Science* 47, 1892-1894, 1907.

Godfrey, T. (1996). Protein modification in industrial enzymology. 2nd Ed., Macmillan Publishers Inc, New York, N.Y edited by Godfrey, T. and West, S. Chapter 2.18., p.301- 325. ISBN 0-333-59464-9.

Gonzalez- Siso, M.I. (1996). The biotechnological utilization of cheese whey: a review. *Bioresource Technology* ,57, 1-11.

Goodno, C.C., Swaisgood, H.E. and Catignani, G.L. (1981). A flurometric assay for available lysine in proteins. *Analytical Biochemistry*, 115, 203-211.

Gormley, R and Holm, F. (2010). Functional Foods: Some Pointers for Success. e-Publication, UCD Institute of Food and Health, Belfield, Dublin 4, Ireland, 1 December 2010. ISBN: 978-1-905254-53-8.

Gough, S., Flynn, O., Hack, C.J. and Marchant, R. (1996). Fermentation of molasses using a thermotolerant yeast, *Kluyveromyces marxianus* IMB3: simplex optimisation of media supplements. *Applied Microbiology and Biotechnology*, 46, 187-190.

Grba, S., Stehlik-Tomas, V., Stanzer, D., Vahcic, N. and Skrlin, A. (2002). Selection of yeast strain *Kluyveromyces marxianus* for alcohol and biomass production on whey. *Chem. Biochem. Eng*. Q. 16, 13-16.

Guadix, A., Camacho, F. and Guadix, E.M. (2006). Production of whey protein hydrolysates with reduced allergenicity in a stable membrane reactor. *Journal of Food Engineering*, 72, 398-405.

Guimarães, P M R, Teixeira, J A and Domingues, L (2010). Fermentation of lactose to bio-ethanol by yeasts as part of integrated solutions for the valorisation of cheese whey. *Biotechnology Advances*, 28, 375-384.

Hack, C.J. and Marchant, R. (1998). Characterisation of novel thermotolerant yeast, *Kluyveromyces marxianus* var. *marxianus*: development of an ethanol fermentation process. *Journal of Industrial Microbiology and Biotechnology*, 20, 323-327.

Halász, A. and Lásztity, R. (1991a). Extraction of yeast proteins, yeast protein concentrates and isolates. In: *Use of yeast biomass in food production*. CRC Press, Inc., 2000 Corporate Blvd. Florida, Chapter 5, p 115-189. ISBN 0-8493-5866-3.

Halász, A. and Lásztity, R. (1991b). Chemical composition and biochemistry of yeast biomass. In: *Use of Yeast Biomass in Food Production*. CRC Press, Inc., 2000 Corporate Blvd. Florida, Chapter 3, p. 23-44. ISBN 0-8493-5866-3.

Halász, A. and Lásztity, R. (1991c). Chemical composition and biochemistry of yeast biomass. In: *Use of Yeast Biomass in Food Production*. CRC Press, Inc., 2000 Corporate Blvd. Florida, Chapter 8, p. 215-281. ISBN 0-8493-5866-3.

Hannon, J.A.,Wilkinson, M.G., Delahunty, C.M., Wallace, J.M., Morrissey, P.A. and Beresford, T.P. (2005). Applications of descriptive sensory analysis and key chemical indices to assess the impact of elevated ripening temperatures on the acceleration of cheddar cheese ripening. *International Dairy Journal,*15. 263-273.

Hannon, J.A., Beresford, T.P., Wilkinson, M., Delahunty, C.M. and Morrissey, P.A. (2005). The effect of manipulating ripening temperatures on cheddar cheese flavour. In Food Flavours and Chemistry, Advances of the New Millenium, Edited by Spanier, A.H. Royal Society of Chemistry, p. 97-107. ISBN 0-85404-875-8.

Hay, J.D. (1993). Novel Yeast Products from Fermentation Processes. *Journal of Chemical Technology and Biotechnology*, 58, 203-205.

Hayward, A. (2008). On-line, *In-situ*, Measurements within fermenters.. In Practical Fermentation Technology, Chapter 9, pp. 271-288. Editors Brian McNeil and Linda M. Harvey. John Wiley and Sons Ltd.

Herbert, D., Philips P.J. and Strange, R.E. (1971). Chemical analysis of microbial cells. In Norris J. and Ribbons DW (Ed.) *Methods in Enzymology*, London Academic Press.

Herbertz, G. (2001) in foreword of Proceedings of the 3rd International Whey Conference. Munich 2001- The importance of whey and whey components in food and nutrition. Behrs-Verlag GmbH. ISBN 3-86022-890-0.

Hernawan, T. and Fleet, G. (1995). Chemical and cytological changes during the autolysis of yeasts. *Journal of Industrial Microbiology,* 14, 440-450.

Hill R.L. (1965). Hydrolysis of proteins. *Advances in Protein chemistry*, 20:37-107.

Hong, Z.H.I., M., P., Shaw, K.J., Didomenico, B. (1994). 'Analysis of beta glucan and chitin in a *Saccharomyces cervisiae* cell wall mutant using high performance liquid chromatography'. *Yeast*, 10: 1083-1092.

Hough, J.S. and Maddox, I.S. (1970). Yeast autolysis. *Process Biochemistry*, 50-52.

Hrckova, M., Rusnakova, M. and Zemanovic, J. (2002). Enzymatic hydrolysis of defatted soy flour by three different proteases and their effect on the functional properties of resulting hydrolysates. *Czech Journal of Food Science*, 20 (1), 7-14.

