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Development of Value Added Products from Spent Kluyveromyces Marxianus Yeast

Rahul Sharma
Department of Biological and Pharmaceutical Sciences, Institute of Technology, Tralee, Kerry, Ireland

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Phosphorylation of yeast protein led to a reduction in nucleic acid content (55%) of yeast protein concentrates with an appreciable increase in protein extraction (10%) from yeast extract. The phosphorylated yeast protein produced was rich in essential amino acids except for sulfur containing amino acids.

Enzymatic digests of phosphorylated yeast protein using pepsin, pancreatin, and papain resulted in angiotensin-I-converting enzyme inhibitory indices within the range of 54-62 %. Of all the hydrolysates tested, the 26 % DH pepsin generated hydrolysate demonstrated greatest ACE inhibitory potency with a measured IC_{50} value of 1.38 mg/ml. Further purification of pepsin digested yeast protein resulted in a peptide mixture with IC50 value of 19.31μg/ml. This inhibitory activity was comparable to values

reported for a variety of ACE inhibitory food protein hydrolysates. The peptide content of this potent fraction was characterized by electrospray LC-MS-MS and resulted in large numbers of peptides with peptide sequence shown to have amino acid residues and active amino acid motifs that are previously reported in ACE inhibitory peptide.

Yeast cell wall extracts that retained their native structure were enzymatically processed to produce different soluble β-glucan preparations. The cell wall preparations obtained were assessed for their immunomodulatory activity using both *in vitro* murine J774.A1 cell line and *ex vivo* human whole blood assays models. This study showed that cell wall preparations (particulate and soluble glucan) from spent *Kluyveromyces marxianus* enhanced the production of T helper cell type 1 cytokines under both in vitro and *ex vivo* conditions. This indicated that cell wall preparations from *Kluyveromyces marxianus* may enhance the protective Th1 mediated innate immune responses in both *in vitro* mammalian cell line and *ex vivo* human whole blood assay model. Simple chemo-enzymatic treatments of yeast cell wall also resulted in protein enriched secondary yeast extracts from cell wall waste.

This study has shown that recovery of functional and bioactive extracts from spent yeast waste presents opportunities to innovate, add value and provide a more sustainable approach to better utilisation of Irish food industry waste streams. Such an approach is one step closer towards an amelioration of the economic and environmental problems facing the Irish food industry today.

Development of value added products from spent *Kluyveromyces marxianus* yeast

Submitted by

Rahul Sharma

B. Tech (Gujarat Agricultural University, Anand, India)
MSc. (Central Food Technological Research Institute, Mysore, India)

Submitted For Degree of Doctor of Philosophy
Institute of Technology Tralee

Supervisors

Mr. Brendan McCarra
&
Dr. Daniel J. Walsh

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Declaration

I, Rahul, confirm 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.

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Abbreviations

ACE:	Angiotensin-I Converting Enzyme.
CGP:	Crude β-glucan particulate insoluble
CβG:	Crude Glucan Particulate
DH:	Degree of hydrolysis DH
ExPASy:	Expert Protein Analysis System
FAPGG:	Furanacryloyl-L-phenylalanylglycylglycine
FBS:	Bovine serum.
GRAS:	Generally Regarded as Safe
HPLC:	High Pressure Liquid Chromatography
HWBA:	Human Whole Blood Assays
IC50:	Half maximal inhibitory concentration
LC-MS-MS:	Liquid Chromatography Mass Spectrometry
m/z:	Mass to Charge ratio
NMWCO:	Nominal Molecular Weight Cut Off
PVDF:	Polyvinylidene difluoride
PYP:	Phosphorylated Yeast Protein
PβG:	Alkali and Acid Insoluble Glucan Particulates
RP-HPLC:	Reverse phase High Pressure Liquid Chromatography
YPC:	Yeast Protein Concentrate

List of own presentations and papers

SHARMA, R., MCCARRA, B. & WALSH, D. J. Development of value added products from spent *Kluyveromyces marxianus* yeast waste *In:* SMITH, C. & TIWARI, B. K., eds. International Conference on Food Safety, Quality and Nutrition (Greening The Food Industry), April 11th to 13th, 2012 Manchester Food Research Centre, Hollings Faculty, Manchester Metropolitan, University, M14 6HR, UK, . 97-109.

ISLAM, N., SHARMA, R., HALL, M., MACARRA, B., WALSH, D. J., CEREDIG, R. & BRADLEY, B. Establishment of clinically relevant 'Human Whole Blood Assay' for automated screening of Immunomodulatory natural biomolecules. At Shannon ABC's Open Innovation Conference 4th and 5th April 2012 at Limerick Institute of Technology.

Publication under consideration

Paper: 01

Tile:	SHARMA, R., WALSH, D. & MCCARRA, B. Effect of pH and temperature of autolysis on chemical and sensory attributes of yeast extract produced from spent *Kluyveromyces marxianus* yeast.
Targeted Journal:	Bioresource Technology
Stage of Publication	Advance stage. To be submitted in May 2013

Paper: 02

Tile:	SHARMA, R., WYNNE, K., MCCARRA, B. & WALSH, D. J. Angiotensin-I-converting enzyme inhibitory activity of yeast protein hydrolysates derived from spent *Kluyveromyces marxianus* yeast
Targeted Journal:	Journal of Agricultural and Food Chemistry
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Paper: 03

Tile:	SHARMA, R., ISLAM, N., WALSH, D. J., CEREDIG, R. & BRADLEY, B & MACARRA, B. Development of bioactive β-glucan enriched cell wall preparations from spent *Kluyveromyces marxianus* yeast.
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Chapter 1.0

Review of literature on development of value added products from spent yeast.

1.1 Introduction

Yeast was the first microorganism domesticated by humankind for production of food and beverages. It was described as a living biochemical agent of transformation by Louis Pasteur. The fermentation of sugars by yeast is the world's oldest and by far the largest application of biotechnological processes at commercial level. With advances in the field of food science and technology the application of fermentation by yeast has been utilized extensively to produce high value commercial products such as microbial enzymes, baker's yeast, and primary and secondary metabolites like ethanol, natural pigments and flavonoids (Péter *et al.,* 2006). Likewise, in the near future, ethanol produced by yeast fermentation will remain a leading biotechnological commodity "in the form of first generations biofuels". However, one of the major by-products of industrial ethanol production from various feed-stocks is yeast waste otherwise known as spent yeast. Spent yeast serves as a sustainable source of high value added by-products.

1.2 Spent brewer yeast

During the process of brewing, food grade yeast multiplies by four to six times of its inoculum volume. Some of the yeast is reused in the next brewing cycle. This excess yeast is recovered from the liquid by filtering, settling and alcohol recovery systems. This excess yeast recovered from the brewing process is known as brewer spent yeast. It is generally recovered as slurry with 10 - 14% of total solid content. The yield of spent yeast generated during the brewing process ranges between 2 - 3 % of total beer production (Christina Galitsky, 2003, Ferreira *et al.*, 2010). Generally spent brewer yeast is primarily sold as an inexpensive animal feed.

1.3 Spent dairy yeast

During the process of cheese manufacturing 90 % of the milk original volume is recovered as a by-product known as cheese whey. This whey is a valuable by-product of cheese manufactures. It is also a potential pollutant which has in past endangered the environment when not disposed appropriately (Zafar and Owais, 2006). An integrated approach is to convert whey to ethanol by employing yeast, particularly *Kluyveromyces* species. Industrially this approach is known as

the whey to ethanol process or **Carbery process**, which was developed in Ireland for making potable alcohol and was later adopted for production of industrial alcohol (Murtagh, 1986, Ferrari *et al.,* 1994, Doyle, 2005, Charles Ling, 2008, Guimarães *et al.,* 2010). However, this process yielded a substantial amount of excess *Kluyveromyces marxianus* yeast waste, which is termed as Spent Dairy Yeast (Moresi *et al.,* 1995).

1.4 Status of spent yeast waste in Ireland

Ireland is one of the largest producers of beer in European Union in year 2011. There are four medium and large size brewing companies in Ireland on six brewery sites. There are also at least 20 micro-breweries. Total beer production of Ireland in year 2011 was 8.514 million hectolitre (Lichota, 2012, Brian *et al.,* 2011) and on basis of theoretical calculation, production of brewers spent yeast by Irish brewing industry is approximately 26,000 tonnes per annum from the beer industry alone.

There is no precise data available on the amount of spent dairy yeast produced in Ireland. However, to the author's best knowledge, the Carbery plant, Ballineen, Co. Cork, Ireland operates with eleven cylindro-conical fermentation vessels. Whey permeate is fermented by *Kluyveromyces maxirunus* in batch mode for 12 to 20 h, depending on the initial concentration of whey and the yeast activity. At the end of the fermentation process, ethanol titre typically reaches in range of 2.5 - 4.2% (v/v). Following fermentation, ethanol is concentrated to 96 % v/v using a continuous distillation process (Pesta *et al.,* 2007, Ling, 2008). The yeast recovered at the end of fermentation is generally reused a number of times before it is discarded as spent dairy yeast.

Carbery produces 11,000 tonnes of ethanol per annum (Doyle, 2005). On the basis of theoretical calculation, the amount of dairy spent yeast produced per annum is in the range of 5500 to 6000 tonnes per annum.

Waste streams from the Irish alcohol and Dairy industries with "generally recognized as safe" (GRAS) status offer an opportunity to utilise a large and sustainable by-product stream to produce high value added products. Spent

yeast waste stream has been briefly reviewed in Section 1.5 and 1.6 for its composition and sustainability of value added products that are derived from this emerging waste resource. A wide range of industrially desirable components are present in this waste resources are outline below.

1.5 Chemical composition of yeast biomass

The gross composition of spent yeast biomass depends on the species and method of cultivation. Several data concerning gross composition of yeast biomass have been published (Table 1.1). The vital characteristics of yeast biomass on a dry matter basis can be summarised as:

- High protein content
- Moderate carbohydrate content
- High nucleic acid content
- High ash content and associated minerals and trace elements
- Low fat content

1.5.1 Proteins

Yeast is an efficient producer of protein having 40 - 55 % protein content on a dry matter basis. Composition and content of protein depends on the strain, medium and condition of growth. In numerous reports, the protein content of yeast was described as both crude protein and true protein. The crude protein was calculated on the basis of total nitrogen content of the yeast multiplied by a factor 6.25. True protein content was calculated on the basis of a value from the Lowry method of protein estimation or total amino acid content of yeast.

Protein content of primary *Saccharomyces cerevisiae*, baker's yeast and spent brewer yeast grown in different medium varies and has been reported to be in the range of 57 %, 31 - 48 % and 39 - 59 %, respectively (Halasz *et al*., 1991, Otero *et al.*, 2002, Lukondeh *et al*., 2003a, Thammakiti *et al.*, 2004, Liu *et al.*, 2008). Similarly protein content of *Kluyveromyces spp.* yeast grown on whey and on lactose varies between 48 % and 40 %, respectively (Orban *et al.*, 1994, Yamada and Sgarbieri, 2005). Biologically yeast proteins play an important physiological role and can be classified as storage proteins, cell wall proteins and metabolically active enzyme proteins.

Table 1.1. Composition *of Saccharomyces* and *Kluyveromyces* species biomass.

Component	Protein (% w/w)	True Protein (% w/w)	Carbohydrate (%w/w)	Nucleic acid (% w/w)	Ash (% w/w)	Lipid (% w/w)	References
Baker's Yeast	47 - 48		28 – 30	8 – 9	1.3 - 1.8		Roshkova *et al.*, 1986
Baker's Yeast	38 - 60	31 - 48	25 – 35	25 - 35	6 - 7	4 - 10	Halász and Lásztity, 1991
Baker's yeast	41.28 ± 1.53	33.51 ± 1.06	40.28 ± 1.02	6.03 ± 0.77	6.22 ± 0.69		Otero *et al.*, 2002
Saccharomyces cerevisiae	53	46	18	7		10	Halász and Lásztity, 1991
Saccharomyces cerevisiae 1907		57	30	9.2			Lukondeh *et al.*, 2003a
Spent brewer's yeast	43.47 ± 0.30		59.61 ± 0.51		10.31± 0.17	1.20 ± 0.57	Thammakiti *et al.*, 2004
Spent brewer's yeast	48		36		3.8		Nigam *et al.*, 2009
Brewer's Yeast		48	36		8	1	Halász and Lásztity, 1991
Spent brewer's yeast	59.67		35.09		7.07	4.9	Liu *et al.*, 2008
Spent brewer's yeast	39.6			9	4.6	0.5	Yamada and Sgarbieri, 2005
Kluyveromyces fragilis sugarcane molasses	50.76 ± 1.83	40.80 ± 0.96	30.21 ± 1.65	7.54 ± 0.61	5.98 ± 0.43		Vasallo *et al* ., 2001
Kluyveromyces fragilis (grown lactose)	49.75 ± 1.81	40.37 ± 0.84	40.96 ± 2.89	7.67 ± 0.65	6.54 ± 0.37	0.65 ± 0.06	Orban *et al.*, 1994
Kluyveromyces fragilis (grown on Whey)	57.57 ± 0.33	48.42 ± 3.12	40.96 ± 2.89	9.46 ± 0.44	8.09 ± 0.32	0.763 ± 0.10	Moresi *et al.*, 1995
Kluyveromyces marxianus FII510700		56	26	12.7			Lukondeh *et al.*, 2003a
Kluyveromyces fragilis	50.76 ± 1.83	40.80 ± 0.96	31.21 ± 1.65	7.54 ± 0.61	5.98 ± 0.43		Otero *et al.*, 2002
Kluyveromyces fragilis dried biomass	37		34.3	4.82	16	7.8	Paul *et al.*, 2002

Storage proteins of yeast cells are mainly derived from two types of amino acid pools. One pool of amino acids is present in cytoplasm of cell and has a rapid turnover of reuse. The second pool of amino acids has a slow turnover and is mainly composed of basic amino acids (Halasz *et al.*, 1991).

Proteins present in yeast cell walls are known as **Cell Wall Proteins (CWPs)**. These are complex in nature and their content ranges between 5 - 15 % w/w of yeast biomass. Chemically they are glycoproteins containing glucomannan and mannan carbohydrate moieties. CWPs play an important role in yeast morphogenesis, adhesion, pathogenicity, antigenicity and are present in low abundance. These CWPs are largely located on the outside of the β-1→3-glucan network and to a lesser extent throughout the cell wall (Javier Pastor *et al.,* 1984, Halasz *et al*., 1991, Lipke and Ovalle, 1998, Posch *et al* ., 2008). Such a variety of proteins are desirable from an amino acid and nitrogen point of view.

Yeast cells are good sources of various commercial enzymes. These include enzymes which play important role in spent yeast processing like proteolytic enzyme, glucanase and nucleases (Shetty, 1980, Achstetter and Wolf, 1985, Halasz *et al.,* 1991, Satyanarayana *et al.,* 2009).

1.5.2 Carbohydrate

Carbohydrate content of yeast varies over a wide range depending on the strain, medium and condition of growth. Generally the content varies in the range of 20 - 30 % of dry weight of yeast. The largest amount of yeast carbohydrate is present as polysaccharides and to a lesser extent as monosaccharides and oligosaccharides (Halasz *et al.,* 1991, Liu *et al.,* 2008).

In most yeast, glycogen serves as an important energy reservoir and is present as soluble and insoluble pools. Insoluble glycogen is linked to β-1→3 glucan of the cell wall, via β-1→6 linkages (Arvindekar and Patil, 2002).

In addition to the above carbohydrates, large portions (16 - 20 %) of the total carbohydrate of yeast are present in the cell wall in the form of different classes of polysaccharide (Halasz *et al.,* 1991). Major classes of polysaccharides are polymers derived from of glucose and mannose. These are called glucan or β-

1→3 glucan and mannan respectively. Both β-glucan and mannan are highly desirable value added products which can be derived from spent yeast.

➲ **β-Glucan**

This is non-ionic homopolysaccharide comprised of D-glucopyranosyl units. There are two types of β-glucan depending on (a) its solubility properties under different conditions and (b) its linkages. Thus β-glucan can be either alkali & acid insoluble (β-1→3 glucan), or alkali soluble (β-1→6 glucan) (Javier Pastor *et al.*, 1984, Halasz *et al.*, 1991). β-1→3 glucan is a major constituent of the cell wall carbohydrates and constitutes around 50 % of the yeast cell wall. Much of β-1→3 glucan has a helical conformation which is composed of a single polysaccharide chain or a triple helix chain. On the other hand, β-1→6 glucan which constitute 10 % of cell wall is a highly branched polysaccharide and is linked to other cell wall components. The average degree of polymerization and corresponding mean molecular weight of β-1→6 glucan are 150 and 24 kDa, respectively (Lipke and Ovalle, 1998).

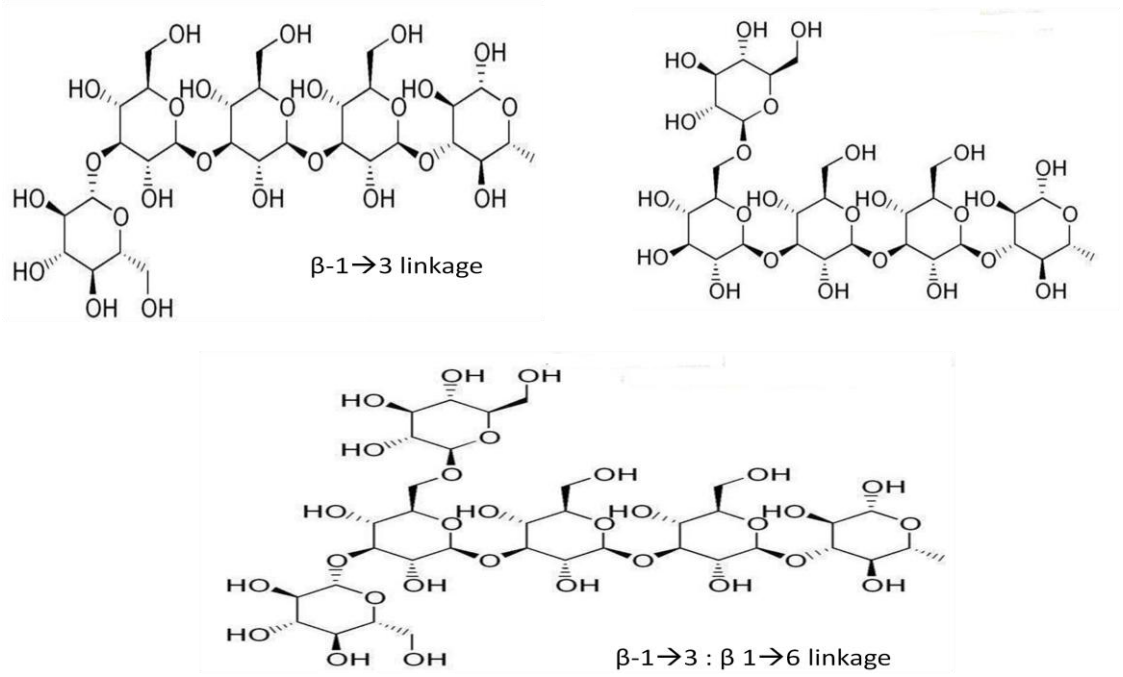


Figure 1.1 Schematic diagram of cell wall β-1→3 linkage and β-1→6 linkage of β-glucan in yeast well wall.

➲ **Mannan**

In the yeast cell wall, mannan exists as a polymer composed of mannose units which are covalently linked to core peptide chains generally referred to as mannoproteins (Halasz *et al.*, 1991). In the mannoprotein complex, the mannose units are linked by α-1→6, α-1→3 linkages (Sedmak, 2006). In general, mannoproteins contribute to 20 - 50 % of the dry weight of the yeast cell wall and is composed of 5 - 50 % of proteins.

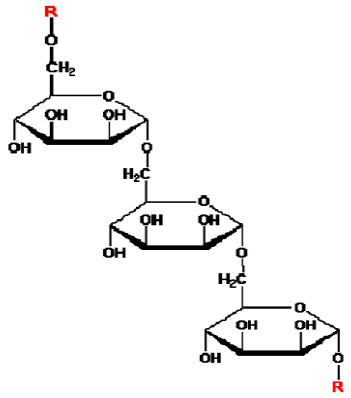


Figure 1.2 Schematic diagram of mannan in yeast cell wall.

Yeast cell wall polysaccharides also contain a minor chitin component, which is a polymer of N-acetyl glucosamine monomeric units. This makes β-1→3 glucan as acid and alkali insoluble compound. Chitin constitutes around 1 - 3 % of the cell wall. Its average degree of polymerization and corresponding mean molecular weight are 120 and 25 kDa, respectively (Lipke and Ovalle, 1998).

1.5.3 Nucleic acids and nucleotides

A high content of nucleic acids is typical of yeast. The amount of nucleic acid is affected by different factors such as medium composition (carbon sources), growth rate and stage, processing of mRNA (Kim *et al.*, 1999). Yeast cells contain 5 - 15 % RNA and 0.1 - 1.5 % DNA on a cell dry weight basis (Halasz *et al.*, 1991).

Both ribonucleic acids (RNA) and deoxyribonucleic acid (DNA) occur in yeast cells. However, the content of RNA can be 50 to 100 times greater in quantity than the DNA (Halasz *et al.*, 1991, Hernawan and Fleet, 1995, Zhao and Fleet, 2005, Zhao and Fleet, 2003, Lukondeh *et al.*, 2003). Therefore, the DNA content

of yeast is generally not determined separately for chemical and nutritional evaluation of yeast biomass.

These nucleic acids are rich source of nucleotide, nucleosides and nucleobases (cytosine, guanine, adenine, thymine and uracil) that are essential for animal nutrition and act as flavour enhancer in savoury food system.