Hromádková, Z., Ebringerová, A., Sasinková, V., Šandula, J., Hříbalová, V. and Omelková, J. (2003). Influence of the drying method on the physical properties and immunomodulatory activity of the particulate (1→3)-β-D-glucan from *Saccharomyces cerevisiae*. *Carbohydrate Polymers*, 51, 9-15.

Hung, N.D., Vas, M., Cheke, E. and Bolcsi, S.Z.A. (1984). Relative tryptic digestion rates of food proteins. *Journal of Food Science*, 49, 1535-1542.

Inchaurrondo, V.A., Yantorno, O.M. and Voget, C.E. (1994). Yeast growth and β-galactosidase production during aerobic batch cultures in lactose-limited synthetic medium. *Process Biochemistry,* 29, 47-54.

Jaehrig, S.C., Rohn, S., Kroh, L.W., Wildenauer, F.X., Lisdat, F., Fleischer, L-G. and Kurz, T. (2008). Antioxidative activity of (1→3), (1→6)-β-D-glucan from *Saccharomyces cerevisiae* grown on different media. *LWT,* 41, 868-877.

Jamas, S., Rha, C., Sinskey, A. J. (1989). 'Glucan composition and process for preparation thereof'. *United States patent application, 4,810646.*

Jamas, S., Easson, D.D. and Bristian, B.R. (1990). Glucan dietary additives. *United States patent application*, 4.962.094 Oct. 9, (1990).

Kakes, Erik. (2008) Scada Systems for Bioreactors. *In Practical Fermentation Technology*, Chapter 10, p. 289-322. Editors Brian McNeil and Linda M. Harvey. John Wiley and Sons Ltd.

Kammoun, R., Bejar, S. and Ellouz, R. (2003). Protein size distribution and inhibitory effect of wheat hydrolysates on Neutrase. *Biosource Technology,* 90, 249-254.

Kapteyn, J. C., Van den Ende, H., Klis F.M. (1999). The contribution of cell wall proteins to the organization of the yeast cell wall. *Biochimica et Biophysica Acca (BBA) General subjects*, 1426(2): 373-383.

Kath F. and Kulicke W.M. (1999). Mild enzymatic isolation of mannan and glucan from yeast *Saccharomyces cerevisiae*. *Die Angewandte Makromolekulare Chemie,* 268, 59-68,

Kelly, M. (1983*). Yeast Extract in Industrial Enzymology*, 1st Ed., Macmillan Publishers Inc, New York, edited by Godfrey, T. and Reichelt, J., Chapter 4.24, p.457-465, ISBN 0-943818-00-1.

Keogh, S. (2006). *The Development of Functional Food Ingredients from Yeast Cell Wall β-glucans*. Master thesis, Institute of Technology, Tralee, Ireland.

Kim, J.H., Lee, B.H. and Lee, J.S. (2002). Production of ribonucleotides by autolysis of *Hansenula anomala* grown on Korean ginseng steaming effluent. *Journal of Bioscience and Bioengineering,* 93, 318-321.

Kim, S.K. and Yun, H.S. (2006). Production of soluble β-glucan from the wall of *Saccharomyces cerevisiae*. *Enzyme and Microbial Technology* ,39, 496-500.

Klis, F. M. (1994). 'Review: Cell wall assembly in yeast. *Yeast*, 10: 851-869.

Klis, F.M., Mol, P., Hellingwerf, K. (2002). Dynamics of cell wall structure in *Saccharomyces Cervisiae. FEMS Microbiology Reviews,* 26: 239-259.

Kogan G. and Kocher A. (2007). Role of yeast cell wall polysaccharides in pig nutrition and health protection. *Livestock Science,* 109 (1-3), 161-165.

Kogan, G. (2000). (1→3, 1→6)-β-D-glucans of yeast and fungi and their biological activity. Atta-ur-Rahman (Ed.). *Studies in Natural Products Chemistry,* 23, 107-152.

Kollar, R., Petrakova, E., Ashwell, G., Robbins, P.W., Cabib E. (1995). Architecture of the yeast cell wall: the linkage between chitin and β- (1-3)-glucan. *Journal of Biological Chemistry,* 270: 1170-1178.

Kollar, R., Reinhold, B.B., Petrakova, E., Yeh, H.J.C., Ashwell, G., Drgonova, J.,

Kong, X., Zhou, H. and Qian, H. (2007) Enzymatic preparation and functional properties of wheat gluten hydrolysates. *Food Chemistry,* 101, 615-620.

Koutinas, A.A., Wang, R.H. and Webb, C. (2005). Development of a process for the production of nutrient supplements for fermentation based on fungal autolysis. *Enzyme and Microbial Technology,* 26, 629-638.

Kourkoutas, Y., Dimitropoulou, S., Kanellaki, M., Marchant, R., Nigam, P., Banat, I.M. and Koutinas, A.A. (2002) High-temperature alcoholic fermentation of whey using *Kluyveromyces marxianus* IMB3 yeast immobilized on delignified cellulosic material. *Bioresource Technology,* 82, 177-181.

Kratochvilova, Anna Kockova (1990). Chemical composition of yeast cells in yeasts and yeast-like organisms. VCH Publishers, p. 131-221, ISBN No. 0-89573-229-7.

Kristiansen Bjorn, (1994). Integrated design of a fermentation plant. VCH and Weinheim. ISBN 3-527-30029-5.

Kurtzman, C.P. (1990). Classification and general properties of yeasts. *Bioprocess Technology,* 5, 4-5.

Kurtzman, C. and Fell, J. (2006). Yeast systematic and phylogeny - implications of molecular identification methods for studies in ecology. Available: http://www.ars.usda.gov/research/publications/publications.htm?SEQ_NO_115=176765 (Last accessed: 03/02/2012).

Lahl, J.W. and Grindstaff, D.A. (1994). Spices and seasonings: hydrolysed proteins. In: *Proceedings of the 6th SIFST Symposium on Food Ingredients- Applications, Status and Safety*, Institute of Food Science and Technology, Singapore, p. 27-29.

Lane, M.M. and Morrissey J.P. (2010). *Kluyveromyces marxianus:* A yeast emerging from its sister's shadow, *Fungal Biology Reviews,* p1-10.

Lawther, M. (2010). β-Glucans and cereal soluble fibres: their role in functional foods. In functional, foods: Some Pointers for Success. Edited by Gormley and Holm, e-Publication, UCD.

Lemieux, L., Puchades, R. and Simard, R. E. (1989). Size-Exclusion HPLC separation of bitter and astringent fractions from cheddar cheese made with added lactobacillus strains to accelerate ripening. *Journal of Food Science,* 54, 1234-1237.

Lemieux, L. and Simard, R., E. (1992). Bitter flavour in dairy products. II. A review of bitter peptides from caseins: their formation, isolation and identification, structure masking and inhibition. *Lait,* 72, 335-385.

Lenney, J F (1975). Three yeast proteins that specifically inhibit yeast proteases A, B, and C. *J. Bacteriol,* 122, 1265-1273.

Lenney, J F and Dalbec, J M (1969). Yeast proteinase B: Identification of the inactive form as an enzyme-inhibitor complex. *Archives of Biochemistry and Biophysics,* 129, 407-409.

Leung, M.Y.K., Liu, C., Koon, J.C.M. and Fung, K.P. (2006). Polysaccharide biological response modifiers. *Immunology Letters,* 105, 101-114.

Limtong, S., Sringiew, C., and Yongmanitchai, W. (2007). Production of fuel ethanol from sugar cane juice by a newly isolated *Kluyveromyces marxianus*. *Bioresource Technology*, 98, 3367-3374.

Ling, K.C. (2008). Whey to Ethanol: A biofuel role for dairy cooperatives. USDA rural development, Rural Business and Cooperative Programs, Research Report 214.

Lipke, P.N. and Ovalle, R. (1998). Cell wall architecture in yeast: New structure and new challenges. *Journal of Bacteriology,* 180, 3735-3740.

Liu, X., Wang, Q., Cui, S.W. and Liu, H. (2006). A new isolation method of β-D-glucans from spent yeast *Saccharomyces cerevisiae*. *Food Hydrocolloids,* 22, 239-247.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry,* 193, 265-275.

Lukondeh, T., Ashbolt, N.J. and Rogers, P.L. (2003a). Evaluation of *Kluyveromyces marxianus* as a source of yeast autolysates. *J Ind. Microbial Biotechnology,* 30, 52-56.

Lukondeh, T., Ashbolt, N.J. and Rogers, P.L. (2003b). Evaluation of *Kluyveromyces marxianus* FII 510700 grown on a lactose-based medium as a source of a natural bioemulsifier. *J Ind. Microbial Biotechnology,* 30, 715-720.

Lukondeh, T., Ashbolt, N.J., Rogers, P.L. and Hook. M. (2003). NMR confirmation of an alkali-insoluble glucan from *Kluyervomyces marxianus* cultivated on a lactose-based medium. *World Journal of Microbiology and Biotechnology,* 19, 349-355.

Madsen, J.S., Ahmt, T.O., Otte, J., Halkier, T. and Qvist, K.B. (1997). Hydrolysis of β-lactoglobulin by four different proteinases monitored by capillary electrophoresis and high performance liquid chromatography. *International Dairy Journal*, 7, 399-409.

Manners, D. J., Mason, A. J., Patterson, L.C (1973a). The structure of a β- (1,3)-D-glucan from yeast cell walls. *Biochemical Journal*, 135, 19-30.

Manners, D. J., Mason, A. J., Patterson, L.C (1973b). The structure of a β- (1-6)-D-glucan from yeast cell walls. *Biochemical Journal*, 135, 31-36.

Market&Markets (2012) Global yeast market by types, applications, & geography trends & forecasts (2011 - 2016). http://www.marketsandmarkets.com/Market-Reports/yeast-industry-268.html.

Martinez-Rodriguez, A.J., Polo, M.C. and Carrascosa, A.V. (2001). Structural and ultrastructural changes in yeast cells during autolysis in a model wine system and in sparkling wines. *International Journal of Food Microbiology*, 71, 45-51.

Matern, H, Hoffmann, M and Holzer, H (1974). Isolation and characterization of the carboxypeptidase Y Inhibitor from yeast. *Proceedings of the National Academy of Sciences of the United States of America,* 71, 4874-4878.

Matsushita, I. and Ozaki, S. (1994). Purification and sequence determination of tasty tetrapeptide (Asp-Asp-Asp-Asp) from beer yeast seasoning and its enzymatic synthesis. *Peptide Chemistry*, 32, 249-251.