1.5.4 Lipids

Total lipid content of yeast varies widely with the species. The majority of yeast contain about 7 - 15 % lipids on dry weight basis. The lipid content and composition of yeast is dependent on growth conditions. Yeast contains triglycerides (20 - 50 % of total lipid), mono & diglycerides (1 - 15 % of total lipid), free fatty acids (1 - 20 % of total lipid) and phospholipid (15 - 60 % of total lipid) (Halasz *et al.*, 1991). Sterols such as Ergosterol are other classes of lipids that are also present in yeast. The Ergosterol content of brewer yeast is in the range of 0.5 - 0.7% on a dry matter basis (Lamacka and Sajbidor, 1997). Ergosterol is the biological precursor to vitamin D_2, cortisone and progesterone that are essential for animal nutrition and physiology (He *et al.*, 2000).

1.5.5 Minerals

The mineral content of yeasts varies over a wide range and can be regarded as relatively high. Typical values are 10 % of dry matter of cell biomass. Phosphorus, potassium, magnesium, calcium and sulphate are the main components of the ash. Growing conditions have a significant influence on the quantity and composition of ash of yeast (Halasz *et al.*, 1991). Yeast would not be regarded as commercial source of minerals. However, as a food additive, its mineral content would be a beneficial attribute.

1.5.6 Vitamins

Spent yeast is a rich source of several vitamins particularly those of vitamin B complex grouping. Vitamin content varies according to yeast species and culture conditions. The basic vitamin content of brewer's yeast is p-amino-benzoic acid 9 - 120, Biotin 0.5 - 2.0, Choline 4850, Folic acid 3.0, inositol 2700 - 500, Niacin 310 - 1000, Pantothenic acid 100, Pyridoxine 25 - 100, Riboflavin 36 -

42, Thiamine 50 - 360 µg per g of dry yeast (Eddy 1958). These vitamins help in breakdown of carbohydrates, fats, and proteins of animal nutrition that provide the body with energy (Halász and Lásztity, 1991).

1.6 Current status of research and development for production of value added products from spent yeast biomass

As per the discussion in Section 1.5, it can be concluded that food grade spent yeast is a rich source of proteins, carbohydrates, nucleic acids, lipids and vitamin B complex. Together these components of spent yeast biomass contribute 85 - 90 % of its dry matter. The desirable properties of the aforementioned yeast components for use in varieties of food and feed products are:

- **Proteins**: rich in variety of essential amino acids that are important for nutrition as source of dietary nitrogen and act as precursor molecules for creation of reaction flavours.
- **Cell wall carbohydrates:** rich sources of β-glucan and mannan oligosaccharides which have reported immunomodulatory, antioxidant, food emulsifying and stabilising properties.
- **Nucleic acids:** rich sources of nucleotide, nucleosides and nitrogenous bases cytosine, guanine, adenine, thymine and uracil that are essential for animal nutrition and also act as flavour enhancer in savoury food system.
- **Vitamins especially vitamin B group**: essential for physiological role in organism and is important ingredient in formulation of nutritional supplement.

Importantly spent yeast offers a sustainable supply of desired components with GRAS status as compare to other sources of raw material (Halasz *et al.*, 1991).
In Figure 1.3 the main products of yeast biomass processing, potentially applicable in food and feed production are summarised. There are mainly five main pools of value added products which may be derived from industrial spent yeast. These are

(i) Dried food yeast,
(ii) Heat treated yeast slurry
(iii) Yeast extract,
(iv) Yeast protein concentrates and
(v) Yeast cell wall preparations.

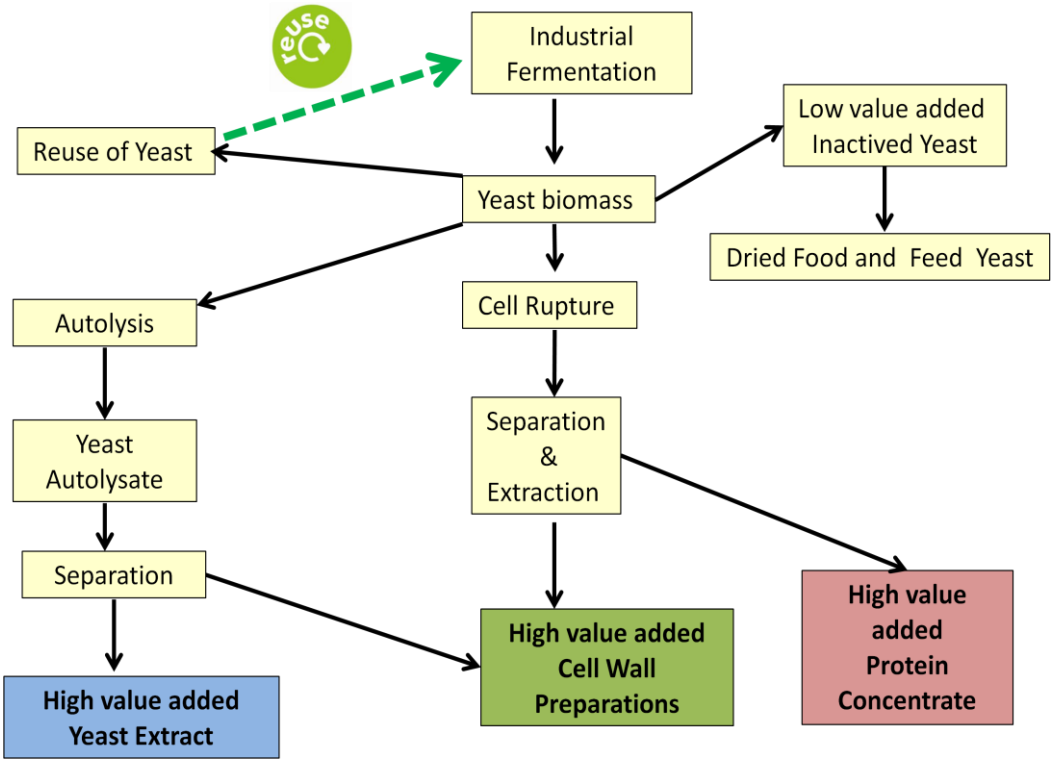


Figure 1.3 Main products of yeast biomass processing in food production. The diagram depicts the process which may be applied to yeast biomass following fermentation. The biomass could be sustainably reused or processed into both low and / or high value added products (Adopted from Halasz *et al.*, 1991).

The simplest use of spent yeast is to use it as inactive yeast cell slurry after centrifugal separation from beer. Generally, spent yeast slurry (10 - 15 %) is pasteurised, then dried and sold to the food and feed industry. The commercial revenues from these product is low (1.0 - 1.5 $ / Kg). Additionally, the high content of nucleic acids and cell wall contents in whole yeast cells limits its food applications. Therefore, further processing of spent yeast is required to extract and purify proteins, cell wall carbohydrates and nucleic acids producing high value ingredients.

The actual value of yeast and resulting derived potential products is highly dependent on the type, quantity and activity of the high value nutritional components.

The choice of processing method depends on the type of product required for the end application and the amount of commercial benefit to be achieved. For

example processor wish to produce high value flavour enhancers and reaction flavours, then spent yeast can be used to produce yeast extract. Similarly, spent yeast can be used to produce protein concentrate and cell wall preparations for use as nutritional supplements in animal feeds.

Several studies had been accomplished at the academic as well as at the commercial level for production and characterisation of these high value added products. These are briefly reviewed in Section 1.6.1.

1.6.1 Yeast extract

Yeast extracts are the concentrates of the soluble fraction of yeast biomass and are composed of free amino acids, peptides, nucleotides and reducing carbohydrates. Yeast extracts are commercially used in food industry as savoury ingredients in soups, sauces, and snack products to impart a broth-like meaty taste impression, referred to as **umami,** (Nagodawithana, 1992). The yeast extracts available in the market differ in cost as well as flavour profile, depending upon the starting material such as spent brewer's yeast, baker's yeast and the manufacturing process, such as autolysis.

The flavour and taste enhancing properties of yeast extracts are mainly due to the interaction of various amino acids in combination with 5' nucleotides, peptides and organic acids (Nagodawithana, 1992, Festring and Hofmann, 2010). This synergistic effect results in the need of low dosages of extracts with surprisingly strong flavour impact. As a result, the usage level of yeast extracts in food products varies in the range of 0.1 - 0.5 % on the basis of weight (Nagodawithana, 1992).

Yeast extracts are produced by **autolytic, plasmolytic and hydrolytic** processes. Traditionally, hydrolytic processes were the most efficient methods of solubilising yeast biomass using hydrochloric acid. However during this process some carcinogenic compounds such as mono- and dichloropropanols and monochloropropanediols are produced (Nagodawithana, 1994). Additionally the salt content in the resulting yeast extract may reach 40 % (Manley, 1994). Theses disadvantages can be overcome by using autolytic processes to achieve

similar and more desirable flavour attributes of yeast extract products (Sugimoto, 1976, Babayan and Bezrukov, 1985, Nagodawithana, 1992, Orban *et al.*, 1994).

Commercially yeast extracts are mainly produced by autolysing either baker's yeast or debittered spent brewer's yeast under controlled conditions of temperature, pH and time with or without addition of certain enzymes and other agents.

1.6.1a Autolysis and affecting factors

Autolysis is a physiological and irreversible breakdown process of yeast cell constituents due to the action of endogenous hydrolytic enzymes such as proteinases, ribonucleases and glucanases (Babayan *et al.*, 1981, Babayan and Bezrukov, 1985, Alexandre and Guilloux-Benatier, 2006).

During initial stages of autolysis the cell endostructures degrade, releasing the vacuolar proteases into the cytoplasm. Released proteases hydrolyse the intracellular polymer components into hydrolytic products which accumulate in the space restricted by the cell wall.

During this process,

a. Endogenous glucanases solubilise the cell wall glucan and release mannoproteins. This results the cell wall in becoming partly digested and more permeable.

b. The hydrolytic products are released from yeast cells when molecular masses of hydrolysed product within yeast cells are small enough to pass through the pores of cell wall (Figure 1.4).

c. Although there are morphological changes to the cell, the cell will remain intact throughout the autolysis. (Amrane and Prigent, 1996).

d. At the end of autolysis, the extracellular extract consist of peptides, amino acids, nucleotides, amino acid derivatives and suspended cell wall materials and is known as **yeast autolysate** (Nagodawithana, 1992).

e. When yeast autolysates are separated by mechanical or chemical method to yield supernatant and sediments, they are known as yeast extract and cell wall respectively.

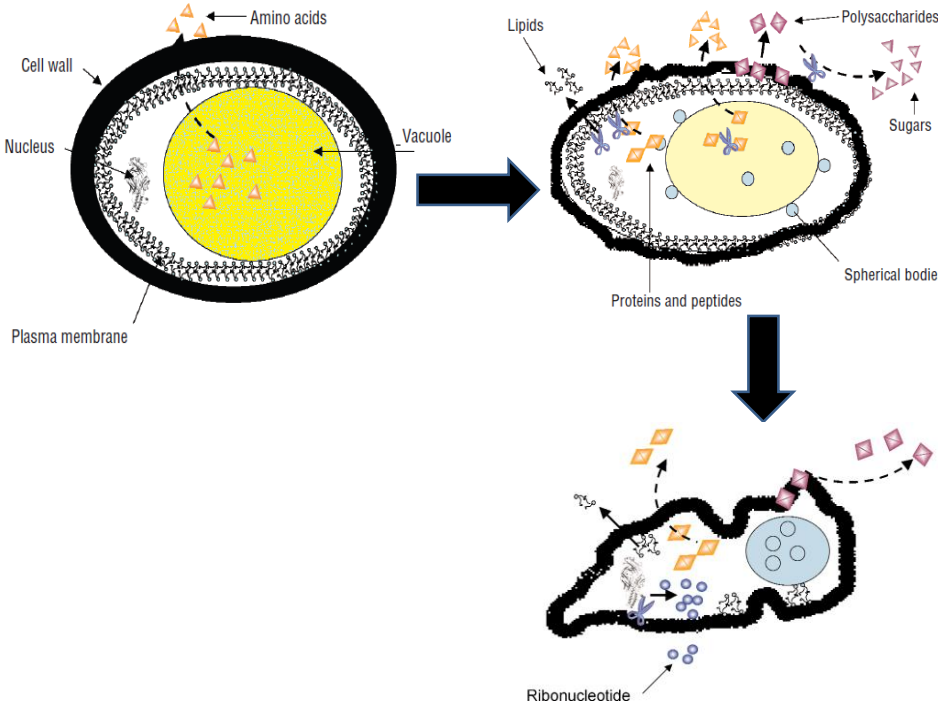


Figure 1.4 Schematic representation of yeast cell autolysis diagram (Adopted from Alexandre and Guilloux-Benatier, 2006).

Autolysis may be regarded as a bioprocess, which can be controlled by manipulating extrinsic control parameters to yield value added products such as yeast extracts and cell wall preparations. Nevertheless factors such as mechanical disintegration of the yeast cell, temperature & pH and additives like exogenous enzyme, salts and chemical solvents can induce or control the autolysis in yeast. These factors have been extensively investigated and commercially exploited for production of yeast extract and can be summarised as:

➲ **Effect of mechanical disintegration on autolysis**

A number of methods have been reported for partial and total cell wall disintegration of yeast cells before and during the autolysis process (Baldwin and Robinson, 1990). These methods rely on use of glass bead cell disintegrators, colloidal and ball mills and two stage homogenizers. Among these methods, two stage homogenization has been most widely studied for its effect on autolysis recovery. The autolysis of homogenized yeast suspension is faster and results up to 70 - 80 % cell biomass solubilisation as compared with autolysis process of non homogenized yeast suspension (Běehalová and Beran, 1986, Moresi *et al.*, 1995, Verduyn *et al.*, 1999, Vasallo *et al.*, 2001). A high degree of hydrolysis of protein was observed for yeast extracts derived from baker yeast autolysate following homogenization (Verduyn *et al.*, 1999). Běhalová and Beran, 1979 reported high proteolytic activity of disintegrated yeast in comparison to chloroformed plasmolysed yeast cell and intact cells. Therefore, homogenization of yeast cell prior to autolysis presumably leads to rapid liberation of endogenous proteases and increases their accessibility to their substrate.

➲ **Effect of temperature and pH on autolysis**

The process of yeast autolysis is mainly governed by the activity of different endogenous enzymes with their own specific temperature and pH optima (Běhalová and Beran, 1979, Běehalová and Beran, 1986).

An experimental study of the autolysis of disintegrated yeast biomass was conducted at various temperatures and pH 5.5 in the presence of sodium chloride (Běhalová and Beran, 1979, Běehalová and Beran, 1986). It was observed that pH 5.5 and temperatures above 50 °C had a detrimental effect on endogenous protease activity, whereas at 40 °C proteases yielded the highest degree of hydrolysis. It was also noted that the taste of products generated at 50 ºC was more acceptable in terms of flavour and aroma, as compared to those generated at 40 ºC which possessed an unpleasant sour taste.

It was concluded that autolysis at 40 °C and 50 °C could be regarded as two different processes since different sets of enzymes were active, which was reflected in the rate of autolysis, chemical composition and organoleptic properties of the yeast extract generated (Běhalová and Beran, 1979, Běehalová and Beran, 1986).

In a study by the group of Mauro Moresi 1995 and Orban *et al.,* 1994 an unstructured kinetics model of the yeast autolysis process was proposed, where the autolysis (reaction) rate constant *k*a was a function of temperature, i.e. as the temperature increases the rate of autolysis increases (Arrhenius equation). However, the researchers found that the molecular activation energy of the yeast autolysis process was similar to the large number of hydrolytic enzyme inactivation reactions. It was concluded that as the temperature of autolysis was increased from 40 to 55 ºC, the rate constant *k*a increased two fold. It was reported that, at a temperature exceeding 60 ºC, the *k*a reduced by 84 % which implied that the enzymatic activity was reduced drastically above its optimum temperature.

In later published reports, autolysis of brewer spent yeast was conducted at different temperatures (45 - 60 ºC) over a period of 72 h (Tanguler and Erten, 2008). It was observed that elevated temperatures had a significant influence on the solid content of yeast extract. Also α amino nitrogen content of yeast extract obtained at autolysis temperature of 50 ºC was higher than that obtained at 45 ºC. This in turn was significantly ($p<0.01$) higher than that obtained at 55 ºC and 60 ºC. This study also revealed that the yeast proteases were active at 45 ºC and 50 ºC but were inactive at 55 ºC and 60 ºC.

When brewer spent yeast autolysis was performed at various pH in the presence of additives, it was observed that an optimal yeast extract yield was obtained at pH 5.5 and 4.5 (Champagne *et al.,* 1999). However the effect of pH was much greater on total nitrogen content than on total yield solids. While the highest level of α amino nitrogen was attained at pH 5.5 followed by pH 4.0, pH 7.0 and pH 8.5. The degree of hydrolysis was reported to be higher at pH 5.5 followed by pH 7.0, pH 8.5 and pH 4.0.

From these results it may be assumed that proteinases had less activity at pH 4.0, pH 7.0 and at pH 8.5 than pH 5.5, whereas peptidase had good activity at pH 5.5, pH 7.0 or at slightly alkaline conditions. In addition, the correlation between total nitrogen and α-amino nitrogen was not very high (R^2 = 0.76). Consequently, Champagne *et al.,* 1999 concluded that proteases, peptidases do not react exactly the same as proteinases at the various pH conditions of autolysis.

As hydrolysis of RNA and DNA during autolysis is a key reaction, a detailed study concerning effects of various pH values (4.0 - 7.0) at 40 ºC and various temperatures (30 - 60 ºC) at pH 7.0 was published (Zhao, J and Fleet, G, 2003 and Zhao, J and Fleet, G, 2005). It was observed that the temperature and pH of autolysis influences the degradation pattern and products of RNA and DNA. The rate and level of reduction of yeast RNA is higher at higher temperatures (60 °C) than lower temperature values (40 °C). Similarly, the level of reduction of the RNA content of yeast biomass was observed to be higher at autolysis of pH 7.0, followed by pH 4.0, pH 5.0 and pH 6.0. In relation to the composition of RNA degradation products, it was observed that 3'-ribonucleotides were by far the most predominant components for all autolysis conditions and comparatively ribonucleosides and nucleobases levels were lower. Zhao, J and Fleet, G (2005), also observed that formation of 5'- ribonucleotides, were highest in temperature 40 - 50 ºC at pH 7.0.

On the basis of this data and pattern of degradation products of RNA, it was assumed that endo-nucleases and exo-nucleases were principally responsible for the degradation of RNA. In the same study it was also reported that the highest amount of DNA degradation occurred at 40 ºC and pH 7.0, where the highest DNase activity was recorded. The degradation products of DNA during autolysis mainly comprise of 3'and 5'-deoxyribonucleotides, and lesser amounts of polynucleotides.

➲ **Effect of additives on autolysis**

Traces of the ethanol in spent brewer yeast and additives like ethyl acetate, sodium chloride are sometimes added to autolysis reactions for dual purpose, (i)

to prevent the growth of contaminating microbes and (ii) to act as plasmolysing agents (Peppler, 1960, Sugimoto, 1976, Akin, 1981, Champagne *et al.*, 1999).
It was assumed that these additives may influence the process of autolysis at different stages. In one case they may affect the release of lytic enzymes in the process of the rearrangement of endostructures of the cell prior the hydrolysis of the cellular material. Alternatively, they may affect the interaction of cell components with the released enzymes, which results either in their activation or in their inhibition (Babayan *et al.*, 1979).

The **addition of sodium chloride** during autolysis enhances the activity of the proteolytic enzyme of yeast and also helps to attain maximum solubilisation of yield (Sugimoto, 1976, Běehalová and Beran, 1979, Běehalová and Beran, 1986, Kollár *et al.*, 1991, Orban *et al.*, 1994, Moresi *et al.*, 1995).

The **addition of exogenous enzymes** is one of the new approaches to enhance the autolysis of heat treated yeast biomass obtained from the alcohol recovery system in breweries (Biocatalysts, 2007).

The use of exogenous enzymes, particularly **plant proteases** (papain, ficin and bromelain) and mixtures of enzymes (pancreatin or *Aspergillus* proteases) are effective through their synergistic effect on the autolysis process. Among all proteases, papain was found to more effective in aiding yeast autolysis (Chao, 1980, Verduyn *et al.*, 1999, Conway J., 2001). It was observed that in the presence of papain, the yeast cell wall structure gradually disappeared during extended period of the autolysis. Therefore it was assumed that small sized enzymes like papain were able to penetrate the yeast cell wall and cell membrane followed by hydrolysis of intracellular components including the natural cytoplasmic inhibitors. This resulted in disorganization of the cytoplasmic structure and increased the availability of substrate components to endogenous enzymes that in turn accelerate the autolysis process (Chao, 1980).

Addition of **β-1→3 glucanase** to yeast does not increase the yield of yeast extract significantly (Conway J., 2001). However when used along with papain,

it improved the yield of yeast extract significantly with additional advantage of a reduction of autolysis time (Ryan and Ward, 1985).

In another study, the autolysis of heat treated yeast (80 ºC for 15 minutes) resulted into 50 % reduction in yeast extract yield. Although addition of **fungal protease** enhances the yields of yeast extracts and total nitrogen contents of one of yeast extract. Enzyme such as **flavourzyme** gave the highest increase in α-amino nitrogen of yeast extracts (Conway J., 2001).

In another study, dried brewer's yeast when treated with a combination of various enzymes, it was concluded that the enzyme to substrate ratio of exopeptidase influenced the protein yield, degree of hydrolysis of yeast and sensory attributes of yeast. However, optimal sensory attributes were obtained with a combination of endo and exopeptidase enzymes (Chae *et al.*, 2001).

Additionally, yeast extracts rich in 5'nucleotides flavour enhancers has been produced by treating yeast autolysate with 'specialty' enzymes such as **5'phosphodiesterase** and adenosine monophosphate (**AMP-deaminase)** derived from different sources (Benaiges *et al.,* 1990, Belem *et al.,* 1997, Belem and Lee, 1999, Chae *et al.,* 2001, Sombutyanuchit, 2001, Jae-Ho *et al.,* 2002, Jong-Soo *et al.,* 2004).

1.6.2 Yeast protein concentrate

Yeast protein isolation is carried out primarily through the breakdown of cell wall integrity. Several methods have been proposed for the disintegration of microbial cells but only a few have proven to be efficient enough at large scale. Among them, the high pressure homogenizers are suitable for yeast biomass (Halasz *et al.*, 1991). Following the disintegration of the yeast cells, the next step is to extract the yeast biomass cell component with dilute acid or alkali, since extractability of different components is highly dependent on the pH. This was concluded that optimal extraction of yeast proteins may be attained under alkaline pH conditions between pH 9.0 - 12.0 (Figure 1.5).

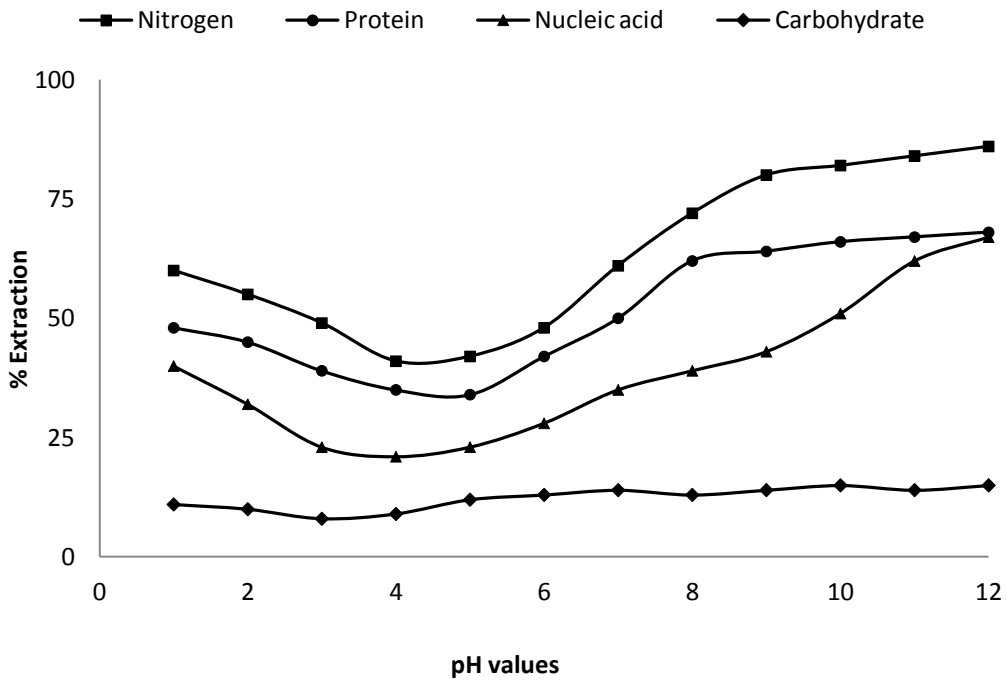


Figure 1.5 Extractability of soluble intracellular components from disintegrated yeast biomass depending on pH (Halasz *et al.*, 1991b).

Moreover, it was also reported that yeast proteins inside the cell are native and during extraction these proteins are extracted as a nucleoprotein complex (Huang and Kinsella, 1986a). That in turn were extracted by above mentioned methods were concentrated by isoelectric precipitation (pH 4.2 to 4.5), ammonium sulfate precipitation and ethanol precipitation. The disadvantage of such methods is the high amount of nucleic acid residue in the obtained protein as high as 22 % (Shetty and Kinsella, 1982). Previous studies have shown that the nucleic acid intake per day of an individual should not exceed 2 g/day (Maul *et al.*, 1970). Therefore it is necessary to reduce nucleic acid content in yeast protein isolates to make it suitable for human consumption.

Numerous methods such as application of chemical modification and chemo-enzymatic treatment of yeast nucleoprotein complexes have been proposed to reduce the nucleic acid content. These methods are briefly described in Table 1.2.

Table 1.2 Summary of various reported methods for reduction of nucleic acid content from yeast protein extracted from yeast biomass.

Methods	Reference
➲ Alkaline extraction of protein under drastic condition (0.2 M NaOH) followed by acid precipitation of protein at 80 °C resulting in reduction of nucleic acid. However, this results in appreciable loss of protein yield and denaturation and formation of an artefact.	Lindblom, 1974, Vananuvat and Kinsella, 1975a, Shetty and Kinsella, 1980
➲ Activation of intracellular RNase by heat shock is an efficient procedure to reduce RNA in yeast cells. This also activates endogenous proteolytic enzyme wherein sodium chloride supplementation during process may provide synergist effect.	Maul *et al.*, 1970, Damodaran and Kinsella, 1983, Bueno *et al.*, 1985
➲ Addition of exogenous nuclease to a soluble fraction from yeast biomass to hydrolyze the nucleic acid. The preferred source of exogenous nuclease was malt sprouts. However, this process on large scale is not economically feasible.	Newell, 1975
➲ Addition of 4 % NaCl to homogenized yeast biomass and incubation at 48 - 62 ºC with pH 9.0 followed by slow acid precipitation of yeast protein. Yeast protein thus obtain only had 1 - 3 % of nucleic acid.	Lindblom, 1976
➲ Addition of Zinc in alkaline cell homogenate followed by incubation at pH 6.0 and 60 ºC, resulted reduction of nucleic acid. Zinc metal may enhance RNase activity during the alkaline treatment that in turn reduces nucleic acid of protein.	Schuldt Jr., 1979
➲ Extraction of nucleic acid by treating yeast biomass in salt suspension (0.5 M NaCl) at pH 1.4 (3.5 N HCl) followed by heating and incubation for 2 h. This is not feasible on larger scale due to process safety concern and high solid loss during process.	Trevelyan, 1978
➲ Precipitation of Nucleic acid from yeast protein suspension in citrate phosphate buffer (0.1 M, pH 7.0) with acetyl-trimethyl – ammonium bromide. After treatment, nucleic acid content of protein was as low as 3 %.	Lawford *et al.*, 1979
➲ Succinylation of yeast protein resulted in reduction of nucleic acid content with increase in yeast protein yield. However succinylated protein thus obtained cannot be used as a source of dietary protein.	Kinsella J and Shetty K, 1979, Vananuvat and Kinsella, 1978

Table 1.2 continued

Methods	Reference
➲ Isolation of yeast protein with a reduced level of nucleic acid content was achieved by use of reversible acylating reagents like maleic anhydride and citraconic anhydride	Shetty and Kinsella, 1982
➲ Phosphorylation of yeast nucleoprotein with phosphorus oxychloride ($POCl_3$) causes dissociation of nucleoprotein complexes. This upon subsequent precipitation at pH 4.2 yields phosphorylated protein with low nucleic acid. 80 % reduction of nucleic acid in yeast protein was attained when ratio of $POCl_3$ to protein (1:5) was use for Phosphorylation. However, there was an appreciable decrease in the recovery of proteins upon phosphorylation with marked improvement in functional property of yeast protein.	Damodaran and Kinsella, 1984, Huang and Kinsella, 1986a
➲ Another way to destabilize nucleoprotein interactions is by using sodium trimetaphosphate (STMP) to yield phosphorylated yeast protein. Optimal conditions for process were pH 11 / 38 °C / 3 % w/v of STMP with holding for 3 h. RNA content of final product was reduced by 60 % with marked improvement in functional property of yeast protein.	Giec *et al.*, 1989, Pacheco and Sgarbieri, 1998, Yamada and Sgarbieri, 2005
➲ Nucleic acid content of yeast protein had been minimize by incubating disintegrated yeast biomass at pH 6.0 for 1 h at 50 °C followed by isoelectric precipitation. This process may activate native nucleases that act on yeast nucleic acid and in turn reduces nucleic acid content of yeast protein.	Otero *et al* ., 1996

Considering all the methods detailed in Table 1.2, phosphorylation of yeast protein results in reduction of nucleic acid due to protein modification which in turn improves functional properties of obtained protein (Damodaran and Kinsella, 1984, Huang and Kinsella, 1986a, Huang and Kinsella, 1986b, Giec *et al.*, 1989, Pacheco and Sgarbieri, 1998, Yamada and Sgarbieri, 2005). This may be attributed to the phosphorylation of protein which increases the protein net negative charge thus increasing the electrostatic repulsion between the nucleoprotein complexes (Damodaran and Kinsella, 1984). These enhanced net negative charges result in increased viscosity, emulsion capacity and foam stability of protein solution. Phosphorylated yeast protein also has reported better water holding capacity than yeast nucleoprotein complexes alone, which may be attributed to an increase in hydration of added phosphoryl groups.

1.6.2a Functional properties of yeast protein

Native proteins from yeast cell were reported to be water and/or salt soluble protein and their denaturation during extraction and concentration process results in change of solubility (Halasz *et al.*, 1991). The typical solubility curve of yeast protein at different pH value is illustrated by Figure 1.6.

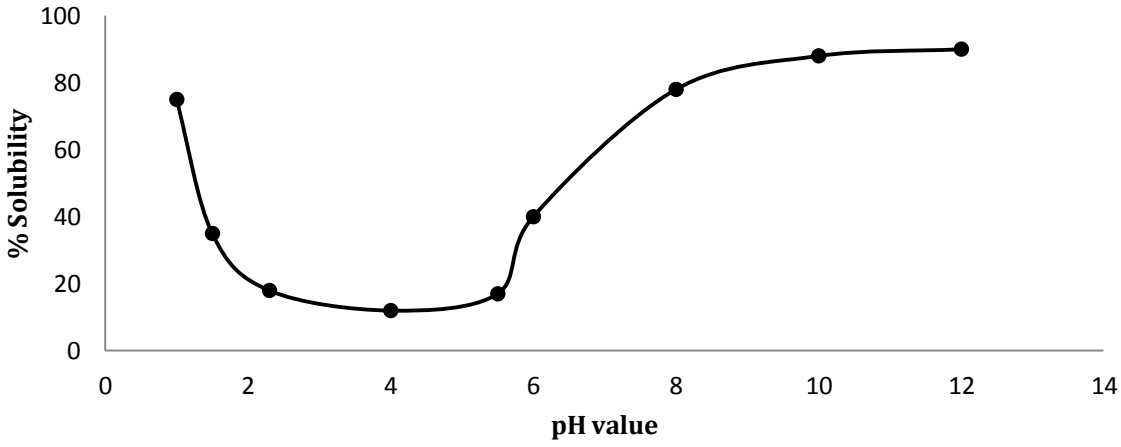


Figure 1.6 Solubility of yeast protein at different pH values (Halasz *et al.*, 1991).

It can be seen from Figure 1.6 that the yeast protein exhibits lowest solubility in the range of pH 4.0 - 4.5 and highest solubility in the alkaline range. Water holding capacity (WHC) values for yeast proteins are in the range of 5 - 10 mL of water per g of protein which is at par with plant protein (soybean, cottonseed

and sunflower). The hydration properties of *Saccharomyces cerevisiae* and *Kluyveromyces fragilis* protein extracted in mild alkaline condition has been reported by Otero *et al.,* 2002 (Table 1.3).

Table 1.3 Functional properties of yeast protein from *Saccharomyces cerevisiae* and *Kluyveromyces fragilis* biomass (Otero *et al.*, 2002).

Sample	% Solubility	WHC, mL/g of protein
Saccharomyces cerevisiae	36.60 ± 0.23	6.78 ± 0.12
Kluyveromyces fragilis	37.42 ± 0.11	6.23 ± 0.16

Additionally, the emulsion capacity of yeast protein varies depending on its process of production. Heat treatment of yeast protein emulsion did not change the emulsion characteristic. Likewise, the presence of sodium chloride does not influence or damage the yeast protein emulsion stability and activity (Halasz *et al.*, 1991).

The functional properties of different yeast proteins were also reported to have **emulsifying activity** value in range of 51 to 59 % and comparable to a value of 46 % for soy protein (Vananuvat and Kinsella, 1975b).

Apart from the storage protein of yeast, the cell wall protein from *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* are reported to be good sources of natural emulsifiers (Cameron *et al.,* 1988, Barriga *et al.,* 1999, Lukondeh *et al.,* 2003b, Del Carmen Vasallo *et al.,* 2006) and is generally referred to as cell wall emulsifier.

This cell wall emulsifier can be extracted easily by heat treatment, purified by ultrafiltration, with a yield of 16 % of the original dry weight and with composition of 44 % mannose and 17 % protein (Cameron *et al.,* 1988).

This product has been stabilized in kerosene-in-water emulsions using a broad range of conditions, from pH 2.0 to 11.0 in the presence of 5 % sodium chloride or up to 50 % ethanolic aqueous phase. Furthermore such emulsions were stable

after three cycles of freezing and thawing in the presence of different solutes in the aqueous phase.

The same group of researchers further resolved the same emulsifying material into two major fractions by capillary electrophoresis. One of the fractions was rich in protein (46 %) with some carbohydrate (9 %) and was assumed to be the protein backbone of the phosphor-manno-protein complex of cell wall proteins. This fraction has more emulsifying ability than the original product obtained from the yeast biomass. The other fraction was rich in carbohydrate and phosphorous and assumed to be phospho-mannan fragments of the cell wall protein. This phospho-mannan rich fraction was very water soluble and believed to enhance the emulsification properties of the first fraction (Barriga *et al.*, 1999).

Similarly, the cell wall protein was extracted from *Kluyveromyces marxianus* as a crude and partially purified emulsifier with yield of 7 - 14 % and 4.2 - 7 % of the cell dry weight, respectively. Partially purified emulsifier was rich in carbohydrate (90 %) and low in protein (4 - 6 %) content. The partially purified emulsifier of 1.2 % (w/v) concentration can stabilize corn oil-in-water emulsion for 3 months over a pH range of pH 3.0 – 11.0 as well as in the presence of sodium chloride (0.2 % - 5 %) in aqueous phase (Lukondeh *et al.*, 2003b).
In addition to this, the cell wall protein from *Kluyveromyces fragilis,* was separated by Sephadex G-50 gel chromatography into three fractions namely FI, FII, FIII (Del Carmen Vasallo *et al.*, 2006). It was found that Fraction FI had a molecular weight greater than 30 kDa and a higher yield with protein content more than the two other fractions. Fraction FI was further resolved on Sephacryl S300 HR gel chromatography into three fractions namely A, B and C with molecular weights in the range of 10 kDa to 150 kDa. Furthermore, sub-fractions B and C showed highest emulsifying activities. Emulsions made from these fractions were stable against creaming and coalescence. The cream phase of fraction B exhibited gel like behaviour.

1.6.2b Nutritional properties of yeast protein

Nutritional aspects of yeast proteins have been extensively studied with respect to their amino acid composition (Table 1.4). Halasz *et al* 1991 have reviewed the nutritive value and safety of yeast protein. It was concluded that the biological value of yeast protein was relatively low as compared to other standard proteins such as casein or egg. However, supplementation of L-methionine significantly increased the biological value (Smith and Palmer, 1976). Moreover, based on amino acid composition of yeast protein, it is accepted that yeast protein contain a high amount of essential amino acid especially a high lysine content (Halasz *et al* 1991).

The only limitation of these yeast proteins are their relatively low content of sulfur-containing amino acids. It was concluded that the type of growth media used for yeast influenced the amino acid composition. It was observed that natural more complex media like whey protein were better at promoting lysine content (Halasz *et al* 1991). Additionally, different conditions of protein extraction and precipitation were observed to influence the amino acid composition of yeast protein concentrates derived from the same yeast biomass (Vananuvat and Kinsella, 1975, Sergeev *et al.,* 1984).

The essential amino acid content of yeast protein meets most of the requirements recommended by FAO/WHO of United Nations (FAO/WHO, 1973) with the exception of the two sulfur-containing amino acids.

Table 1.4. Amino acid content in different yeast biomass, yeast protein preparation and yeast extracts.

Descriptions	**Essential amino acid**								**Non-essential amino acid**										**References**
g of Amino acids per 100 g of protein	Threonine	Valine	Methionine	Isoleucine	Leucine	Phenylalanine	Lysine	Tryptophan	Aspartic acid	Glutamic acid	Serine	Glycine	Histidine	Arginine	Alanine	Proline	Tyrosine	Cystine	
Saccharomyces fragilis cells	5.19	6.72	0.86	5.43	8.21	4.77	8.97		11.40	14.90	5.55	5.68	2.93	5.03	8.33	4.23	1.88	0.08	(El-Samgragy, 1988)
Kluyveromyces marxianus	5.82	6.77	0.86	5.82	8.44	4.69	9.33		10.40	13.20	5.88	5.04	2.76	7.73	7.53	4.34	1.59	0.01	
Candida tropicalis	5.60	5.95	1.51	5.78	9.42	4.24	8.66		9.72	16.84	5.66	4.72	2.91	4.84	6.11	4.67	2.83	0.43	
Kluyveromyces fragilis grown on whey	4.45	5.02	0.38	3.82	5.47	3.98	6.91	1.07					1.98	4.30					(Paul *et al*., 2002)
Kluyveromyces fragilis cells	4.41	4.69	0.62	4.26	6.50	3.78	5.46	1.30	10.36	12.93	4.62	3.25	1.70	4.83	5.01		3.25	1.26	(Orban *et al*., 1994)
Kluyveromyces fragilis grown on whey	4.40	4.84	1.54	4.31	8.10	3.64	8.60	1.40	10.61	13.60	4.58	3.51	1.96	4.68	5.00		3.39	1.52	
Saccharomyces fragilis	2.98	4.07	Cys+Met	4.80	9.50	3.56	6.79	0.70	7.06	26.00	3.43	5.48	1.22	2.80	11.6	4.36	2.93	2.70	(Sergeev *et al*., 1984)
Yeast Protein Extract / pH 3.0	4.24	4.66		4.85	10.5	4.07	7.83	0.74	9.41	19.40	4.62	5.14	1.55	4.60	8.14	4.45	3.53	2.37	
Yeast Protein Extract / pH 8.0	4.76	4.65		4.60	11.0	5.36	9.50	0.81	10.50	15.40	5.77	4.89	1.50	1.60	7.69	5.08	4.3	2.62	
Yeast Protein Extract / pH 9.5	4.40	5.17		4.20	7.46	4.09	10.0	0.80	10.50	19.90	4.44	5.90	2.52	4.18	7.52	3.38	3.54	1.99	
Yeast Protein Extract / pH 12.0	4.18	5.88		3.58	9.30	1.21	8.13	0.83	13.40	11.30	5.63	7.72	3.92	4.02	10.3	5.86	0.62	0.12	
Saccharomyces fragilis grown in a whey medium	4.60	4.90	1.30	4.30	6.00	3.00	6.90		7.20	10.90	4.20	4.00	2.50	5.40	5.50	4.00	3.30	1.53	(Vananuvat and Kinsella, 1975)

Table 1.4 continued

Descriptions	Essential amino acid								Non-essential amino acid										References
g Amino acids per 100 g of protein	Threonine	Valine	Methionine	Isoleucine	Leucine	Phenylalanine	Lysine	Tryptophan	Aspartic acid	Glutamic acid	Serine	Glycine	Histidine	Arginine	Alanine	Proline	Tyrosine	Cystine	
Yeast protein isolates extracted with 0.4 % NaOH and precipitated at pH 4.0	3.95	4.37	1.20	3.33	7.25	3.09	6.86		10.20	10.60	5.11	4.27	0.50	3.75	5.10	3.46	3.42	1.05	
Yeast protein isolates extracted with 0.4 % NaOH and precipitated at 80 C, pH 6.3	4.73	5.34	1.51	4.00	9.11	3.89	8.54		12.00	12.70	6.19	4.66	0.72	4.57	6.22	4.16	3.42	1.17	(Vananuvat and Kinsella, 1975)
Yeast protein isolates extracted with water and precipitated at pH 4.0	4.38	4.96	1.38	3.84	8.18	3.48	7.91		11.20	10.90	5.60	4.62	0.64	4.35	6.03	3.88	3.42	1.31	
Yeast protein isolates extracted with water and precipitated at 80 C, pH 6.3	4.95	5.57	1.46	4.03	9.43	3.90	9.75		12.70	12.40	6.33	4.98	0.87	4.70	7.18	3.42	3.42	1.31	
Phosphorylated yeast protein from *Saccharomyces cerevisiae*	5.00	6.00	2.40	5.10	8.50	9.20	9.20	1.80					2.40						(Yamada and Sgarbieri, 2005)
Yeast Extract from spent brewer's yeast	2.21	5.87	0.67	2.98	4.97	3.82	8.81	0.98	7.63	7.80	3.04	3.27	7.29	1.90	5.90				(Chae *et al*., 2001)
Yeast extract from autolysed *Kluyveromyces fragilis* grown on whey with NaCl	4.12	4.74	0.90	3.72	5.98	3.22	5.36	0.90	10.09	11.84	4.15	2.86	1.78	4.41	4.29		3.41	0.85	
Yeast extract from autolysed *Kluyveromyces fragilis* grown on whey with NaCl	3.95	4.71	0.87	3.71	5.86	3.18	5.03	0.88	9.87	11.28	4.39	2.92	1.91	4.43	4.02		3.12	0.56	Orban *et al*., 1994)
**FAO/ WHO profile	4.0	5.0	3.5[A]	4.0	7.0	6.0[B]	5.5	1.0	Not quoted								[B]	[A]	

**Recommended by FAO/WHO of United Nations (FAO/WHO, 1973). [A]Phenylalanine + Tyrosine. [B]Methionine +Cystine

1.6.2c ACE inhibitory properties of protein hydrolysates

In recent years it has been recognized that dietary proteins provide a rich source of biologically active peptides of potential health benefiting interest. One such activity of specific interest is the **angiotensin-I-converting enzyme** (**ACE**) inhibitory activity of peptides. However such peptides are inactive and or within the sequence of the parent protein and can be released in three ways: a) through hydrolysis by proteolytic microorganisms, b) through the action of proteolytic enzymes derived from microorganisms or plants and c) through hydrolysis by digestive enzymes.