Matoba, T. and Hata, T. (1972). Relation between bitterness of peptides and their chemical structures. *Agriculture and Biological Chemistry,* 36, 1423-1431.

McCarra, B. (2006) The characterisation of commercial protease enzymes for the production of protein hydrolysates using soya protein, casein, and wheat gluten as subtrates. *Enterprise Ireland & Kerry Food Ingredients Innovation Partnership Program* (2005-2006). *IP / 2005 / 0664,* Final Technical Report.

McCarra, B., (1983) Production of Xanthan Gum from Whey. MSc Thesis, National University of Ireland, NUIG, 18-23.

McCarra, B. (2002). Yeast research at IT Tralee can prevent millions going to waste. Available:http://www.irishscientist.ie/2002/contents.asp?contentxml=02p128a.xml&contentxsl=is02pages.xsl (Last accessed: 13/03/2012).

McCarra, B. (2001). Development of added value products from brewery wastes. *Enterprise Ireland & Dromkeen Food Ingredients funded Applied Research Program* (1998-2001), AR /97/182, Final Technical Report, p 1-8.

McCarra, B. (2005). Bioprocessing of Natural Products, in Irish Scientist, Editor Geraldine Van Esbeck, Oldbury Publishing, Vol. 13, p 58-59.

MacFie,H.J, Bratchell, N.,Greenhoff, K, Lloyd,K. ,Vallis, V.(1989). Designs to balance the effect of order of presentation and first-order carry- over effects in hall test. *Journal of Sensory Studies,* Volume 4, Issue 2, 129–148.

Milić, T.V., Rakin, M. and Šiler-Marinković, S. (2007). Utilization of baker's yeast (*Saccharomyces cerevisiae*) for the production of yeast extract: effects of different enzymatic treatments on solid, protein and carbohydrate recovery. *Journal of Serbian Chemistry Society*, 72, (5), 421-457.

Miller, G.L. (1959). Use of dinitrosalicyclic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 31, 426-428.

Mion-Du Crest. 1998. Bakers Yeast Products – Enhancing the flavour. *The European Food and Drink Review,* (Autumn 1998), 69-71.

Mckinstry, P. J., Indky, H. E. and Kim, N. D. (1999). The determination of major and minor elements in milk formula by slurry nebulisation and inductivity coupled plasma – optical emission spectrometry (ICP-OES). *Food Chemistry*, 65, 245-252.

Molano, J., Bowers, B., Cabib, E. (1980). Distribution of chitin in the yeast cell wall. *The Journal of Cell Biology*, 85, 199-212.

Molimard, P. and Spinnler, H. E. (1996). Review: Compounds involved in the flavor of surface mould-ripened cheeses: Origins and properties. *Journal of Dairy Science*, 79, 169-184.

Monti D., Ferrandi, E.E., Righi, M., Romano, D. and Molinari F. (2008). Purification and characterization of the enantioselective esterase from *Kluyveromyces marxianus* CBS 1553. *Journal of Biotechnology*, 133, 65-72.

Moore, S. and Stein, W.H. (1948). Photometric ninhydrin method for use in the chromatography of amino acids. *Journal of Biology and Chemistry*, 176, 367-388.

Moresi, M. (1994), Cost/benefit analysis of yeast and yeast autolysate production from cheese whey. *Italian Journal of Food Science*, 6, 357-370.

Moresi, M., Orban, E., Quaglia, G.B. and Casini, I. (1995). Effect of some physico-chemical treatments on the kinetics of autolysed - yeast extract production from whey. *J. Sci. Food Agri.*, 67, 347-357.

Mosqueira, F.G., Higgins, J.J., Dunnill, P. and Lily, M.D. (1981). Characteristics of mechanically disrupted bakers yeast in relation to its separation in industrial centrifuges. *Biotechnology and Bioengineering*, 23, 335-343.

Mrsa, V, Klebl, F and Tanner, W (1993) Purification and characterization of the *Saccharomyces cerevisiae* BGL2 gene product, a cell wall endo-beta-1,3-glucanase. *J. Bacteriol*, 175, 2102-2106.

Murray, J. M., Delahunty, C. M. and Baxtrer, I. A. (2001). Descriptive sensory analysis: past, present and future. *Food Research International*, 34, 461-471.

Nagodawithana, T. (1992). Yeast-derived flavors and flavor enhancers and their probable mode of action, *Food Technology* ,11, 138–144.

Nandakumar, R., Yoshimune, K., Wakayama, M. and Moriguchi, M. (2003). Microbial glutaminase: biochemistry, molecular approaches and applications in the food industry. *Journal of Molecular Catalysis B: Enzymatic*, 23, 87-100.

National Collection of Yeast Cultures, (2012). Available at http://www.ncyc.co.uk/research.html. (Last accessed 28/5/2012).

Ney, K.H. (1979). Bitterness of peptides: amino acid composition and chain length. *American Chemical Society (ACS) Symposium Series*, *Food Taste Chemistry*, 115, 149-173.

Nilsang, S., Lertsiri, S., Suphantharika, M. and Assavanig, A. (2005). Optimisation of enzymatic hydrolysis of fish soluble concentrate by commercial proteases. *Journal of Food Engineering*, 70, 571-578.

Nishimura, T. and Kato, H. (1988). Taste of free amino acids and peptides. *Food Reviews International*, 4, 175 - 194.

Nitsawang, S., Haiti-Kaul, Rajni. and Kanasawud, P. (2006). Purification of papain from *Carica papaya* latex: Aqueous two-phase extraction versus two-step salt precipitation. *Enzyme and Microbial Technology*, 39, (5) 1103-1107.