ACE is one of the several physiologically indigenous enzymes that play an important role in the renin–angiotensin system (RAS), which regulates arterial blood pressure as well as salt and water balance (Murray *et al.*, 2005). The main effector molecule of the RAS, angiotensin II, is produced through an enzymatic cascade consisting of renin. It first cleaves angiotensinogen to form the deca-peptide angiotensin, and ACE, which further cleaves angiotensin I into the octapeptide angiotensin II by removing the C-terminal dipeptide His-Leu. The resulting angiotensin II is a potent vasoconstrictor, which stimulates the release of aldosterone and antidiuretic hormone or vasopressin (FitzGerald *et al.*, 2004). This increases the retention of sodium, water and the regeneration of rennin. In addition, ACE, also termed kininase II, inactivates the vasodilators bradykinin and kallidin in the kallikrein-kinin system by cleaving the C-terminal dipeptide Phe-Arg. These effects directly act in concert to raise blood pressure.

Therefore, inhibition of ACE by ACE inhibitory drugs like captopril and natural ACE inhibitory peptides has been shown to result in an antihypertensive effect in hypertensive human subjects and animals (Ondetti *et al.*, 1977, Takano, 1998).

Furthermore, several studies have been conducted on the production and isolation of ACE inhibitory peptides from animal and plant-derived food proteins such as milk, egg, soy, corn, groundnut and fish (FitzGerald *et al.*,

2004, Vercruysse *et al.*, 2005: Matsui *et al.*, 2006, Miguel and Aleixandre, 2006, Guang and Phillips, 2009, Jakala and Vapaatalo, 2010) .

Among these protein sources a large number of potent peptides were liberated from milk proteins. As for the ACE inhibitory drugs, structure–activity correlations between different peptide inhibitors of ACE indicate that binding to ACE is strongly influenced by the C-terminal tri-peptide sequence of the substrate. However, the precise substrate specificity for ACE is not fully understood and it appears that ACE prefers competitive inhibitors containing hydrophobic amino acid residues at the three C-terminal positions. Moreover, a C-terminal lysine or arginine, with a positive charge on the ε-amino group, also seems to contribute substantially to the inhibitory potency (Korhonen and Pihlanto, 2006).

In recent years only a few studies have been reported which outline the ACE inhibitory activity of yeast-derived peptides. Table 1.5 summarises the reported ACE inhibitory activities associated with yeast to date. Moreover, a recent report on the large scale proteomic analysis of *Saccharomyces cerevisiae* yeast by tandem mass spectrometry (LC/LC-MS/MS) identified the presence of 7,537 peptides and 1,504 proteins during a completely automated analysis (Peng *et al.*, 2002). This demonstrated the complexity and wide variety of peptides and enzymatic peptide sources present in yeast that may be of potential multifunctional bioactivity.

Table 1.5 Reported ACE inhibitory activities associated with yeast to date.

Yeast organism	Description	IC_{50}[a] / ACE[b] inhibition Index	References
Brewer's yeast	**AF(Ala - Phe),** dipeptide generated by hydrolysis of brewer's yeast by alkaline proteases	3	Kanauchi, 2005
Brewer's yeast	**GF (Gly - Phe),** dipeptide generated by hydrolysis of brewer's yeast by alkaline proteases	3.4	
Saccharomyces cerevisiae	Yeast autolysate obtained after 121 h of autolysis in model wine system. ACE inhibition Index was obtained when 0.63 mg/ mL of peptide nitrogen was present test reaction.	65 %	Alcaide-hidalgo *et al.*, 2007
Saccharomyces cerevisiae	Yeast autolysate obtained after 24 h of autolysis in synthetic wine medium. ACE inhibition Index was obtained when 0.21 mg / mL of peptide nitrogen was present test reaction.	22 %	Aredes Fernández *et al.*, 2010
Oenococcus oeni (Lactic acid bacteria)	Extract obtained after 48 h secondary fermentation of yeast autolysate in synthetic wine medium. ACE inhibition Index was obtained when 0.29 mg/ mL of peptide nitrogen was present test reaction.	36 %	
Saccharomyces cerevisiae (isolated from red wine)	LIPPGVP	17.5	Takayanagi & Yokotsuka, 1999
	YYAPFDGIL	83.0	
	YYAPF	26.4	
	SWSF	76.3	
	WVPSVY	25.7	
	AWPF	18.3	

[a]IC_{50}: The amount of peptide that mediated a 50 % inhibition of ACE activity (μM). [b] ACE inhibition Index is the value for the percentage ACE inhibition observed based when amount of peptide is added to test reaction compare to control (mg /mL peptide nitrogen present in test reaction)

1.6.3 Yeast cell wall preparations

After the extraction of yeast protein as a concentrate from yeast biomass, the remaining components are cell walls residues of yeast biomass. As discussed in Section 1.5.2, the cell wall components are rich sources of polysaccharide and glycoprotein. To date several methods have been proposed through publications and patents (Table 1.6) for extraction of value added products from cell wall components.

The classical method of the cell wall extraction is predominantly based on the solubility of different cell wall components under alkaline and acidic conditions. In short, it involves alkali treatments of the disintegrated yeast biomass or cell wall waste from the yeast extract industry, for the dissolution of manno-protein. This is followed by acid treatment to dissolve certain residual non-glucan residues and hydrolysis of the glycosidic linkages, principally the β-1→6 linkage. Finally the product after acid treatment is subject to heat and solvent treatment to remove lipids (Bacon *et al.*, 1969, Manners, 1974, Jamas, 1989, Williams *et al.*, 1991, Klis, 1994, Nguyen *et al.*, 1998).

However, these methods result in the appreciable chain length degradation of β-glucan and with appreciable loss of cell wall protein fraction as waste stream. Consequently quite a few methods are proposed with the milder condition to extract β-glucan and manno-proteins from yeast. These proposals are briefly summaries in Table 1.6.

Table 1.6 Various reported methods for the fractionation of cell wall components into value added products.

Methods for cell wall fractionation into value added products	β- Glucan (% w/w)	Mannan (% w/w)	References
➲ Ethylene diamine, sodium hydroxide, buffers of citrate salts were used to solubilize mannoproteins and subsequently precipitated with Fehling's solution or methanol. Extraction was done for characterization of mannoprotein.			Sentandreu and Northcote, 1968, Nakajima and Ballou, 1974
➲ Extraction with 2 % boiling sodium-dodecyl-sulfate (SDS) in the presence or absence of reducing agents, like mercaptoethanol, represents a widely used approach to free glucan from mannoproteins and other proteins. Extraction was done for characterization of mannoprotein.			Valentin *et al.*, 1984, Javier Pastor *et al.*, 1984
➲ Treatment of whole cells with pure water at temperature135 °C was also applied, yielding a highly contaminated mannoprotein fraction. Extraction was done for characterization of mannoprotein.			Shibata, 1983, Peat *et al.*, 1961
➲ Treatment of disintegrated cell in hot water at 125 ˚C for 5 h, followed by treatment of crude glucan with protease and lipases for further purification. Extracted (1→3), (1→6)-*β*-d-glucan subsequently used for ascertain the antioxidant activity of different fraction.	75	Waste product	Jaehrig *et al.*, 2008
➲ Mild, step wise fractionation of yeast cell wall powders into β-glucan and mannoprotein with yield of 25 % and 14 %, respectively. Fraction method includes hot water treatment to cell wall followed by protease treatment to heat treated cell residue and solvent extraction. Aqueous extract obtained after hot water was precipitated with ethanol to have mannoprotein.	83 - 92	76 - 78	Freimund *et al.*, 2003, Sauter, 2004

Table 1.6 continued

Methods for cell wall fractionation into value added products	β- Glucan (% w/w)	Mannan (% w/w)	References
➲ Spent brewer yeast was autolysed and cell wall residues then homogenized, extracted with alkali followed by acid treatment and subsequently spray dried.	50.5 - 53.0	Waste product	Thanardkit *et al.,* 2002 and Suphantharika *et al.,* 2003
➲ Extraction of mannoprotein in citrate buffer followed by either ultrafiltration or precipitation with acidic ethanol. The product obtained was subsequently tested for emulsifying properties. The approximate yield of bioemulsifier was in range of 4 - 16 %	Waste product	44 - 90	Cameron *et al.*, 1988, Barriga *et al.*, 1999, Dikit *et al.*, 2010
➲ Cell wall residues after protein extraction were heated (75 ˚C for 20 minutes) in alkaline conditions. The soluble fraction after alkaline extraction was subjected to isoelectric precipitation to yield cell wall protein fraction and insoluble fraction was termed crude glucan preparation. The yield of cell wall proteins and glucan preparation was 15.2 and 18 % respectively.	88	19	Otero *et al* ., 1996
➲ Mild and sequential treatment of yeast biomass was proposed to produce glucan and mannan preparations with application of autolysis process, followed by enzymatic treatment with one or more enzymes of proteases, glucanase and lipases. The insoluble fraction is glucan and soluble fraction yields mannoprotein as retentate when subjected to ultrafiltration.	45 - 54	15 – 62	Sedmak, 2006 , Wheatcroft, 2002

1.6.3a Functional properties of yeast cell wall carbohydrates

β-glucan preparations are of commercial interest to the food industry due to its demonstrated advantages in improving physical properties of foods and also due to its multiple beneficial effects on human and animal health. Belem and Lee, 1998 reviewed the applications of yeast cell wall polysaccharides and concluded that yeast β-glucan can be used to improve the physical properties of foods via their thickening and water holding abilities. These polysaccharides are good emulsifying stabilizers and can also be used as fat replacers. This fat replacing property is due to mouthfeel effects.

In addition β-glucan from yeast cell wall is nutritionally defunct in the human digestive tract and also acts as a crude fiber source. It has ability a) to alter the freezing temperature of frozen food, b) to inhibit starch retro-gradation and c) to improve moisture-retaining capacity.

1.6.3b Biological properties of yeast cell wall carbohydrates

Yeast β-1→3 glucan or simply β-glucan is a class of compounds that are known as **biological response modifiers (BRMs).** These are capable of stimulating the immune system by upregulation or downregulation of the specific pathways of immune response as shown in Figure 1.7.

In general β-glucan targets various cell types in the immune system i.e. a) macrophage activation, b) T-cell stimulation, c) activation of natural killer (NK) cells, d) activation of classical and alternative complement pathways and e) increased antibody production (Bohn and BeMiller 1995).

Among these, macrophages are the best characterised targets for the β-glucan in the mammalian system which plays a significant role in elicitation of the immune system (Bohn and BeMiller 1995, Zekovi *et al.,* 2005, Chan *et al.,* 2009, Petravić-tominac *et al.,* 2010). The molecular mechanism by which β-glucan interacts with receptors on macrophages to stimulate the synthesis of cytokines, chemokines and reactive oxygen intermediates was reported previously (Gantner *et al.,* 2003).

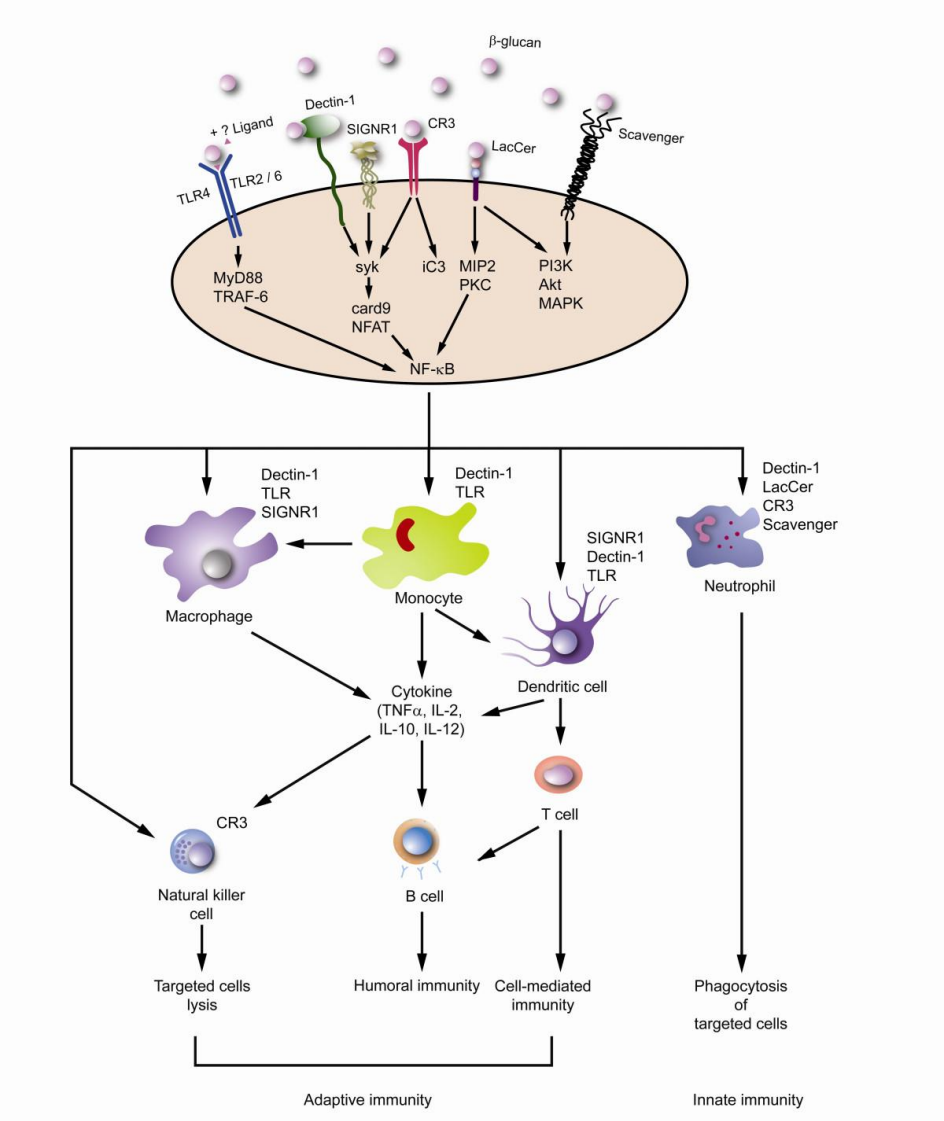


Figure 1.7 Schematic pathway of immune activation by β-glucans. β-glucans can act on a variety of membrane receptors found on the immune cells. It may act singly or in combination with other ligands. Various signaling pathway are activated and their respective simplified downstream signaling molecules are shown. The reactors cells include monocytes, macrophages, dendritic cells, natural killer cells and neutrophils. Their corresponding surface receptors are listed. The immunomodulatory functions induced by β-glucans involve both innate and adaptive immune response (Adopted from Chan *et al.*, 2009).

The major receptors reportedly involved in 1→3 glucan recognition/binding were identified to be a phagocytic, non-CR3 receptor on macrophages specific for β-1→3 glucan (Czop and Austen, 1985, Czop and Kay, 1991), a glycosphingolipid β-1→3 glucan receptor on human neutrophils (Wakshull *et al.*, 1999), and recently, the dectin-1 receptor found of human macrophages (Brown and Gordon, 2001).

Significant barriers to the effective use of naturally occurring yeast β-glucan as immune modulating agents are their insolubility in water at acidic pHs. When orally administered, they pass through the stomach nearly intact mainly due to their large molecular weight (Zhang and Hamaker, 2010). In contrast, oral administration of water soluble glucans was shown to increase systemic levels of interleukin IL-12 following their absorption by gastrointestinal (GI) tract (Rice *et al.*, 2005).

Also, systemic administration of insoluble glucan to rats was shown to increase their susceptibility to endotoxin shock (Cook *et al.*, 1980), while soluble β-glucan had no such adverse effects (Bowers *et al.*, 1986). Conversely, low molecular weight, water soluble β-glucan was demonstrated to protect against infection and shock in rats and mice (Vereschagin *et al.*, 1998, Hetland *et al.*, 2000). Clinical studies suggested that administration of soluble glucans to post-surgical/trauma patient's stimulated conversion from leukocyte anergy, decreased septic complications and improved host survival (Williams *et al.*, 1995, Williams *et al.*, 1996). However, little is known about the precise mechanisms by which these glucans alter the septic state and affect the cell biology of leukocytes.

In consideration of scientific findings outlined above a major focus in recent times has been at developing methodologies to increase the solubility of β-1→3 glucan. In order to improve β-glucan solubility, several derivatization procedures such as sulfation, phosphation, and carboxymethylation have been applied (Williams *et al.*, 1991, Demleitner *et al.*, 1992, Williams *et al.*, 1992). Additionally, many different methods including acid and alkaline hydrolysis, enzymatic digestion, and ultrasound treatment have been applied to depolymerise the insoluble macromolecular structure to native soluble β-1→3 glucan (Zekovi *et al.*, 2005).

In present study, a focus has been on the physiological *in vitro* assessment of water soluble β-1→3 glucan preparation obtained from chemo-enzymatically treated cell wall of *Kluyveromyces maxiaunus*.

In general, the ability of β-glucans to modulate immunity has a number of beneficial applications (Peat *et al.*, 1961, Manners, 1974, Halasz *et al.*, 1991, Dritz *et al.*, 1995, Belem and Lee, 1998, Lipke and Ovalle, 1998, Freimund *et al.*, 2003, Kogan, 2002, Sedmak, 2006, Kogan and Kocher, 2007) which can be summarised as follows:

- Enhance innate host defences by binding to specific macrophage receptors and activating macrophage, resulted in antitumor, antibacterial, and wound-healing activities.
- β-Glucan can also directly influence the activity of other immune cells, including T and B cells, NK cells, eosinophils and neutrophils.
- Potent immunomodulatory, anti-inflammatory, anti-microbial, anti-infective, antiviral, antitumoral, hepatoprotective, antidiabetic and wound-healing properties.
- Possesses potent antioxidant properties and radioprotective effect.
- Increases the effectiveness of antibiotics and reduces LDL cholesterol level (cholesterol-lowering).
- Inhibition of pathogen like *E. coli* and *Salmonella* spp. adhesion to epithelial tissue.
- Stimulation of immunocompetent cells.
- Suppress toxic activity of mycotoxins probably by interacting with their toxic radical.

1.7 Current market scenario of value added products derived from yeast biomass

According to the European Association for Specialty Yeast Producers (EURASYP) value added products derived from yeast biomass are termed as specialty yeast products derived from inactivated, plasmolysed, autolysed or hydrolysed food yeasts. This includes the food yeasts, autolysed yeasts, yeast extracts, yeast cell walls and yeast β-1→3 glucan (Eurasyp, 2010).

As per the BCC research company report (März, 2010), the global market value for yeast and yeast specialty products will reach close to $ 5 billion by 2015, an increase from $ 3 billion in 2009. This represents a compound annual growth rate (CAGR) of nearly 8 %. The largest contributor in this segment are yeast

autolysate and extracts with the estimated value of $ 950 million in 2009, and this is expected to grow with a CAGR of 6 % to reach nearly $ 1.4 billion in 2015.

Baker's yeast, the second-largest contributor, is expected to reach $ 1.4 billion in 2015, after increasing from the 2009 estimate of $ 904 million at a CAGR of 7.9 %. The segment made up of active feed yeast is projected to increase at a CAGR of 9.6 %, growing from an estimated $ 144 million in 2009 to $ 250 million in 2015.

The market for ethanol yeast in comparison to bakery and brewery yeast is very new but is projected to experience a CAGR of more than 18 % through the forecast period, and to increase from $ 87 million in 2010 to $ 240 million in 2015 (Figure 1.8).

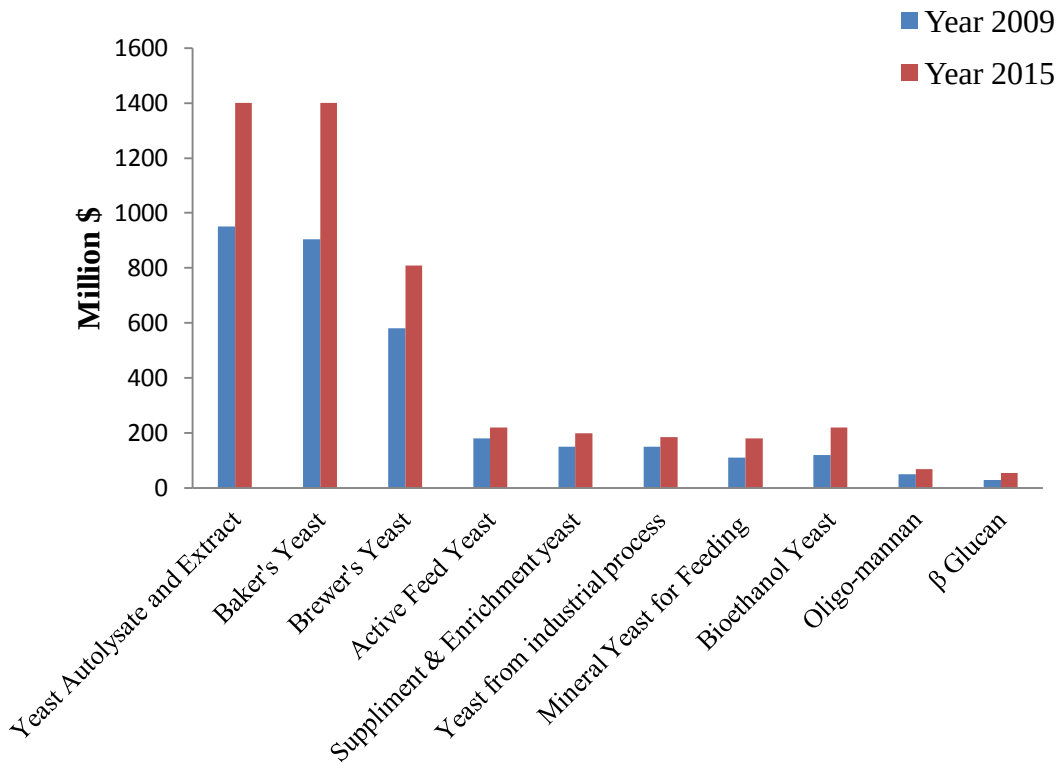


Figure 1.8 Development of worldwide market of yeast by value (März, 2010).

The market for β-glucan ingredients has an estimated value of $ 80 to 100 million (März, 2010). The global market for β-glucan holds significant growth potential as a result of European Food Safety Authority (EFSA) permission to

use it as novel food supplement. This will be further driven by the fact that food ingredients that enhance the immune system have been one of the best performing functional food sectors with recorded projected market values of $ 21 billion by 2015 from about $ 13 billion in 2011 (Starling S., 2012).

Additionally as per Consultancy Leatherhead International the yeast extract market will grow between 3 to 4 % per annum and growth rate will remain high in the future due to a strong demand from Asian markets. Asian Food makers are replacing monosodium glutamate and hydrolysed vegetable proteins (HVP) in their formulations with yeast extract flavour enhancers due to the natural perception and cleaner labels, i.e. no E number (Halliday, 2010, Fletcher, 2005).

According to the Frost & Sullivan consultancy report in the year 2007, the European market for yeast was estimated to be worth $ 559.6 million and approaching maturity (Telles, 2008). Historically, Europe has dominated the production of speciality yeast products and a handful of European suppliers, including BioSpringer, DSM, and Bel Industries supply the two-third of the world's consumption 100,000 MT per year (Fletcher, 2005). Yeast speciality production is primarily based on baker's yeast that uses sugar molasses as its feed stock.

Currently, the major concern for the European yeast market is the shortage of molasses due to a) restriction of sugar trade in Europe and on-going implications of the European Union regime, b) emergences of the biofuels industry which also uses molasses. This has increased the molasses prices by 50 per cent and a corresponding 10 per cent increase in yeast prices. Thus brewers and spent dairy yeast will be in great demand for production of speciality yeast products (Telles, 2008).

The emergence of promising new specialty yeast sector with high growth rate has added a much needed revenue boost to the yeast industry. To remain competitive in the market, constant innovation, application and product development is required (Telles, 2008). Most of the global and European manufacturers are looking into developing customer specific solutions as an

opportunity to expand their product and business portfolio, by introducing a range of newer products and applications. For example, DSM Food Specialties developed "Savorkey" range of reaction flavour from yeast extract and Maxarome Select range of neutral 5' nucleotide rich yeast extract (Fletcher, 2006).

1.8 Objectives and layout of this study

After a detailed analysis with respect to composition of yeast biomass, technological developments in yeast processing and market trends of value added products from yeast biomass, it was determined that an alternative source of yeast biomass for production of yeast speciality products was needed. This was vital as traditional sources such as baker yeast may not be economically feasible in the future due to advent of bio fuel industry.

Therefore this present study is based on following the ***hypotheses to utilise spent Kluyveromyces marxianus yeast waste as an alternative source for production of value added products.***

- That the intrinsic factors like pH and temperature can influence the process of autolysis of *Kluyveromyces marxianus* and can be optimised to extract soluble constituents of yeast biomass.
- That flavour precursors (peptides, amino acid, and nucleotide) derived from yeast during autolysis may undergo various reactions such as Maillard reaction to yield yeast extract flavouring with quantifiable sensory attributes.
- Yeast protein hydrolysates with elevated anti-hypertensive activity can be purified and isolated by bioactivity guided fractionation.
- That yeast cell wall preparation(s) with immunomodulatory activity can be better characterized by mammalian cell culture models and *in vitro* assays.

➲ **Overall objectives of the current project are:**

1. To develop a process to generate yeast extracts with unique sensory attributes. This was addressed as per methodology outlined in Chapter 2 ***"Effect of pH and temperature of autolysis on chemical and sensory attributes of yeast extract produced from spent Kluyveromyces marxianus yeast".***

2. To develop a process to generate a glucan enriched product(s) with characterised immunomodulatory activities. This was addressed as per methodology outlined in Chapter 3 "***Phosphorylated yeast protein concentrate and bioactive β-glucan enriched cell wall preparations from spent Kluyveromyces marxianus yeast***".

3. To purify and characterize the anti-hypertensive peptide(s) in a novel prepared yeast protein hydrolysate. This was addressed as per methodology outlined in Chapter 4 "***Angiotensin-I-converting enzyme inhibitory activity of yeast protein hydrolysates derived from spent Kluyveromyces marxianus***".

➲ **Research strategy overview**

The main research strategies being applied are,

1. Application of descriptive sensory analysis and chemical indices to study the effect of pH and temperature of autolysis on yeast extract attributes.

   Deliverable: *Generation of a yeast extract with unique quantified sensory attributes for potential application in culinary seasoning.*

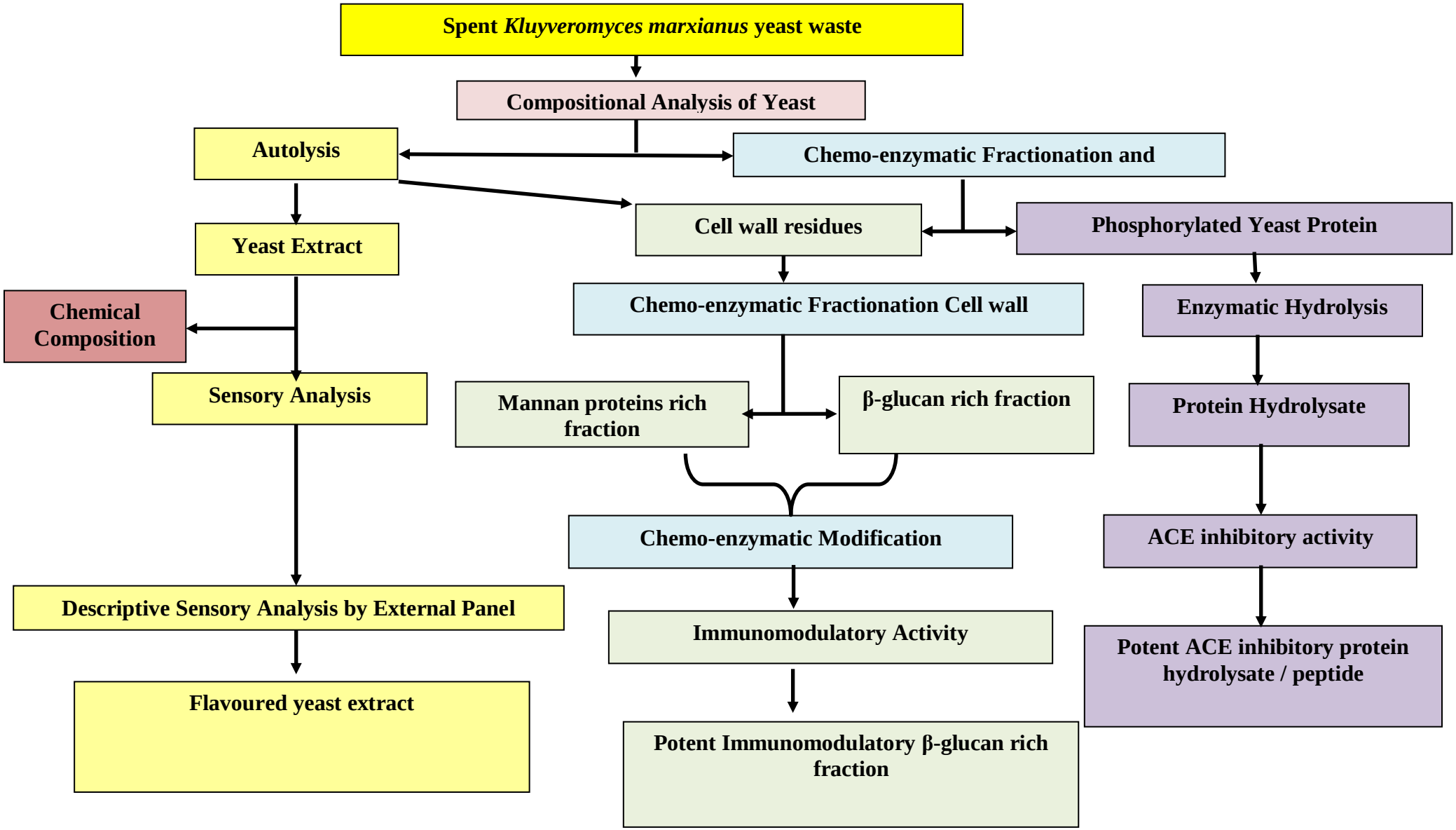
2. Yeast protein hydrolysate with potent anti-hypertensive activity will be fractionated by semi-preparative reversed phase HPLC. Active fractions will be selected, pooled and lyophilized for further peptide purification using reversed phase HPLC. Potent peptide(s) will be subjected to amino acid LC-MS-MS sequencing.

   Deliverable: *Identification of a novel anti-hypertensive peptide from yeast protein (Kluyveromyces marxianus).*

3. Using training supports within Shannon ABC an *in-vitro* murine macrophage cell and whole human blood culture model will be employed to ascertain the immunomodulatory activity of the glucan extracts.

Deliverable: *Generation of a glucan enriched product with immunomodulatory properties for application in animal feed.*

Figure 1.9 Schematic Flow chart over viewing the main approach to addressing the objectives of the current research study



Chapter 2.0

Effect of pH and temperature of autolysis on chemical and sensory attributes of yeast extract produced from spent *Kluyveromyces marxianus* yeast.

2.1 Introduction

Yeast extracts are predominantly produced by controlled autolysis of *Saccharomyces cerevisiae* such as baker's yeast and debittered spent brewer's yeast. However, yeast extract production from food grade *Kluyveromyces marxianus* is not well studied (Amrane and Prigent, 1996, Lukondeh *et al.*, 2003a) and limited information is available about the effect of autolytic conditions like temperatures and pH on the type of yeast extract produced.

Yeast extracts are used directly in a wide variety of food applications such as flavouring bases. The previous studies of yeast extract were focused mainly on chemical and odour characterization (Hajšlova *et al.*, 1980, Ames and Leod, 1985, Dziezak, 1987, Munch *et al.*, 1997, Munch and Schieberle, 1998, Champagne *et al.*, 1999, Chae *et al.*, 2001, Mahadevan and Farmer, 2006). The information on the taste active, non volatile constituents of yeast extracts are relatively limited (Festring and Hofmann, 2010).

Modern sensory techniques such as 'sensory descriptive analysis' is well established and used by the cheese industry to evaluate changes in taste active (sensory) attributes of cheese produced at various ripening conditions (Murray *et al.*, 2001, Mirarefi *et al.*, 2004, Hannon *et al.*, 2005). Such sensory discriptive analysis has been used herein to objectively determine and describe the sensory attributes of yeast extracts arising from the different autolytic treatments used in this study.

Therefore, current study was aimed at investigating the effect of pH and temperature of autolysis on sensory and chemical characteristic of yeast extract produced from spent *Kluyveromyces marxianus* yeast as per schematic experimental design shown in Figure 2.1.

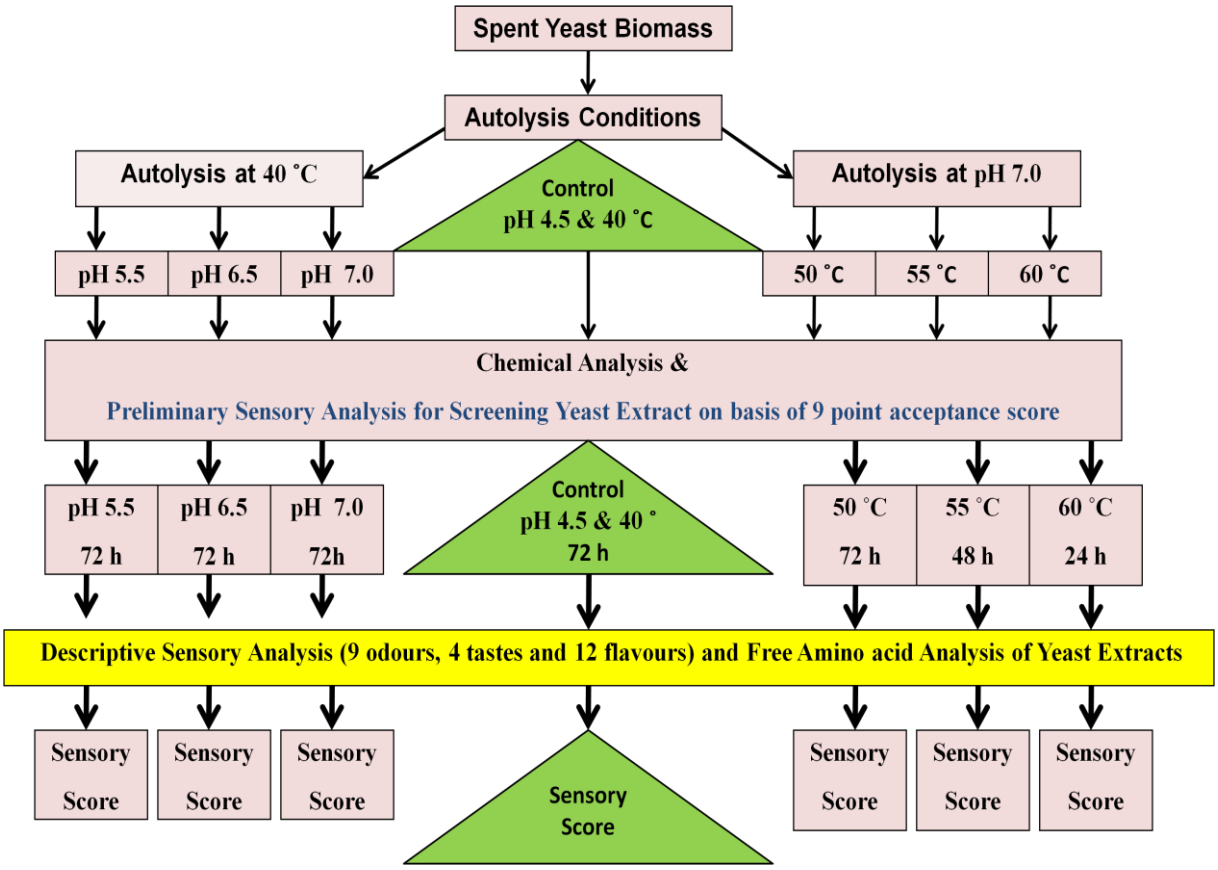


Figure 2.1 Overview of the experimental design used for determining the effect of various autolysis conditions on sensory and chemical characteristic of yeast extract produced from spent *Kluyveromyces marxianus* yeast.

2.2 Materials and methods

2.2.1 Raw material supply

Spent yeast biomass of *Kluyveromyces marxianus* was obtained as a by-product of an ethanol fermentation process of whey permeates. It was kindly provided by Carbery Group, Ballineen Co. Cork, Ireland. The spent yeast was obtained in small batches and was stored upon receipt at -20 ^{o}C prior to use. All other chemicals used were of analytical grade.

2.2.2 Yeast autolysis procedure

Investigations into yeast autolysis at various initial pH (4.5 to 7.0) and temperature (40 to 60 ˚C) conditions were carried out at 40 ˚C and pH 7.0, respectively as described by Zhao and Fleet, 2005 with some modifications as per Moresi *et al.*, 1995 (Figure 2.1).

In brief, the experiment was conducted as three independent trials from three different batches of spent yeast slurry brought from industry. The average value of pH and total solid (dry matter) of each batch of spent yeast slurry varies in the range of 4.5 ± 0.5 and 15.0 ± 5 %, respectively. Therefore in order to avoid batch wise variation; the ***spent yeast control*** *for autolysis experiments was standardized to pH 4.5 and 10 % of dry matter*.

Similarly, for each independent autolysis experiments, pH of a 200 mL diluted aliquots (10 % w/w) of spent yeast was adjusted to either *pH 4.5 (control*), 5.5, 6.5 or 7.0 by addition of 2M NaOH or 2M HCL. The precise biomass concentration in each suspension was determined by dry weight measurement (Section 2.2.3).

To initiate autolysis, the yeast suspensions were incubated at 40, 50, 55 and 60 ºC in an orbital shaker rotating at 200 rpm for 96 h. Samples of the autolysing yeast biomass were withdrawn at fix time intervals up to 96 h and were separated by centrifugation (3000 g, 10 min, 4 ºC) into cell pellet and yeast extracts supernatant. The yeast extract obtained at pH 4.5 at 40 ˚C for each autolysis treatment was considered as control to ascertain the effect of various pH and temperature conditions. The yeast extract obtained in triplicates from three independent trials performed for each autolytic condition were pasteurized at 80 ºC for 30 min, cooled and stored at -20 ºC prior to analytical analysis.

2.2.3 Autolysis process analysis

The cellular breakdown of spent yeast cells and progress of autolysis process were monitored by assaying the percent total solid, total nitrogen, and α- amino nitrogen content of yeast extracts. These determinants correspond to the release of protein nitrogen, hydrolyzed protein, peptide, and free amino nitrogen as breakdown products of yeast cell during autolysis.

In brief, total solids in spent yeast suspension and yeast extracts were determined by dry weights following drying at 105 ºC for 24 h until constant weight is achieved **(Appendix I: Methods 1.0).**

Total nitrogen was analysed by using the micro-Kjeldahl method as per International Dairy Federation method (I.D.F., 1993, **Appendix I: Methods 2.0**). The α-amino nitrogen content of yeast extract was determined by ninhydrin method (Doi *et al.,* 1981) with some modification as per Baer *et al.,* 1996 using Leucine as standard. In brief, 40 µL standards of Leucine (0.1 mM to 0.25 mM) solution and appropriately diluted yeast extract samples were placed into each well of microtiter plate respectively. Then 80 µL of ninhydrin stannous chloride reagent was added to each well, using a multichannel pipette. The mixing was done by repeated pipetting and was incubated at 90 ºC for 20 minutes by carefully floating the microtiter plate in water bath. Subsequently for cooling, the plates were transferred in ice water bath. Then 120 µL diluent was added to each reaction mixture. Afterwards, the microtiter plate was wiped dry to take the absorbance of these standards and samples at 570 nm in VarioskanFlash 96-well plate reader. Using the standards a standard curve was plotted to determine the sample concentrations as mM Leucine **(Appendix I: Methods 3.0).**

The individual amino acids content in selected yeast extracts were analyzed in duplicates using an amino acid analyser based on ion-exchange chromatography (Biochrom 30, Pharmacia Biochrom Ltd). In brief, free amino acids in yeast extract were purified by mixing 200 µL of 10 % sulfosalicylic acid with 800 µL yeast extract to precipitate high molecular weight proteins according to the method of Pharmacia Biochrom Ltd (Cambridge, United Kingdom). Subsequently, the amino acids were separated by using standard lithium citrate buffers of pH 2.80, 3.00, 3.15, 3.50, and 3.55. The post-column derivatization was performed with ninhydrin (Stenberg *et al.,* 2002). Total amino acids in yeast extract were determined in duplicates after hydrolysis of samples at 110 °C for 24 h with 6 M HCl prior to the amino acids analysis.

2.2.4 Definitions

1. Yield of solids in autolysis is defined as the ratio of total solids in yeast extract to total initial yeast solids.

2. The degree of hydrolysis (DH) is defined as the percentage of peptide bonds hydrolysed. For an autolysis experiment this is calculated by estimating the

percentage ratio of α-amino nitrogen in relation to the total nitrogen corresponding value.

3. Peptide-bound amino acids of selected yeast extract were calculated as the difference of total and free amino acids.

4. The ratio of free amino acid to total amino acid ($R_{faa: taa}$) that indicates how much of each amino acid is still present as peptides, was calculated by dividing free amino acid content to total amino acid content of selected yeast extract.

2.2.5 Descriptive sensory analysis of yeast extract – methodology

To evaluate difference in sensory characteristic, the yeast extract samples produced at various autolytic treatments in triplicates, were pasteurized and frozen at -20 ˚C. Prior to a final descriptive sensory analysis of yeast extract by a trained external sensory panel, a large number of samples were screened out on basis of a **9 point hedonic scale** (Drake, 2009). This scale is defined as, 1 for dislike extremely, 2 for dislike very much, 3 for dislike moderately, 4 for dislike slightly, 5 for neither like nor dislike, 6 for like slightly, 7 for like moderately, 8 for like very much and 9 for like extremely.

For evaluation, composite samples were prepared by blending equal volumes of the liquid yeast extracts from all three independent like trials. The blended samples were pasteurized at 80 ºC for 30 minutes, cooled and frozen at -20 °C until the final tasting.

All selected yeast extracts samples in duplicates were then analysed for odour and flavour using a one balanced design experiment. Prior to descriptive sensory assessment of the samples, the panel members, consisting of nine highly trained assessors performed the sensory evaluation. The sensory profiling was carried out by panel member of the Sensory Laboratory at the Department of Food Science, Mumbai University, India (DFScMU). The panel members were faculty and student of DFScMU, all had followed a basic training as per the ISO standard 8586-1:1993, Sensory Analysis - General Guidance for Selection, Training and Monitoring of Assessors and ASTM STP 758 Guidelines for the Selection and Training of Sensory Panel Members.

Focus groups were held with the judges prior to descriptive sensory analysis, in order to develop a consensus vocabulary for odour, flavour and mouthfeel attributes. During each discussion, the panel evaluated the odour, taste and flavour of each of the yeast extracts and added new descriptors to the previously existing vocabulary where necessary (Hannon *et al.*, 2005). Using the total seven duplicated shortlisted yeast extracts involved in the study, a list of initial attributes were developed. Panellists were trained during two 1-h sessions to ensure a uniform understanding of the developed sensory attributes. After completing of their training, the panel rated the intensity of the seven products with respect to 9 odours, 4 tastes and 12 flavours attributes (Table 2.1).