Noordam, B. and Kortes J. (2009). Yeast Autolysates. WIPO, Patent Application 2009/007424 A1, PCT /EP2008/059013. p. 1-14.

Noguchi, M. Arai, S. Yamashita, M. Kato, H and Fujimaki, M. (1975). Isolation and identification of acidic oligopeptides occurring in a flavour potentiating fraction from a fish protein hydrolysate. *Journal of Agriculture and Food Chemistry*, 23, 49-53.

Nguyen, T.H., Fleet, G.H. and Rogers, P.L. (1998). Compositions of the cell walls of several species. *Applied Microbiology Biotechnology*, 50, 206-212.

Okiyama, A. and Beauchamp, G.K. (1998). Taste dimensions of monosodium glutamate (MSG) in a food system: role of glutamate in young American subjects. *Physiology and Behaviour*, 65, 177-181.

Okiyama, K.M. and Ueda, Y. (2002). Taste enhancements between various amino acids and IMP. *Chem. Senses*, 27, (8), 739-745.

Orban E., Quaglia, G.B. and Casini, I. (1994). Effect of temperature and yeast concentration on the autolysis of *Kluyveromyces fragilis* grown on lactose-based media. *Journal of Food Engineering*, 21, 245-261.

Orberg P.K and Sandine.W.E, 1984. Autolysate of whey-grown *Kluyverornyces fragilis* as a substitute for yeast extract in starter culture media. *Journal of Dairy Science*, 67, 37-43.

O'Shea, D.G. and Walsh, P.K. (2000). The effect of culture conditions on the morphology of the dimorphic yeast *Kluyverornyces marxianus var. marxianus* NRRLy2415: a study incorporating image analysis. *Appl Microbiol Biotechnol*, 53, 316-322.

Osumi, M. (1998). The ultrastructure of yeast: cell wall structure and formation *Micron*, 29, 207-233.

Ozmihci, S. and Kargi, F. (2007). Kinetics of batch ethanol fermentation of cheese-whey powder (CWP) solution as function of substrate and yeast concentrations. *Bioresource Technology*, 98, (16), 2978-2984.

Palomero, F., Morata, A., Benito, S., Gonzalez, M.C. and Suarez-Lepe, J.A. (2007). Conventional and enzyme-assisted autolysis during ageing over lees in red wines: Influence on the release of polysaccharides from yeast cell walls and on wine monomeric anthocyanin content. *Food Chemistry*, 105, 838-846.

Pan,B.S., Kuo J.M., Wu C.M. (2007). Flavour compounds in foods in *Chemical and functional properties of food components*. 3 Ed CRC Press edited by Zdzislaw E. Silorski ISBN0-8493-9675-1.

Pawlet, D. and Bruce, G. (1996). Debitteing of protein hydrolysates, *In Industrial enzymology. 2nd Ed*., Macmillan Publishers Inc, New York, N.Y edited by Godfrey, T. and West, S. Chapter 2.9, p.179- 186. ISBN 0-333-59464-9.

Pedersen, B (1994). Removing from protein hydrolysis. *Food Technology*, 45, (10), 96-98.

Peppler, H.J. (1979). Production of Yeasts and Yeasts Products, 2nd Ed/Volume 1, Chapter 5, p157-185. Editors H.J. Peppler and D. Perlman, Academic Press, Library of Congress Cataloging in Publication Data, ISBN 0-12-551501-4.

Pelissier, J.P. (1984) Proteolyse des caseines. *Science Aliments*, 4, 1-35.
Penas, E., Prestamo, G., Gomez, R. (2004). High pressure and the enzymatic hydrolysis of soybean and whey proteins. *Food Chemistry*, 85, 641-648.

Phaff, H.J. (1977). Enzymatic yeast cell wall degradation. In: *Advances in Chemistry*. Feeney, R.C. and Whitaker, J.R., Eds. American Chemical Society, Washington, D.C., p. 244.

Popineau, Y., Huchet, B., Larre, C. and Berot, S. (2002). Foaming and emulsifying properties of fractions of gluten peptides obtained by limited enzymatic hydrolysis and ultrafiltration. *Journal of Cereal Science*, 35, 327-335.

Popolo, L, Gilardelli, D, Bonfante, P and Vai, M (1997). Increase in chitin as an essential response to defects in assembly of cell wall polymers in the ggp1delta mutant of *Saccharomyces cerevisiae*. *The Journal of Bacteriology*, 179 463-469.

Prieto, M.L.C. (2009). The development of functional food ingredients by enzymatic treatment of yeast cell wall polysaccharides. Master Thesis, Institute of Technology, Tralee, Ireland.

Roa, M.B., Tanksale, A.M., Ghatge, M.S. and Deshpande, V.V. (1998). Molecular and biotechnological aspects of microbial proteases. *Microbiology and Molecular Biology Reviews*, 62, (3), 597-635.

Ragasso, J.A., Chalier, P., Crouzet, J. and Ghommidh, C. (2001). Identification of alcoholic beverages by coupling gas chromatography and electronic nose. *In Food Flavours and Chemistry, Advances of the New Millenium*, Edited by Spanier, A.H. Royal Society of Chemistry, p. 404-411. ISBN: 0-85404-875-8.