On the morning of analysis, samples were removed from storage at 4 ºC, (after being previously thawed from storage at -20 ºC) made up to 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. 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. Also reference samples of 0.2 % MSG and yeast suspension were made available. The panel rated the intensity of the yeast extract attributes on a scale of 0 - 9 with extremes of each descriptive term. The samples were evaluated in duplicate over a two-day period, and the order of presentation was balanced to account for the first order and carry-over effects.

2.2.6 Statistical analysis applied

Results were evaluated for statistical significance by one way analysis of variance **(ANOVA),** multiple comparison Tukey test. The dependent variables were means value of composition of yeast extract and sensory attributes of yeast extract while the other variables like initial pH or temperature and duration of autolysis were applied as covariates. All statistics were performed using the program SPSS for Windows, Version 18.0, SPSS Inc., Chicago, IL.

Table 2.1 List of important descriptors for descriptive sensory analysis of yeast extract.

	Odour attributes	**Definition**
1	Pungent	Physically penetrating sensation in the nasal cavity. Sharp smelling irritant. At the high end of this scale, a stinging sensation.
2	Caramel	The taste and aromatics associated with burnt sugar or syrup, toffee made with sugar that has been melted further
3	Creamy	Fatty. creamy tasting, of the nature of, or containing cream
4	Sweet	The taste and aromatics reminiscent of perspiration generated foot odour which are sour, stale and slightly cheesy
5	Fruity	The taste and aromatic blend of different fruity identities
6	Sulphur	The smell of sulphur - in particular that associated with hardboiled eggs or a struck match
7	Chemical	Odour associated with non-specific solvents.
8	Rancid	Odour associated with sour milk and oxidised fats. Having the rank aroma or taste characteristics of fats when no longer fresh.
9	Yeast	Reminiscent of the odour associated with farms/ cow dung/ manure/ slurry. Reference - Yeast sample.
10	Savoury	Odour associated with foods containing salt and/or MSG.
	Taste attribute	**Definition**
1	Sweet	The fundamental taste sensation of which sucrose was typical.
2	Salty	The fundamental taste sensation of which sodium chloride was typical.
3	Sour / Acidic	A sour, tangy, sharp, citrus-like taste. The fundamental taste sensations of which lactic acid and citric acids are typical.
4	Bitter	The fundamental taste sensations of which caffeine and quinine are typical.

Table 2.1 List of important descriptors for descriptive sensory analysis of yeast extract (Contd.)

	Flavour attributes	Definition
1	Buttery	Fatty, buttery tasting, of the nature of, or containing butter.
2	Nutty	The non-specific nut like taste and aromatics characteristic of several different nuts, e.g. peanuts, hazelnuts and pecans
3	Rancid	Flavour associated with sour milk and oxidised dairy fats. Having the rank aroma or taste characteristics of diary fats when no longer fresh.
4	Mouldy	Flavour characteristics associated with earth, raw mushrooms or moulds.
5	Process	A bland, shallow and artificial taste
6	Astringent	A mouth drying and harsh sensation. The complex of drying, puckering and shrinking sensations in the lower cavity causing contraction of the body
7	Balance	Mellow, smooth, clean. In equilibrium, well arranged or disposed, with no constituent lacking or in excess.
8	Strength	Flavour intensity, degree of mildness and maturity
9	Blue Cheese	Flavour associated with acetone or blue cheese.
10	Pungent Sensation	Physically penetrating sensation in the nasal cavity. Sharp smelling irritant. At the high end of this scale, a stinging sensation.
11	Savoury	Chemical feeling factor elicited by certain peptides and nucleotides. Reference - 0.2 % MSG.
12	Fatty	The range of sensations typically associated with fat content.

2.3 Results and discussion

In the present study the effect of pH and temperature on the chemical composition and sensory attributes of yeast extract obtained from autolysis of spent *Kluyveromyces maxirunus* yeast was studied as a function of time.

2.3.1 Effects of initial pH and temperature of autolysis on total solids content and yield of yeast extract.

The influence of initial pH and temperature of autolysis on total solids of yeast extracts and autolysis yield is given in Figure 2.1 and 2.2, respectively.

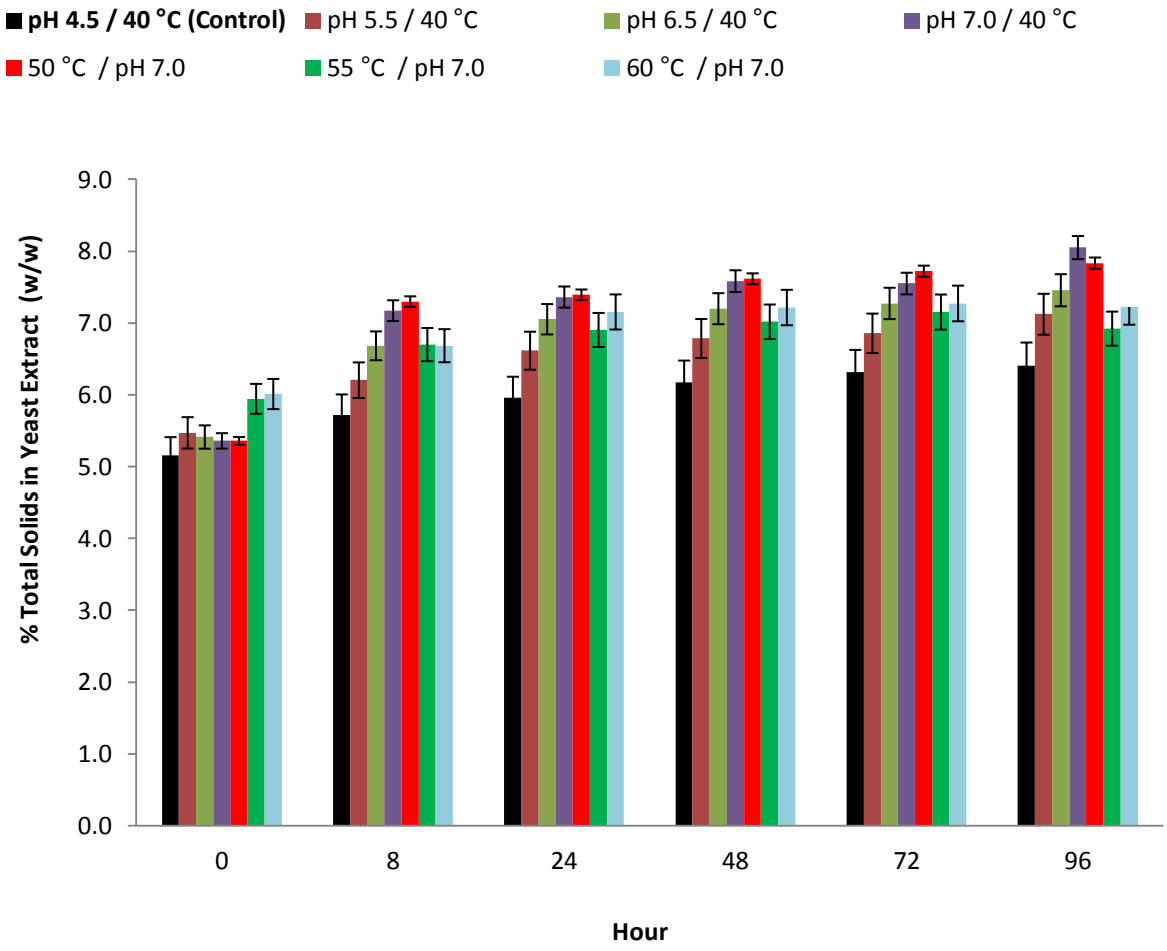


Figure 2.2 Influence of different initial pH and temperatures of autolysis on total solid of yeast extracts obtained from spent *Kluyveromyces marxianus* yeast. The results are reported as means ± standard deviation (n = 9).

The amount of total solids, released from yeast cells during autolysis at pH 7.0 and temperatures 40 °C and 50 °C, was increased considerably after 24 h of incubation as compared to zero h. Within each autolysis treatments after 72 h of

incubation the highest solid contents were obtained as 7.72 % at pH 7.0 and temperature 50 °C.

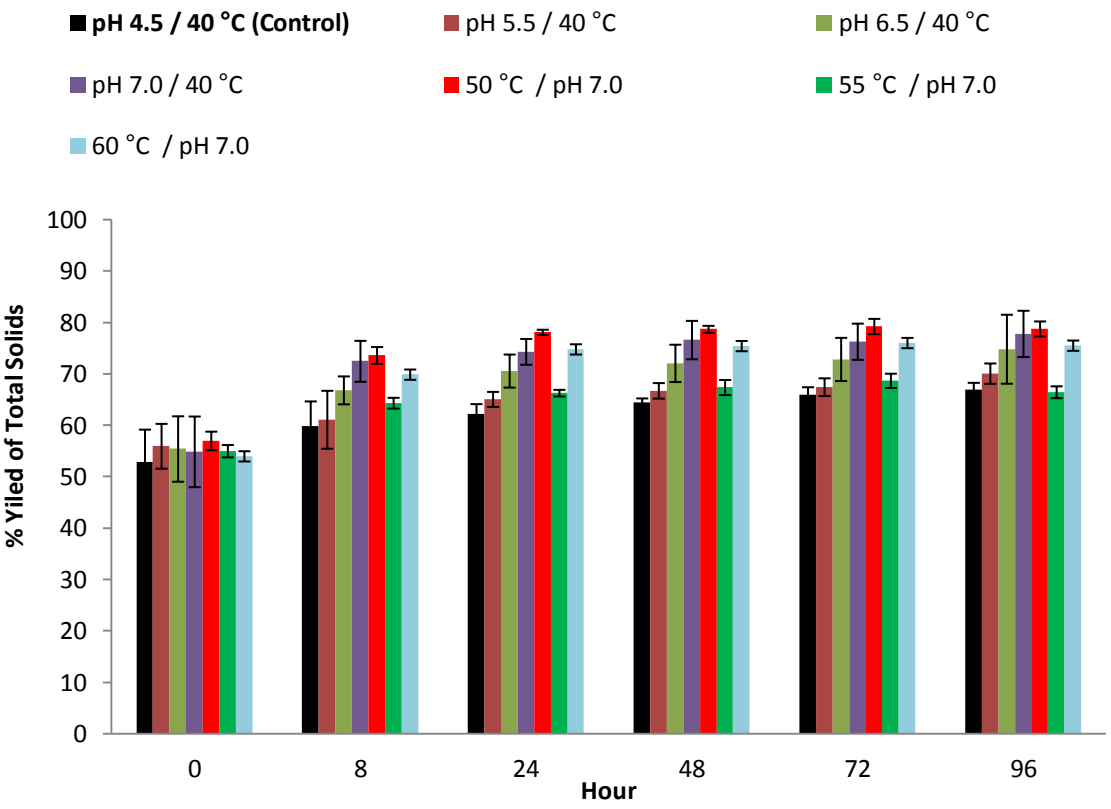


Figure 2.3 Influence of different initial pH and temperature of autolysis on % total solid yield of yeast extract from spent *Kluyveromyces marxianus* yeast. The results are reported as means ± standard deviation (n = 9).

Among the different initial pH values, autolysis at pH 7.0 and 6.5 exhibited the highest total solid yield which reached 78 % and 73 %, respectively. Likewise, among the different temperature at pH 7.0, autolysis at 50 ˚C exhibited the highest total solid yield which reached 79.26 %.

The variance analysis ($p < 0.05$) of total solid of yeast extract indicates that the influence of initial pH and incubation time of autolysis on solids release was significant. This is in agreement with observation by Champagne *et al.*, 1999 and Tranguler & Erten 2009, where the initial pH and incubation time of autolysis of baker's yeast had affected the solid yield of yeast extracts.

2.3.2 Effects of initial pH and temperature of autolysis on the total nitrogen and α- amino nitrogen content of yeast extracts.

As reported previously (Běehalová and Beran, 1986, Halasz *et al.*, 1991, Orban *et al.,* 1994, Verduyn *et al.,* 1999, Vasallo *et al.,* 2001) and confirmed in this present study, the main component of soluble yeast extract were degraded protein and α-amino nitrogen released from yeast biomass during autolysis.

This reaction is mainly governed by the action of proteolytic enzymes system within the yeast biomass (Běhalová and Beran, 1979, Běehalová and Beran, 1986). This proteolytic system in yeast *Saccharomyces cervisiae* is well studied and was reported to have at least thirty proteolytic enzymes of different classes (Saheki and Holzer, 1975, Achstetter and Wolf, 1985).

Although the proteolytic system of *Kluyveromyces marxianus* is not as well studied as that of *Saccharomyces cervisiae*, there have been reports on the characterization of serine carboxypeptidase and lysine aminopeptidase from this yeast (Ramírez-Zavala *et al.,* 2004a, Ramírez-Zavala *et al.,* 2004b) that exhibit activity over a wide range of pH values and narrow range of temperature values.

The effect of initial pH and temperature on the total nitrogen content of yeast extracts on a dry solid basis during autolysis of spent yeast is shown in Figure 2.4. For all treatments the total nitrogen content of yeast extract increased rapidly within 24 h, thereafter the rate of increase slowed until the end of the incubation period.

Among all treatments, for control (pH 4.5 and 40 °C) the highest value of total nitrogen of yeast extract was obtained as 7.99 % w/w. For different pH treatments smaller release of total nitrogen was observed in the range of 7.32 - 7.35 % for pH values between 6.5 and 7.0. This is an indication that proteinase activity in a spent yeast was higher at lower pH values than at near neutral pH (Champagne *et al.,* 1999).

Similarly, among the different temperature conditions of autolysis and after 72 h of incubation, the highest total nitrogen in yeast extract was obtained as 7.24 %

w/w at 50 °C which was lower than control. Moreover, total nitrogen of yeast extract obtained at higher temperatures (55 and 60 °C) was significantly lower than control. These results suggested that there was a marked release of protein in yeast extracts produced at temperatures 40 ˚C and 50 °C as compared to elevated temperatures. This may be due to reduction in proteinase activity at elevated temperatures as was observed by Tanguler and Erten, 2008.

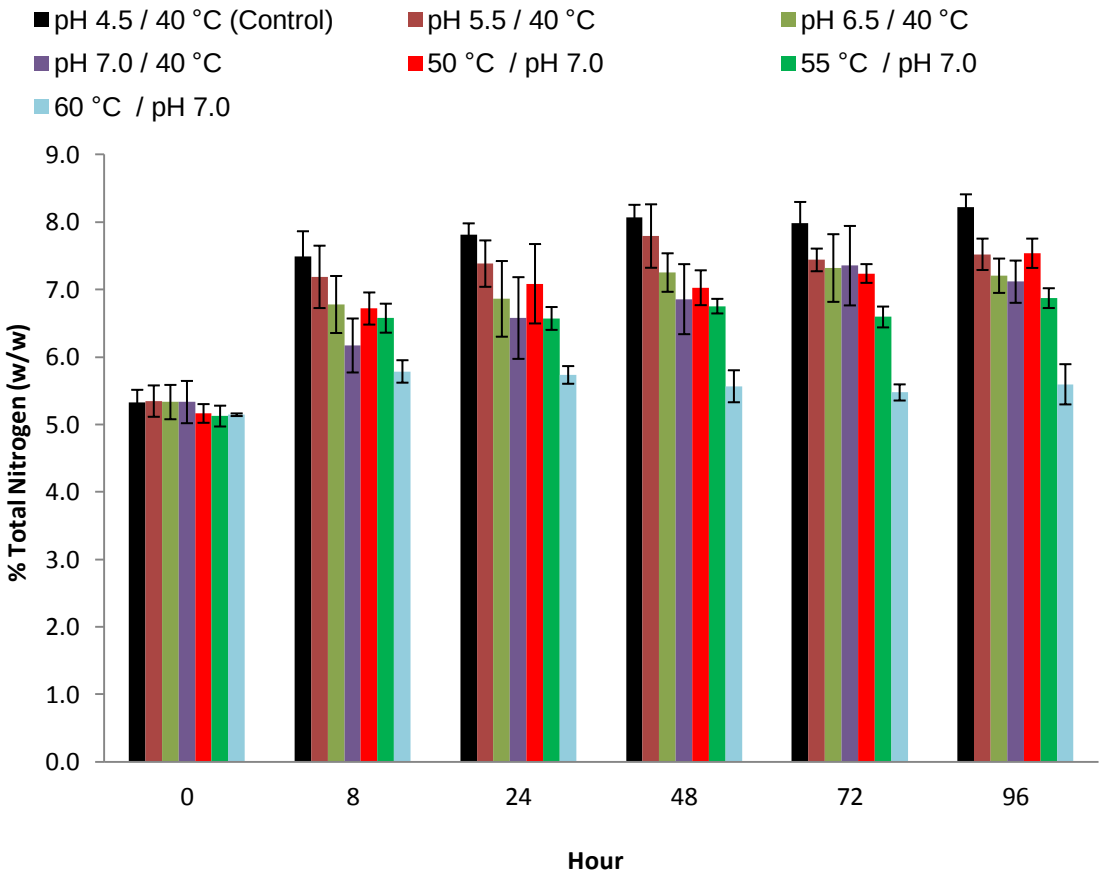


Figure 2.4 Time course of total nitrogen release from spent *Kluyveromyces maxirunus* yeast during autolysis at various pH and temperature conditions. The results are reported as means ± standard deviation (n = 9).

Apart from the requirement of a high nitrogen content of yeast extract intended for food grade application, the amount of α-amino nitrogen and peptide nitrogen content are important quality criteria for yeast extract selection and use (Nagodawithana, 1992, Sommer, 1998). However, relatively less data is available on the amount of α-amino nitrogen released during autolysis of *Kluyveromyces marxianus* (Amrane and Prigent, 1996, Amrane and Prigent, 1998). The level of α-amino nitrogen on a dry weight basis varied with initial pH and temperature of autolysis (Figure 2.5).

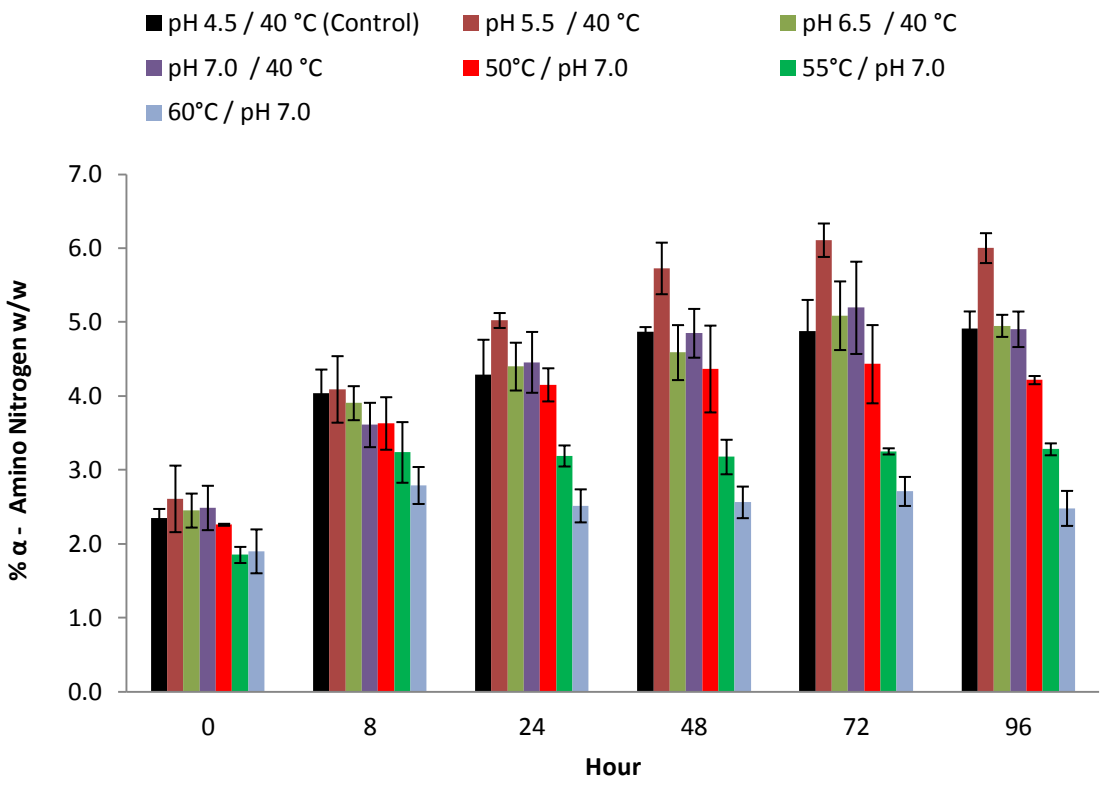


Figure 2.5 Time course of α-amino nitrogen release from spent *Kluyveromyces maxirunus* yeast during autolysis at various pH and temperature conditions. The results are reported as means ± standard deviation (n = 9).

The release of α-amino nitrogen into the extracellular medium was found to be highest at the pH 5.5 and 40 °C with 6.12 % α-amino nitrogen released on a dry solid basis after 72 h of autolysis. Lower amounts of α-amino nitrogen were determined at the other pH conditions including control.

Similarly, among the different temperature conditions of autolysis, the highest content of α-amino nitrogen were obtained at 40 °C and 50 °C and lowest α-amino nitrogen were observed at 55°C and 60 °C. Variance analysis ($p < 0.05$) showed that the autolysis at the initial pH 5.5 and 40 °C significantly increased the α- amino nitrogen content of the yeast extract in comparison with other treatment condition. This suggests that the serine carboxypeptidase and lysine amino-peptidase may be responsible for this effect since it was reported to have good activity at slightly acidic to neutral pH values and in temperature ranges of 40 - 50 °C (Ramírez-Zavala *et al.*, 2004a, Ramírez-Zavala *et al.*, 2004b). Moreover, the lower value α-amino nitrogen of yeast extract as obtained at

temperature 55 °C and 60 °C may be due to inactivation of peptidase enzyme above temperature 50 °C.

2.3.3 Preliminary sensory analysis results of yeast extract

A successful strategy in designing an autolysis process ultimately depends on the sensory attributes of yeast extract produced. Therefore large numbers of samples of yeast extracts (Table 2.2) obtained during various autolysis treatments were subjected to initial screening acceptance test on a 9 point hedonic scale. It was determined that yeast extracts produced at end of 72 h of autolysis were mostly acceptable for all pH treatments at temperature 40 °C. However, for temperature treatment at pH 7.0, the yeast extract obtained at the end of 72 h of autolysis at 50 °C, was found to be acceptable.

Table 2.2 Acceptance score for yeast extracts obtained from different autolysis conditions

Treatments	**Control**	**40 °C**			**pH 7.0**		
Time	**pH 4.5 / 40 °C**	**pH 5.5**	**pH 6.5**	**pH 7.0**	**50 °C**	**55 °C**	**60 °C**
8 h	5.0	5.5	7.2	7.4	7.1	6.2	5.5
24 h	5.0	5.5	7.3	5.5	8.0	6.3	**6.0**
48 h	6.0	6.5	7.4	6.5	8.2	**6.9**	4.0
72 h	**6.0**	**7.5**	**7.6**	**7.5**	**8.5**	5.6	3.4
96 h	5.5	7.0	7.4	7.0	7.5	5.75	3.0

All values were means of duplicate analyses

In contrast, it was observed that when autolysis was conducted at elevated temperatures (> 55 ºC), the acceptability score of yeast extract reduced upon long incubation. Therefore, yeast extract with higher acceptability score produced after 48 h and 24 h of autolysis at 55 and 60 ºC, respectively, were selected for chemical and sensory characterization.

2.3.4 Chemical and sensory characterization of selected yeast extracts produced using various conditions of autolysis.

The yeast extracts selected from acceptance tests were progressed to descriptive sensory analysis by external panel to assess flavour differences arising from different autolysis treatment. They were also characterized for chemical composition mainly free amino acid content, degree of hydrolysis and peptide bound amino acid (Table 2.3, Figure 2.6).

Table 2.3 Compositions of yeast extract produced at various autolysis conditions.

Treatments / **Constituents**	***Control (pH 4.5)***	**40 °C**			**pH 7.0**		
		pH 5.5	***pH 6.5***	***pH 7.0***	***50 °C***	***55 °C***	***60 °C***
Total Nitrogen, % (w/w)[1]	7.99	7.44	7.32	7.35	7.24	6.75	5.79
α- amino Nitrogen, % (w/w)[1]	4.89	6.12	5.10	4.90	4.43	3.18	2.52
% DH[a1]	61.25	82.20	69.69	66.57	61.24	47.11	43.46
Free Amino acid, % (w/w)[2]	19.95	26.58	24.15	23.95	24.40	22.30	19.46
Total Amino Acid, % (w/w)[2]	41.68	43.83	39.20	40.38	37.78	35.23	30.22
Peptide bound Amino acid[a], % (w/w)[2]	21.73	17.25	15.05	16.43	13.38	12.93	10.76

[a]Degree of hydrolysis. [1]All values were means of triplicates analyses. [2]All values were means of duplicate analyses.

The main findings for chemical characterization of yeast extracts are:

- It was observed that the chemical compositions of the yeast extracts produced at various autolytic conditions were close to the composition reported in the literature for yeast extract (Moresi *et al.*, 1995, Munch *et al.*, 1997, Chae *et al.*, 2001).

- It was observed that the amount of free amino acids as a percentage of total protein on a dry matter basis was almost equal for yeast extracts obtained at various autolytic conditions. Nevertheless, it was also noticed for selected yeast extracts produced at 40 °C, within acidic pH range including control (pH 4.5) had higher content of peptide bound amino acid (15 - 21 %) with considerably higher % DH value (between 61 - 82). This indicates the presence of dipeptides (Adler-Nissen, 1986, Aaslyng *et al.*, 1998). This finding may be attributed to the higher activity of endopeptidases at lower temperatures in near acidic conditions.

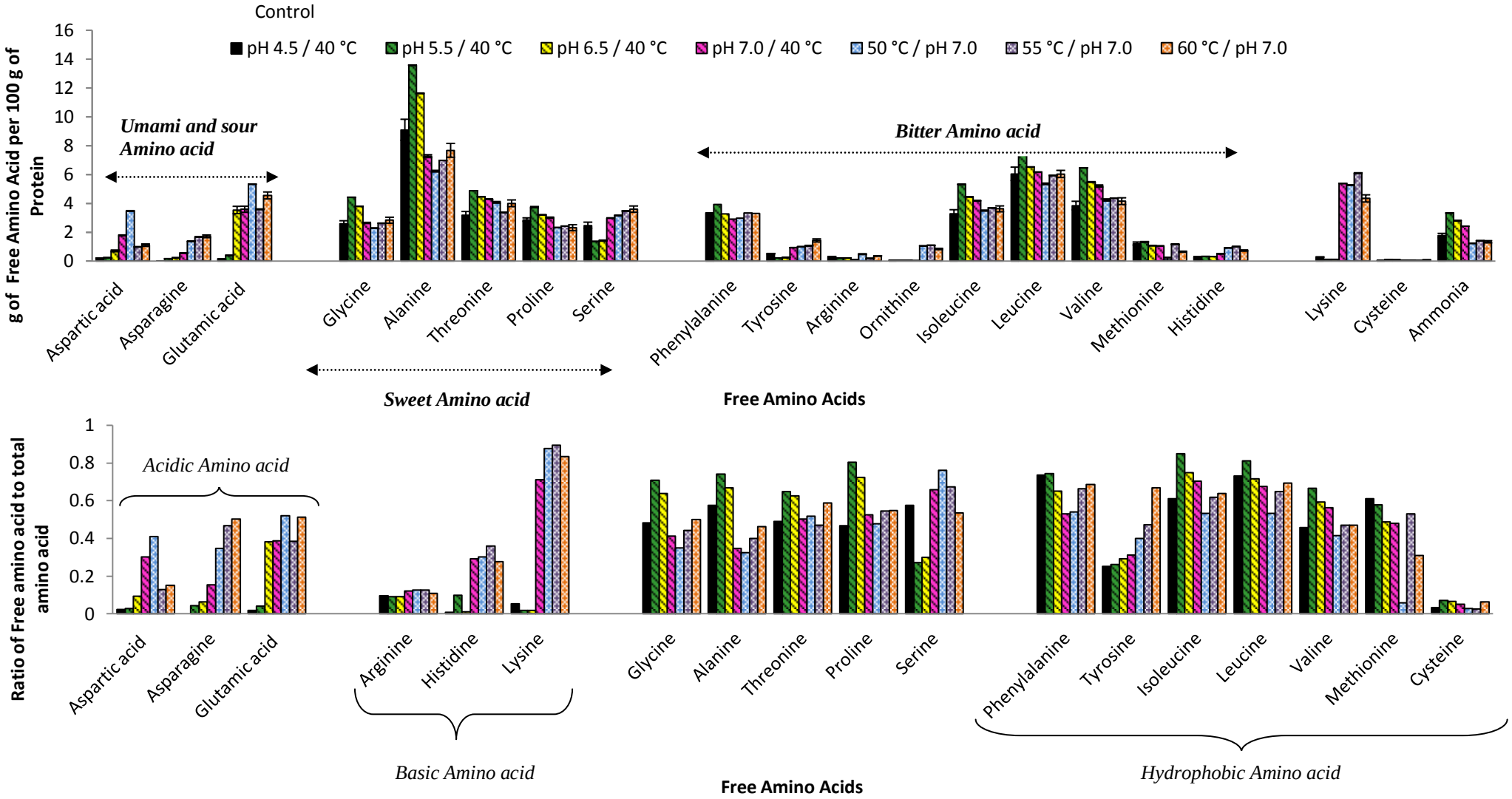


Figure 2.6 Influence of initial pH and temperature conditions of autolysis on free amino acid distribution and ratio free amino acid to total amino acid of yeast extracts. For pH treatment at 40 °C, yeast extracts obtained at end of 72 h of autolysis were analysed. For temperature treatment at pH 7.0, yeast extract at end of 72 h, 48 h and 24 h of autolysis were analysed obtained at 50, 55 and 60 °C respectively. All values were means of duplicate analyses.

- The free amino acid distribution of selected yeast extracts (Figure 2.6) were found to be at par with reported values of yeast extract from brewer's yeast (Chae *et al.,* 2001) and that may influence the flavour attributes of yeast extract.

    - For instance it is well documented that free amino acids have distinctive taste profiles (Nishimura and Kato, 1988) that in turn influence taste of the smaller peptides depending on their amino acid composition. Therefore like free amino acids depending on their amino acid composition the taste characteristics of peptides can be classified into three groups, sour peptides taste, bitter peptides and peptide with little or no taste (Kirimura *et al.,* 1969) and are widely present in fermented food system (Lemieux and Simard, 1992).
    - Furthermore the presence of free amino acids can contribute to overall flavour of yeast by serving as substrates for other flavour-generating reactions, such as deamination, decarboxylation and Maillard reactions, and are precursors of aroma compounds, such as aldehydes, alcohols, acids, esters and thiols as reported in cheese ripening (Yvon *et al.,* 1998).

- It was found that pH and temperature of autolysis had influenced the amino acid distribution in yeast extract such that the free amino acid, alanine, glycine and proline and to a lesser extent leucine and isoleucine were higher in yeast extract produced at acidic condition at 40 °C compared to yeast extract produced at elevated temperature at near neural pH value.

- Glutamic and aspartic were present as free amino acids to a much larger extent in yeast extract obtained at 50 ºC / pH 7.0 and in agreement with observation made by Moresi *et al.,* 1995.

- The ratio of free amino acid to total amino acid ($R_{faa:taa}$) indicates how much of each amino acid may still be present as peptides (Figure 2.6). In general, under acid condition of autolysis, 55 to 68 % of the peptide bonds containing amino acid including the alanine, glycine, threonine, proline, valine and methionine were hydrolysed and present as free amino acid.

- Yeast extract produced in pH range between 4.5 and 6.5 at 40 ºC, had contents of 28 - 35 % for peptide bound hydrophobic amino acids with 65 - 72 % of free bitter tasting amino acid ($R_{faa:taa}$: 0.65 – 0.72).
  - This can be attributed to the action of Carboxypeptidase Y which has pH optima at lower pH and have the ability to release hydrophobic amino residues from C-terminal of peptides (Achstetter and Wolf, 1985, Revillion *et al.*, 2003, Ramírez-Zavala *et al.*, 2004b).

- In contrast, yeast extract in same pH range, $R_{faa:taa}$ value for peptide composed of acidic and basic amino acids was only around 0.07. This indicates that during the autolysis at lower pH, it is difficult to hydrolyse peptide with small basic and acidic amino acids.

- For peptides of yeast extract obtained at 50 - 55 °C at pH 7.0, the $R_{faa:taa}$ for hydrophobic amino acids were in range of 0.42 - 0.68 which is slightly higher than the acidic and basic amino acid. This indicates that peptide bound amino acid may be mainly composed of acid and basic amino acids.

- During autolysis at neutral pH, at higher temperature the peptide bonds including the serine and lysine were mostly hydrolysed as $R_{faa:taa}$ value range between 0.45 to 0.65.
  - This can be assumed that serine carboxypeptidase and lysine amino-peptidase present in *Kluyveromyces marxianus* may be responsible for this activity since both enzymes were reported to be stable under neutral or slightly alkaline pH conditions (Ramírez-Zavala *et al.*, 2004a, Ramírez-Zavala *et al.*, 2004b).
  - Additionally, the $R_{faa:taa}$ value of peptide composed of lysine was around 0.82, which indicates that lysine amino-peptidase were mostly active and released the alkaline residue from amino end with greater efficiency for lysine (Ramírez-Zavala *et al.*, 2004a).

The descriptive sensory analysis of selected yeast extracts screened from acceptance tests are shown in Figure 2.7.

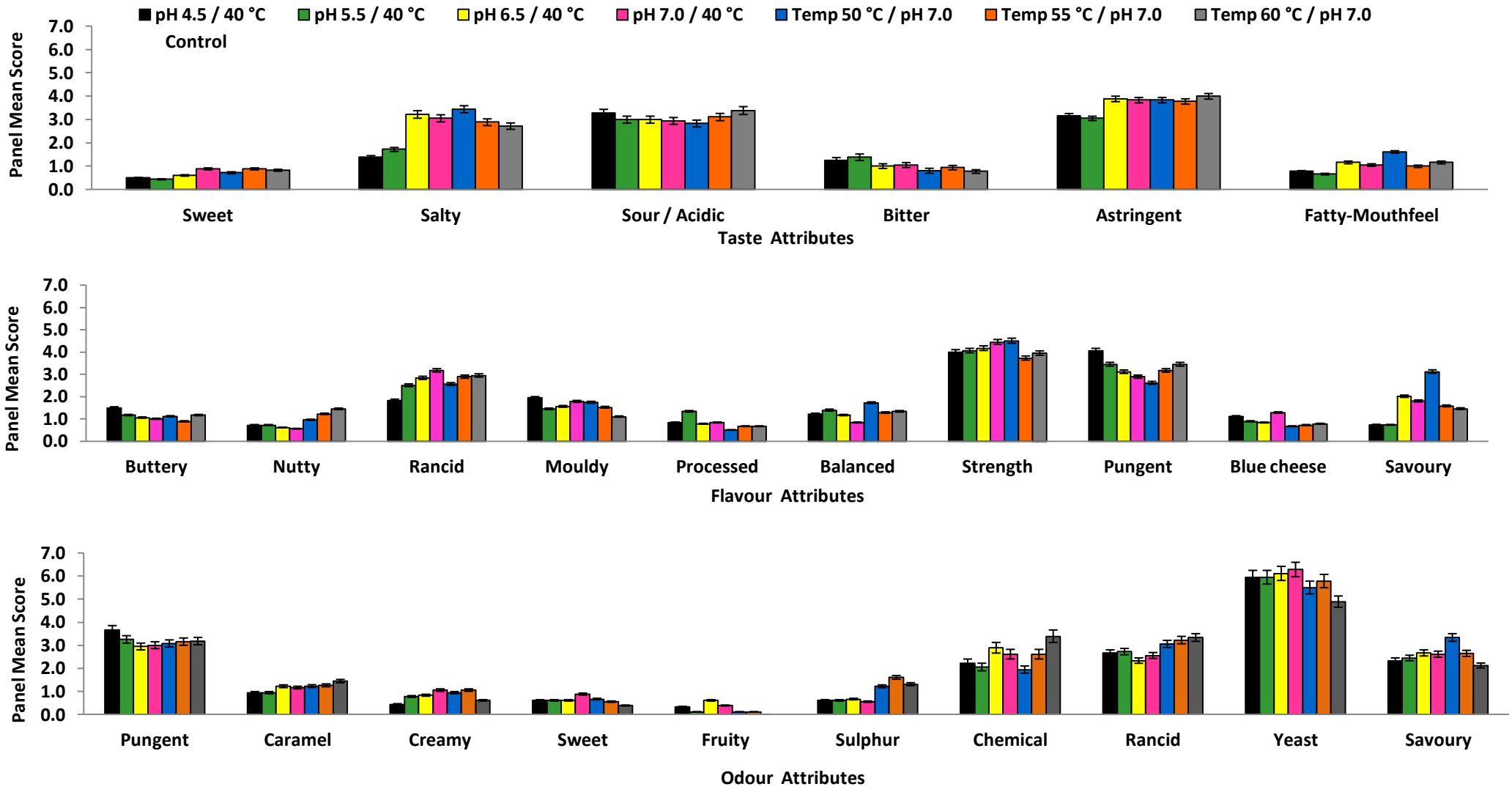


Figure 2.7 Influence of initial pH and temperature condition of autolysis on taste, flavour and odour attributes of yeast extracts. For pH treatment at 40 °C, yeast extract obtained at end of 72 h of autolysis were analysed. For temperature treatment at pH 7.0, yeast extract at end of 72 h, 48 h and 24 h of autolysis were analysed obtained at 50, 55 and 60 °C respectively. All values were means ± SD duplicate analyses

The main findings for chemical characterization of yeast extracts are:

- Major taste attributes of yeast extract obtained after different autolysis treatment were “astringent”, “sour / acidic” and “salty”.
- Similarly, for flavour attributes of yeast extract, the dominating attributes were “strength” followed by “pungent, “rancid”, “mouldy” and “savoury”.
- With regard to odour attributes the main attributes was “yeast” followed by “rancid”, “pungent”, “chemical” and “savoury”.
- Variance of analysis found that 3 of the 25 sensory attributes significantly ($p < 0.05$) discriminated between the yeast extract. This includes “salty” taste, “savoury” flavour and “savoury” odour.
- It was observed that due to increase in intensity of astringency, pungency, sourness / acidity and rancidity of yeast extract. The overall strength of yeast extract had increased.
    - This increase in astringency of yeast extract can be attributed to generation of astringent peptides or amino acid derivatives (Harwalkar and Elliott, 1971, Rothenbuehler *et al.*, 1982, Lemieux *et al.*, 1989) during autolysis process.
    - This increase in sourness and pungency of yeast extract were may be due to formation of organic acid. Hernawan and Fleet (1995) had reported the recovery of organic acids like propionic, succinic and to a lesser extent, acetic and formic acid during autolysis of *Saccharomyces cervisiae*. Though, the biochemical mechanisms by which the acids are produced during autolysis have not been reported, it is most likely related to the metabolism of sugars and lipids.
    - On other hand, the increase in rancidity as well as pungency of yeast extracts can be due to generation of free fatty acids as a result of lipolysis of yeast lipids by action of extracellular lipase present in *Kluyeromyces maxirunus* (Molimard and Spinnler, 1996, Welthagen and Viljoen, 1998, Deive *et al.*, 2003). Since the recovery of a range of free fatty acids in beer and wine have been credited to lipid degradation during the autolysis of brewer's and wine yeasts (Ernest C.-H. Chen, 1980, Troton *et al.*, 1989).

- In the present study a higher bitterness was found in control and yeast extract produced at pH 5.5 (Figure 2.7).
  - This bitterness in yeast extracts can be attributed to presence of higher content of hydrophobic amino acid and smaller peptides leucine at the C-terminals (Ishibashi *et al.,* 1987, Nishimura and Kato, 1988). Also, the higher intensity of bitterness during the autolysis condition at pH 5.5 may be due to the presence of dipeptides with hydrophobic amino acids which are bitterer than free amino acid (Matoba and Hata, 1972).

- The increased mouthfeel of yeast extract obtained by autolysis conducted at 50 °C, can be attributed to release of cell wall polysaccharides especially mannoprotein which is also responsible for increase in mouthfeel of wine during ageing (Alexandre and Guilloux-Benatier, 2006).

- Yeast extract obtained at elevated temperature of autolysis (55 and 60 °C), has a characteristic acidic, salty taste with pronounce pungency, sulfury odour along with distinct note of nuttiness.
  - This complex difference between sensory profiles of yeast extract produced at elevated temperature of autolysis were most likely due to the generation of more volatiles and non-volatile compound than to the amino acids.
  - The type of amino acid present in yeast extract can influence the type of different volatiles developed upon interaction with sugars. For instance, thiamine and cysteine on reaction with sugar are reported to be precursors for 3-mercapto-2-pentanone responsible for sulfury odour in heat treated yeast extract (Munch *et al.,* 1997, Cerny and Guntz-Dubini, 2008).
  - It has also been reported that glycine can increase the formation of a compound like furfural (Whitfield *et al.,* 1988), whereas both glycine and lysine can increase pyrazine formation (Meynier and Mottram, 1995). Munch *et al.,* 1997 also reported the generation of 2-furanmethanethiol (roasty or nutty odour), 2-methoxyphenol (burnt odour), and 4-hydroxy-2, 5-dimethyl-3(2H)-furanone (caramel-like odour) in a heat treated yeast extract.

- Yeast extract produced at near neutral pH at 50 °C had significantly higher savoury profile (salty taste, savoury flavour and odour).

- This may be due to the high content of glutamic acid and aspartic acid along with higher content of basic amino acids which are very important for imparting the umami taste (Figure 2.7).
- Moreover it can be assumed that peptide bound amino acids mainly composed of acid and basic amino acid which acts as low molecular weight savoury peptides, may enhance the umami attributes of yeast extract (Nagodawithana, 1994, Aaslyng *et al.,* 1998).
- Several savoury peptides have been discussed in the literature such as tetra-peptide from enzymatically hydrolysed beer yeast extract, consisting of four aspartic acid residue exhibiting glutamate and bouillon like taste quality (Matsushita and Ozaki, 1994).
- It was also been reported that lactones such as 3-hydroxy-4,5-dimethyl-2(5H)-furanone known as sotolon (curry odour), slowly increased in sparkling wine during ageing, which mainly derived from threonine that is transformed into α-ketobutyric acid which then reacts with acetaldehyde.
- Also the presence of 3-hydroxy-4,5-dimethyl-2(5H)-furanone has been reported as a key odorant in commercial yeast extract (Munch *et al.,* 1997) and which contributes to a savoury like attribute.

➢ Overall the yeast extract obtained at pH 7.0 and 50 °C scored as the best yeast extract with optimum attributes and a balanced score with good strength, mouth feel, and savoury profile when compared with other yeast extract including control yeast extract (pH 4.5).

2.4 Final conclusions

This study showed that pH and temperature of autolysis affects the product yield, proteolysis reaction, and sensory attributes of yeast extracts produced from spent *Kluyveromyces marxianus* yeast.

It was found that the optimum condition for autolysis spent *Kluyveromyces marxianus* yeast for production of yeast extract for food application is 50 ˚C at pH 7.0. At this condition yeast extract produce will have significantly higher savoury attributes (salty taste, savoury flavour and odour) with higher product yield (79.26 %).

In contrast, yeast extract produced at pH 5.5 / 40 ˚C with less sensory acceptability with significantly ($p < 0.05$) higher content of free amino nitrogen (6.12 % w/w) and degree of hydrolysis (82.2 %) will be ideal for application of yeast extract for microbial media preparation (Conway *et al.*, 2001).