Raksakulthai, R. and Haard, N.F. (2003). Exopeptidases and their application to reduce bitterness in food: A review. *Critical Reviews in Food Science and Nutrition*, 43 (4), 401-445.

Ramirez-Zavala, B., Mercado-Flores, Y., Hernandez-Rodriguez, C. and Villa-Tanaca, L. (2004b). Purification and characterization of a serine carboxypeptidase from Kluyveromyces marxianus. *International Journal of Food Microbiology*, 91, 245-252.

Ramirez-Zavala, B., Mercado-Flores, Y., Hernandez-Rodriguez, C. and Villa-Tanaca, L. (2004b). Purification and characterization of a lysine aminopeptidase from Kluyveromyces marxianus. *FEMS Microbiology Letters*, 235, 369-375.

Reed, Gerald and Nagodawithna, Tilak W. (1991) Yeast Derived Products. *In Yeast Technolgy, 2nd Ed*, Chapter 8, p 369-414. Editors AVI, Library of Congress Catalog, Card Number 90-33144 , ISBN 0442-31892-8.

Revillion, Jean P. de Palma, Brandelli, Adriano and Ayub Marco A. Záchia, Production of yeast extract from whey using *Kluyveromyces marxianus*, 2003 *Brazilian Archives Biology and Technology*, 46(1), 121-128.

Roa, M.B., Tanksale, A.M., Ghatge, M.S. and Deshpande, V.V. (1998). Molecular and biotechnological aspects of microbial proteases. *Microbiology and Molecular Biology Reviews*, 62, (3), 597-635.

Ryan, E.M. and Ward, O.P. (1985). Study of the effect of β-1,3-glucanase from *Basiomycete* QM 806 on yeast extract production. *Biotechnology Letters*, 7(6), 409-412.

Ryan, E.M. and Ward, O.P. (1988). The application of lytic enzymes from *Basiodiomycete aphyllophoroles* in production of yeast extract. *Process Biochemistry*, 23, 12-16.

Saha, B.C. and Hayashi, K. (2001). Debittering of protein hydrolysates. *Biotechnology Advances, 19, 355-370.*

Sandbach, D.M.L. (1981) Alcohol production from whey – Methods and Prospects. Paper presented at Marschall International Cheese Conference, Wisconsin, Sept. 15-18, (1981).

Santipanichwong, R. and Suphantharika, M. (2009). Influence of different β-glucans on the physical and rheological properties of egg yolk stabilized oil-in-water emulsions. *Food hydrocolloids,* 23, 1279-1287.

Sathish, H.A..; Kumar, Parigi Ramesh.; and Prakash. (2007). Mechanism of solvent induced thermal stabilization of papain. *International Journal of Biological Macromolecule,* 41, 383-389.

Satyanarayana, T, Kunze, G, Gummadi, S N, Kumar, D S, Dash, S S and Sahu, S K (2009) Industrially Important Carbohydrate Degrading Enzymes from Yeasts: Pectinases, Chitinases, and β-1,3-Glucanases. In: *Yeast Biotechnology: Diversity and Applications*, p. 673-691 Springer Netherlands.

Sedmak, J.J. (2006). Production of β-glucans and mannans. United States Patent Application Publication. US 2006/02634150 A1. Nov. 23, 2006.

Selmer-Olsen, E. and Sorhaug, T. (1998). Comparative studies of the growth of *Lactobacillus plantarum* in whey supplemented with autolysate from brewery yeast biomass or commercial yeast extract. *Milchwissenschaft,* 53 (7) 367-370.

Sharma, R., Mc Carra, B. and Walsh, D.J. (2012). Development of value added products from spent *Kluveromyces marxianus* yeast waste. In *Smith,C. and Tiwari, B.K. eds International Conference on Food Safety, Quality and Nutrition, Greening the Food Industry,* Manchester Food Research Centre, Manchester Metropolitan University, Manchester UK. p. 97-110.

Shotipruk, A., Kittianong, P., Supthantharica, M. and Muangnapoh, C. (2005). Application of rotary microfiltration in debittering process of spent brewer's yeast, *Biores Technol*, 96: 1851–1859.

Silveira, W.B., Passos, F.J.V., Mantovani, H.C., and Passos F.M.L. (2005). 'Ethanol production from cheese whey permeate by *Kluveromyces marxianus*, UFV-3: A flux analysis of oxido-reductive metabolism as a function of lactose concentration and oxygen levels'. *Enzyme and Microbial Technology*, 36,(7) 930-936.

Silvestre, M.C. (1997). Review of methods for the analysis of protein hydrolysates. *Food Chemistry*, 60, (2), 263-271.

Singh, R.H. and Bhermi H.K. (2008). Production of extracellular exoinulinase from *Kluyveromyces marxianus* YS-1 using root tubers of *Asparagus officinalis*. *Bioresource Technology,* 99, 7418–7423.

Slagle, S.P. and Zimmerman, D.R. 1979. Evaluation of a yeast single cell protein with young pigs. *J. Animal Sci.,* 49(5), 1252.

Smithers, (2008). Whey and whey proteins. International Dairy Journal,18, 695-704 Sombutyanuchit, P., Suphantharika, M. and Verduyn, C., 2001, Preparation of 50-GMP-rich yeast extracts from spent brewer's yeast, *World J Microbiol Biotechnol,* 17, 163–168.

Sombutyanuchit, P., Suphantharika, M. and Verduyn, C., (2001). Preparation of 50-GMP-rich yeast extracts from spent brewer's yeast, *World J Microbiol Biotechnol,* 17, 163–168.

Sommer. R. Yeast extracts: production, properties and components, (1996), *Food Australia,* 50, 181-183.

Spellman, D., McEvoy, E., O' Cuinn, G. and Fitzgerald, R.J. (2003). Proteinase and exopeptidase hydrolysis of whey protein: Comparison of the TNBS, OPA and pH stat methods for quantification of degree of hydrolysis. *International Dairy Journal*, 13, 447-453.

Spellman, D., O' Cuinn and FitzGerald, R.J. (2005). Physiochemical and sensory characteristics of whey protein hydrolysates generated at different total solids levels. *Journal of Dairy Research,* 72, 138-143.

Steensma, A., van Dijck, P.W.M. and Hempenius, R.A. (2004). Safety evaluation of phosphodiesterase derived from *Leptographium procerum*. *Food and Chemical Toxicology,* 42, 935-944.

Stone, Herbert and Bleibaum Rebecca.N (2009) Sensory Evaluation . In *Food Science and Technology*, Chapter 13, p 323-340 Edited by Campbell-Platt, Wiley-Blackwell Publication, IUFoST. ISBN 978-0-632-06421-2.

Stratford, M. (1994). "Another brick in the wall" 'Recent developments concerning the yeast cell envelope'. *Yeast* 10, 1083-1092.

Sugimoto, H. (1974). Synergistic effect of ethanol and sodium chloride on autolysis of baker's yeast for preparing food-grade yeast extracts. *Journal of food science,* 39, 939-942.

Sun, S.W., Lin, Y.C., Weng, Y.M. and Chen, M.J. (2006). Efficiency improvements on ninhydrin method for amino acid quantification. *Journal of Food Composition and Analysis*, 19 (2-3), 112-117.

Suphantharika, M., Khrunrae, P., Thanardkit, P. and Verduyn, C. (2003). Preparation of spent brewer's yeast β-glucans with a potential application as an immunostimulant for black tiger shrimp, *Penaeus monodon*. *Bioresource Technology,* 88, 55-60.

Suphantharika, M., Varavinit, S., Shobsngob, S. (1997). Determination of optimum conditions for autolyzed yeast extract production. *ASEAN Journal on Science and Technology for Development*, 14, 21-28.

Surowka, K., Zmudzinski, D. and Surowka, J. (2004) Enzymatic modification of extruded soy protein concentrates as a method of obtaining new functional food components. *Trends in Food Science and Technology*, 15, 153-160.

Tanguler, H. and Erten, H. (2007). Utilisation of spent brewer's yeast for yeast extract production by autolysis: The effect of temperature. *Food and Bioproduct Process*, 86(4), 317-321.

Temelli, F. (1997). Extraction and functional properties of barley β-glucan as affected by temperature and pH. *Journal of Food science,* 62(2), 1194-1197.

Thammakiti, S., Suphantharika, M., Phaesuwan, T. and Verduyn, C. (2004). Preparation of spent brewer's yeast β-glucans for potential applications in the food industry. *International Journal of Food Science and Technology*, 39, 21-29.

Thanardkit, P., Khrunrae, P., Suphantharika, M. and Verduyn, C. (2002). Glucan from spent brewer's yeast: preparation, analysis and use as a potencial immunostimulant in shrimp feed. *World Journal of Microbiology and Biotechnology*, 18, 527-539.

Thornton, J. (1992). Brewers yeast extract - a survey of the types available to the food processor. IFI NR (2) p. 40 – 43.

Tomas-Pejo, E., Garcia-Aparicio, M.,,Negro, M.J.,Olivia,J.M.,and Ballesteros,M. (2008).- Effect of different cellulase dosages on cell viability and ethanol production by *Kluyveromyces marxianus* in SSF processes. *Bioresource Technology*. doi: 10.1016/j.biortech.2008.07.012.

Transfiguracion, J.C., Lee, B.H., Park, S.Y. and Van Der Voort, F.R. (1998). Purification and characterisation of a carboxypeptidase Y from *Kluyveromyces fragilis* JSB95. *Journal of Dairy Science,* 81, 647-654.

Uscanga, A., Francois, J.M. (2003). 'A study of yeast cell wall composition and structure in response to growth conditions and mode of cultivation'. *Letters in Applied Microbiology,* 37:268-274.

USP XX1 (1985). Protein Hydrolysates. *The United States Pharmacopeia,* USP Convention Inc. p. 911-912.

Valderrama, M.J., de Siloniz, M.I., Gonzalo, P. and Peinado, J.M. (1999). A differential medium for the isolation of *Kluyveromyces marxianus* and *Kluyveromyces lactis* from dairy products. *Journal of Food Protection,* 62 (2), 189-193.

Vananuvat, P and Kinsella, J E (1975). Extraction of protein, low in nucleic acid, from Saccharomyces fragilis grown continuously on crude lactose. *Journal of Agricultural and Food Chemistry*, 23 216-221.

Van der Ven, Cornelly. Gruppen, Harry.; De Bont, Dries, B.A.; Voragen, Alphons C.J. (2001). Reversed phase and size exclusion chromatography of milk protein hydrolysates: relation between elution from reversed phase column and apparent molecular weight distribution. *International Dairy Journal*, 11, 83-92.

Van Hai, N. and Fotedar, R. (2009). Comparison of the effects of the prebiotics (Bio-Mos® and β-1,3-D-glucan) and the customised probiotics (*Pseudomas synxantha* and *P. Aeroginosa*) on the culture of juvenile western king prawns (*Penaeus latisulcatus* Kishinouye, 1896). *Aquaculture* 289, 310-316.

Varavinit, S., Shobsnogob, S., Bhidyachakorawat, M. and Suphantharika,M. (2000). Production of meat-like flavour. *Science Asia*, 26, 219-224.

Vasallo, M.C., Puppo, M.C., Palazolo, G.G., Otero, M.A., Beress, L. and Wagner, J.R. (2006). Cell wall proteins of *Kluyveromyces fragilis*: Surface and emulsifying properties. *LWT* 36, 729-739.

Vegarud, G.E. and Langsrud, T. (1989). The level of bitterness and solubility of hydrolysates produced by controlled hydrolysis of caseins. *Journal of Dairy Research*, 56, 375-379.

Vitova, E. Loupancova, B. Zemanova, J. Stoudkova, H. Brezina, P and Babak, L. (2006). Solid-Phase Microextraction for Analysis of Mould Cheese Aroma. *Czech J. Food Science*, 24, 268 - 274

Walker, G.M. (1998a). *Yeast Physiology and Biotechnology*. Chichester: John Wiley & Sons, Dundee, Scotland, Chapter 2, Yeast Cytology, pp. 11-50, ISBN 0-471-96446-8.

Walker, G.M. (1998b). *Yeast Physiology and Biotechnology*. Chichester: John Wiley & Sons, Dundee, Scotland, Chapter 6, Yeast Technology, p.265-320. ISBN 0-471-96446-8.

Wang,C.,Wang, Q. and Tian, J., (2010). Optimization of Enzymatic Production of Oligopeptides from Apricot Almonds Meal with Neutrase and N120P. *Int. J. Mol. Sci*. 11, 4952-4961.

Weigele, M., DeBernado, S.L., Tengi, J.P. and Leimgruber, W. (1972). A novel reagent for the fluorometric assay of primary amines. *Journal of the American Chemical Society*.

Wesolowski-Louvel,M., Breunig, K.D. and Fukuhara, H. (1996). *Kluyveromyces lactis.* In *Nonconventional Yeasts in Biotechnology. A Handbook* (ed. K.Wolf), p. 139-201. Springer-Verlag, Berlin and Heidelberg.

West, G., (1996). Flavour Production with Enzymes, in *Industrial Enzymology. 2nd Ed*., Macmillan Publishers Inc, New York, N.Y edited by Godfrey, T. and West, S. Chapter 2.12., p. 209- 224. ISBN 0-333-59464-9

Wheatcroft, R., Langeris, W., Kulandai, J., Gilbert, R., Sime, K.J. and Smith, C.G. (1997). Production of β-Glucan-Mannan preparations by autolysis of cells under certain pH, Temperature and time conditions. International Patent Application (PCT) WO 97/02356 23 January 1997.

Wheatcroft, R., Langeris, W., Kulandai, J., Gilbert, R., Sime, K.J. and Smith, C.G (2002). Production of β-Glucan-Mannan preparations by autolysis of cells under certain pH, Temperature and time conditions. United States Patent Application Publication. US 6.444.448 B1. Sep. 3, (2002).

WHO/FAO/UNU. (2007) Protein and amino acid requirements in human nutrition. *WHO technical report series,* no. 935, 135-160.

Wikstrom, K., Lennart, L., Rogar, A. and Westerlund, E. (1994). Rheological studies of water-soluble (1–3),(1–4)-β-D-Glucans from milling fractions of Oat. *Journal of Food Science,* 59, (5), 1077-1080.

Wood,BJB and Hodge,M. (1985) Yeast-Lactic Acid Bacteria Interactions and their contribution to fermented foodstuffs in *Microbiology of Fermented Food*, *Volume 1* edited by Brian BJ Wood, Chapter 7 p 263 – 293, Elsevier Applied Science Publishers, London & New York, ISBN 0 85334 33 22.

Yalcin,S.K, Bozdemir, M.T, and Ozhas, Z.Y. (2009). Utilization of whey and grape must for citric acid production by two *Yarrowia lipolytica* strains. *Food Biotechnology*, 23, 266-283.

Yaylayan, V.A. and Wnorowski, A. (2001). Role of L-serine and L-threonine in the generation of sugar-specific reactive intermediates during the maillard reaction. In *Food Flavours and Chemistry, Advances of the New Millenium*, Edited by Spanier, A.H. . Royal Society of Chemistry, pp. 313-317. ISBN: 0-85404-875-8.

Yamaguchi, S. (1991). Basic properties of umami and effects on humans. *Physiology and Behavior,* 49, 833-841.

Yoshida, Y., Yokoi, W., Ohishi, K., Ito, M., Naito, E. and Sawada, H. (2005). Effects of the Cell Wall of *Kluyveromyces marxianus* YIT 8292 on the plasma cholesterol and fecal sterol excretion in rats fed on a high-cholesterol diet. *Biosci. Biotechnol. Biochem*. 69, 714-723.

Zafar, S.and Owais, M. (2006). Ethanol production from crude whey by *Kluyverornyces marxianus. Biochemical Engineering Journal*, 27, 295-298.

Zhao, J. and Fleet. G.H. (2005) Degradation of RNA during the autolysis of *Saccharomyces cerevisiae* produces predominantly ribonucleotides. *Journal Ind Microbial Biotechnol*, 32, 415-423.