It is well understood from the present study that the autolysis processes at pH 5.5 (40 °C) and 50 °C (pH 7.0) were two distinct processes as different biochemical reactions were occurring during autolysis process. This was reflected in the variation in the chemical composition and sensory attributes yeast extract.

The results of this study also provided insight on how to modify the composition of yeast extract to get desirable taste attributes. For example, shifting the pH from 5.5 towards 7.0 during the course of the incubation could enable the production of yeast extract with limited free amino nitrogen and more total solids with higher sensory acceptability. On the other hand, a temperature shift towards 50 °C during incubation may result in yeast extract with higher acidic and basic free amino acid with significantly higher savoury attributes yeast extract as desired in food ingredients for savoury application.

To produce yeast extract with tailored desirable attributes, lab based process outlined in the current study were quite lengthy (72 h). This process would ideally need to be require to be shortened to make the process commercial feasible without the development of microbial contamination during autolysis process (Nagodawithana, 1992).

In this perspective, further studies are desirable on potential interactions between pH and temperature with other autolysis promoters like exogenous enzyme and sodium chloride to yield yeast extract with desirable sensory attributes and shorter autolysis time.

Chapter 3.0

Phosphorylated yeast protein concentrate and bioactive β-glucan enriched cell wall preparations from spent *Kluyveromyces marxianus* yeast

3.1 Introduction

This chapter deals with composition analysis of spent *Kluyveromyces marxianus* yeast and their chemo-enzymatic fractionation to produce value added phosphorylated yeast protein and β-glucan enriched cell wall preparations. These valued added preparations were subjected to immunomodulatory analysis. There are only a few scientific studies published dealing with the immunomodulatory activities of *Kluyveromyces marxianus* extracts (Yoshida *et al.*, 2004, Yoshida, 2005, Yoshida *et al.*, 2007, Maccaferri *et al.*, 2011).

Therefore, the aim of the work was to elucidate the effects of different cell wall preparations from *Kluyveromyces marxianus* yeast on

i) An *in vitro* classically activated murine macrophage cell line J774A.1 and
ii) On human whole blood using a clinically relevant standardized human whole blood assay, (HWBA), which was shown to be an excellent *ex vivo* system for cytokine production (De Groote *et al.*, 1992, Wang *et al.*, 2000, Thurm and Halsey, 2001, Damsgaard *et al.*, 2009).

3.2 Overview of work performed

On the basis of conclusions drawn from literature reviews (Figure1.3, Table 1.2 & 1.6), a simple experimental procedure was designed for complete utilisation of yeast biomass by the application of a non-degrading process. In this, yeast biomass was subjected to autolysis and mechanical disintegration in order to extract protein enriched pools. Yeast protein pools obtained after mechanical disintegration were subjected to phosphorylation to reduce the nucleic acid content. The cell wall residues obtained at the end of autolysis and mechanical disintegration were subsequently subjected to hot water and protease treatment. The aim of these steps was to reduce the protein content of β-glucan enriched products obtained at the end of treatments. This β-glucan enriched product was also subjected to multi-step acid and alkali treatment to remove traces of lipids and protein to yield relatively purer, more potent biologically active alkali and acid insoluble β-glucan particulates. This insoluble glucan particulate was transformed into different soluble β-glucan preparations by enzymatic treatment. This was done with the aim to enhance the solubility of β-glucan preparations in cell culture media to ascertain and enhance their biological activity as advised in

previous reports (Williams *et al.,* 1991, Demleitner *et al.,* 1992, Williams *et al.,* 1992). The treatment procedure is illustrated in Figure 3.1 and is described in detail in the following subsections.

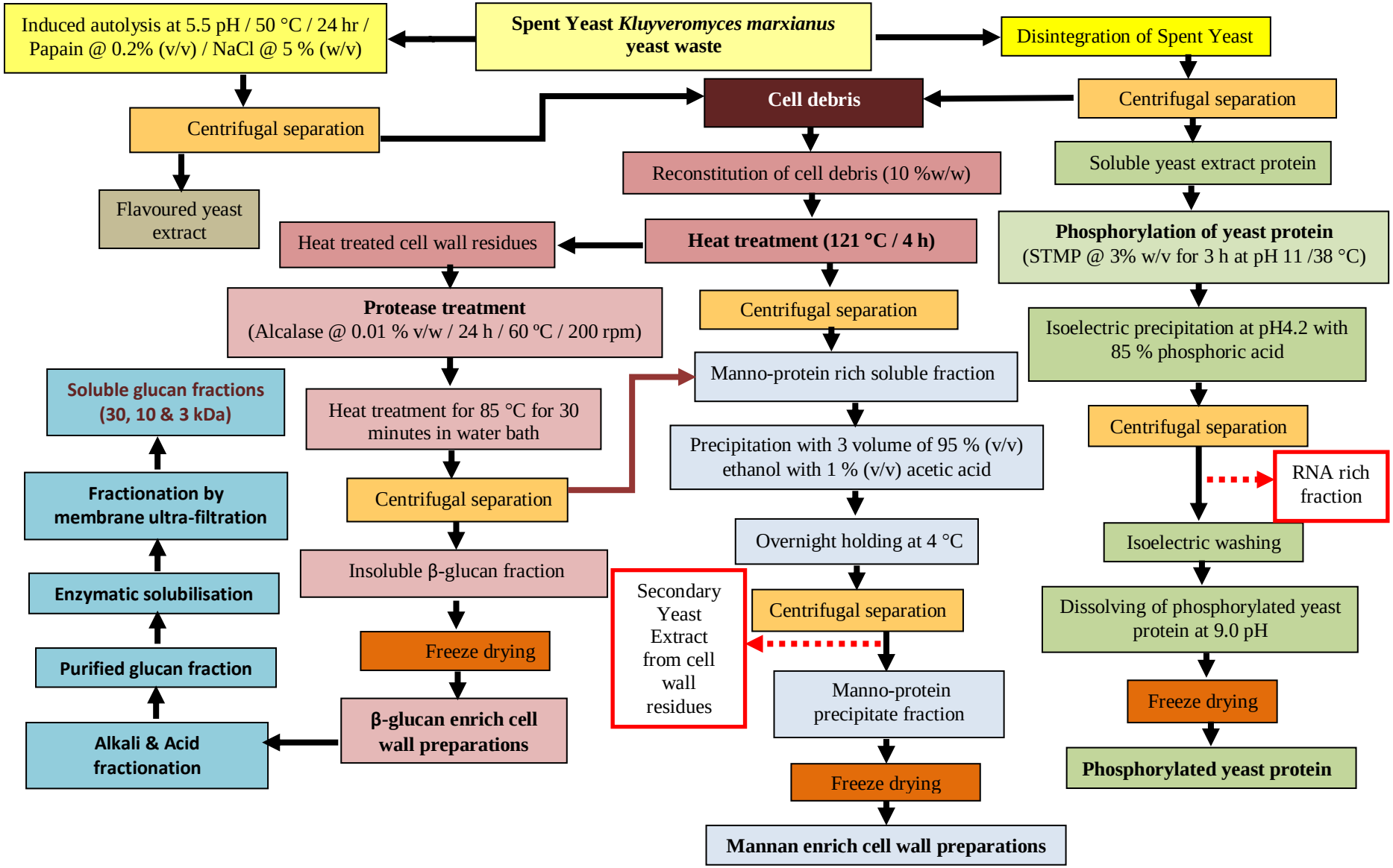


Figure 3.1 Experimental schemes for the preparation of phosphorylated yeast protein and different β-glucans enriched cell wall preparations from spent *Kluyveromyces*

3.3 Materials and methods

3.3.1 Materials

Spent yeast biomass of *Kluyveromyces marxianus* was obtained as described in Section 2.2.1. Sodium trimetaphosphate (STMP), HCL, NaOH, KOH, ethanol, acetic acid, and perchloric acid were obtained from Sigma (Dublin, Ireland) and the enzyme Alcalase was kindly provided by Novozymes, Denmark. The DEPOL 667P (β-glucanase enzyme, 12,000 U/g from *Trichoderma* sp.) was provided by Biocatalyst, UK.

Murine macrophage cell line J774A.1 was obtained from ATCC, Middlesex, UK. Dulbecco's Modified Eagle's Medium (DMEM), RPMI-1641 Medium, Foetal Bovine Serum (FBS), 0.25 % Trypsin-EDTA solution, Resazurin sodium salt, Lipopolysaccharide (LPS), Griess reagent, $NaNO_2$ were purchased from Sigma Aldrich, Ireland. L-Glutamine (2 mM) and phosphate buffer saline (PBS) were purchased from Gibco, Bio Sciences Ltd, Ireland.

Recombinant mouse interferon-gamma (IFN-γ) was obtained from BD Biosciences, Oxford, UK. BD Vacutainer® CPT™ was obtained from Becton Dickinson, Plymouth, UK. Quantikine mouse interleukin 12 (IL-12), mouse interleukin 10 (IL-10) for macrophage J744A.1 cell line and for human whole blood assay human tumor necrosis factor alpha (TNF-α), human interleukin 6 (IL-6), human interleukin 8 (IL-8), human monocyte chemotactic protein 1 (MCP-1), human transforming growth factor β1 (TGF-β) and human interleukin 10 (IL-10) were purchased from R&D Systems (Abingdon, UK). Ultrafilters, Vivaspin 20 were purchased from Vivascience, Sartorius, UK. All other reagents used were of analytical grade.

3.3.2 Preparation of phosphorylated yeast protein

Alkaline spent yeast suspension (2 L, pH 9.0, 10 % w/w) was mechanically disintegrated for a period of 30 minutes using a Silverson L5 laboratory dispenser at 6000 rpm. The homogenised mixture obtained was centrifuged at 3600 g for 15 minutes. The supernatant obtained was then divided into two equal parts. One part was treated with 3% w/v STMP for 3 h at pH 11.0 with gentle mixing using an orbital shaker (50 rpm) at 35 ºC to phosphorylate the

protein, whereas another part without any treatment was used as control. The pH of both parts was subsequently adjusted to 4.2 with 2 M HCl leading to precipitation of proteins. Subsequently the phosphorylated protein precipitate and control yeast protein was separated by centrifugation at 3600 g at 4 ºC for 30 minutes. The sedimented precipitated protein was washed three times with acidified distilled deionised water (pH 4.2) and later suspended in alkaline deionised water (pH 9.0) for subsequent lyophilisation. Yield and composition are shown in Table 3.2.

3.3.3. Preparation of yeast cell wall residues from Yeast biomass

3.3.3.1a Mechanical disintegration

An alkaline spent yeast suspension (2 L, pH 9.0, 10 % w/w) was mechanically disintegrated using a Silverson L5 Series Laboratory dispenser for 30 minutes at 6000 rpm. The insoluble materials were sedimented using a Beckman centrifuge (JS-H2) at 3600 g for 15 minutes at < 10 ºC. The sedimented insoluble materials rich in cell wall were then washed three times with deionised water until the supernatant become clear and subsequently lyophilized. Yield and composition are shown in Table 3.3a.

3.3.3.1b Induced autolysis

The spent yeast suspension (2 L, pH 6.5, 10 % w/w) was added with sodium chloride (5% w/v) and the proteolytic enzyme papain (0.2 % w/w of protein). The spent yeast suspension was autolysed at 50 °C for 24 h with agitation at 200 rpm in an orbital shaker. The autolysate was then heated at 80 °C for 15 min, cooled to room temperature and centrifuged at 3600 g for 10 min to separate the cell wall residues and yeast extract. The cell wall residue paste was then lyophilized for which yield and composition are shown in Table 3.3b.

3.3.3.2 Hot water treatment

The cell wall residue obtained in the above steps (Table 3.3a, 3.3b) was suspended in deionised water (500 mL, pH 9.0, 10 % w/w) and 25 g of glass beads with diameters of 0.3 - 0.4 cm were added to a 2000 mL conical flask for mixing during boiling. The suspension was heated at 121 ºC for 4 h and then cooled to room temperature. The insoluble materials were sedimented using a

Beckman centrifuge (JS-H2) at 3600 g for 15 minutes at < 10 ºC to remove supernatant and other soluble material. The sedimented insoluble material rich in cell wall component was washed thoroughly with deionised water until the supernatant become clear. The cell wall residue paste was then lyophilized for which yield and composition are shown in Table 3.3a and 3.3b.

3.3.3.3. Protease treatment

The dried cell wall residues from the above steps (Table 3.3a, 3.3b) were suspended in deionised water, yielding a suspension containing 15 % (w/w) solids content. After heating the suspension to 60 °C and adjustment to pH 8.0 with 2 M NaOH, the suspension was treated with commercial alkaline proteinase Alcalase (0.01 % v/w) for 24 h in an orbital shaker maintained at 60 ºC and 200 rpm. After enzymatic treatment, the suspension was heated at 80 ºC for 15 min to deactivate the enzyme and cooled to room temperature. The insoluble materials were sedimented using the Beckman centrifuge (JS-H2) at 3600 g for 15 minutes at < 10 ºC to remove supernatant and other soluble material.

The sedimented insoluble materials rich in cell wall component washed at least three times with deionised water until the supernatant become clear. The insoluble crude β-glucan particulate (**CβG**) paste was then lyophilized for which yield and composition are shown in Table 3.3a and 3.3b. For biological assays 10 g of CβG particulate was sterilized by dry heating at 160 °C for 1 h in a glass bottle (Mark Gerald Deacon-Shaw and Koenig, 2011) as wet sterilization of glucans was found to be unacceptable due to the swelling and agglomeration of the glucan particles (a hygroscopic nature phenomenon).

3.3.3.4. Alkali and acid insoluble glucan preparations

The insoluble crude β-glucan particulate (**CβG**) obtained from the above step (Table 3.3a) was further extracted by the procedure adopted from Jamas *et al.*, 1997 with some modification. The first step in the extraction process was an **alkaline extraction** of 10 % w/v crude β-glucan suspension in 1 M NaOH which was heated to 95 °C and maintained at this temperature for 1 h. After completion of the alkaline extraction, the β-glucan remained as an enlarged

particulate mass and was separated by centrifugation at 3500 g for 10 min at room temperature. The collected cell wall fraction was repeatedly (x2) subjected to this extraction process. This treatment solubilized the cellular proteins, nucleic acids, mannans, soluble glucans and polar lipids into the supernatant fraction. The process also deacetylated chitin from the cell wall to chitosan. The alkali insoluble glucan was then further extracted twice at 75 °C for 1 h in water adjusted to pH 4.5 with HCl (**acidic extraction)**. The insoluble extract obtained was then treated with 0.1 M acetic acid for 1 h at 75 °C for removal of glycogen, chitin and residual proteins. After these treatments insoluble glucan was removed and washed three times with deionized water to remove any residual acid as well as any yeast degradation products. The final step in extraction process entailed two sets of sequential **organic extractions**. The first Organic extraction was carried out in 70 % isopropanol and second organic extraction was carried out in 70 % acetone. These two steps removed nonpolar lipids and hydrophobic protein traces from final product. The resulting wet solids were dried under vacuum at 70 ± 2 °C to yield free flowing alkali and acid insoluble glucan (**PβG**) particulates. The PβG particulates were sterilized by dry heating at 160 °C for 1 h in a glass bottle (Mark Gerald Deacon-Shaw and Koenig, 2011).

3.3.3.5. Enzymatic hydrolysis

The **PβG** powder was re-suspended (10 % w/v) in phosphate buffer solution (PBS, pH 7.4) boiled for 1 h and the final volume adjusted to 100 mL with PBS at 20 °C. Then suspension was enzymatically digested with DEPOL 667P (0.5 % w/v) for 24 h at 55 °C in orbital shaker at 200 rpm and subsequently heated at 85 °C for 30 minutes to stop the enzymatic reaction. The unhydrolysed PβG was separated by centrifugation (3500 g for 10 min). The soluble enzymatic hydrolysed PβG was further fractionated by membrane ultrafilter with a range of nominal molecular weight cutoff (**NMWCO**) membranes of 30, 10, and 3 kDa, respectively. The products obtained were coded as S30R, S10R and S3R which correspond to fractions with a molecular weight (MW) distribution of > 30 kDa, 10 - 30 kDa and 3 - 10 kDa, respectively. The soluble β-glucan preparations were in sterile glass bottles for subsequent sterilization by autoclave at 121 °C for 15 min at 15 psi.

3.3.3.6. Precipitation of mannoproteins enriched product

The supernatant and soluble fraction produced as a result of the centrifugation of cell wall residue after hot water treatment and protease treatment, respectively were treated with three volumes of 95 % ethanol containing 1 % (v/v) acetic acid. The solution was stored overnight at < 5 ºC to precipitate mannoproteins. This was then centrifuged at 3600 g for 15 minutes at < 10 ºC to remove the supernatant fraction and other soluble material. The sedimented precipitate was washed twice with aqueous ethanolic solution (70 % v/v) and subsequently lyophilised. Yield and composition are shown in Table 3.3a and 3.3b.

The supernatant after the separation of the precipitated mannoprotein was concentrated and dried at 70 °C in rotatory evaporator to yield a brownish coloured secondary yeast extract. Yield and composition of the dried product is shown in Table 3.3a and 3.3b.

3.3.4 Analytical methods

➲ **Total nitrogen**

Total nitrogen of samples were measured using the micro-Kjeldahl method **(**I.D.F., 1993, **Appendix I: Method 2.0)** and crude protein content was calculated as total nitrogen multiplied by the factor 6.25. All the analyses were done in triplicate.

➲ **Total carbohydrate**

The dried cell wall preparations were solubilised in 2 N H_2SO_4 by wetting the samples for 3 h at room temperature with intermittent mixing (Dallies *et al.*, 1998) and then heating the wetted samples at 95 ºC for 5 minutes. The samples were then subsequently cooled to room temperature and stirred vigorously until a homogenous solution was achieved **(Appendix I: Method 5.0).** Samples were diluted with deionised water if required for subsequent analysis of total carbohydrate by the microplate based phenol sulphuric acid method (Masuko *et al.*, 2005). All the analyses were done in triplicate.

➲ **β-glucan and mannose content**

Yeast β-glucan and mannose content of samples were measured using the Yeast β-glucan, D-mannose, D-fructose and D-glucose kit of Megazyme (Megazyme

International Ireland, Wicklow, Ireland, **Appendix I: Method 6.0 & 7.0).** All the analyses were done in triplicate.

➲ **RNA content**

The RNA contents of samples were determined by the Orcinol method (Herbert *et al.,* 1971) with yeast RNA as standard **(Appendix I: Method 8.0).** All the analyses were done in triplicate.

➲ **Chitin content**

The chitin content of samples was determined on the basis of the reaction of glucosamine with acetylacetone to form a chromogenic complex with p-dimethylaminobenzaldehyde (Popolo *et al.,* 1997, **Appendix I: Method 9.0).** N-acetyl glucosamine was used as standard. All the analyses were done in triplicate.

➲ **Mineral content**

Mineral content was determined by using Inductively Coupled Plasma Atomic Emission Spectrometry (McKinstry *et al.,* 1999). Approximately 0.5 g 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 temperatures and the solution was diluted to the desired volume with water. All the analyses were done in triplicate.

➲ **Free amino acid content**

The individual amino acids were analyzed in duplicates in an amino acid analyzer (Biochrom 30, Pharmacia Biochrom Ltd) using ion-exchange chromatography. The amino acids were separated using standard lithium citrate buffers of pH 2.80, 3.00, 3.15, 3.50, and 3.55. The post-column derivatization was performed with ninhydrin (Stenberg *et al.,* 2002). Total amino acids were determined in duplicate after hydrolysis of samples at 110 °C for 24 h with 6 M HCl prior to the amino acids analysis. All the analyses were done in duplicate.

3.3.5. Mammalian cell line base assays system

3.3.5.1 Cell culturing

Murine macrophage cell line J774 are monocytes derived from the macrophage cell line of BALC/c mice. They were routinely grown in DMEM with 10 % (v/v)

FBS and 2 mM L-Glutamine at 37 °C in an atmosphere of 5 % CO_2 with fully humidified air.

3.3.5.2 Cell viability assay

Cells were trypsinized from sub-confluent cultures by adding 0.25 % Trypsin-EDTA solution to the culture flasks containing confluent cells followed by 2 minute incubation at 37 °C. The trypsin reaction was stopped by adding 10 mL of the appropriate culture medium containing 10 % foetal bovine serum (FBS). The cell suspension was then transferred to a 50 mL tube and centrifuged at 900 g for 3 min. Obtained cell pellet was then suspended in 2 mL of DMEM culture medium supplemented with 10 % (v/v) FBS and 2 mM L-glutamine and mixed thoroughly by repeated pipetting to make a single cell suspension. Cells were then counted with a hemacytometer and prepared at a concentration of 5×10^5 cells/mL using DMEM media. Cells were then seeded into duplicate wells in a 96 well flat bottom plate and incubated for 4 h in a CO_2 (5 %) incubator at 37 °C to allow cell attachment to the plate surface. After 4 h, fresh medium with or without insoluble and soluble β-glucan preparations at various final concentrations were added to the designated wells. After 24 h of culture, Alamar blue reagent dissolved in PBS was added to the test plate at a final concentration of 10 %. After 4 h of incubation at standard condition, test and control wells was read for fluorescence at 540 nm and 630 nm using a varioskan micro-titer plate reader (Thermo Scientific, **Appendix I: Method: 10.0).**

3.3.5.3 Activation of murine macrophages

Murine macrophage cells (J774A.1) were cultured (100 µL) in 96 well plates at a concentration of 50,000 cells per well. To ensure adherence, cells were cultured 6 - 8 h at 37 °C and 5 % CO_2. Then 100 µL of IFN-γ was added to the wells to yield a final concentration 20 IFN- γ units/mL. After 12 h of incubation the supernatant was discarded and 100 µL of LPS was added to yield a final concentration of 10 ηg/mL LPS in DMEM. After 9 h of LPS stimulation, supernatant was removed from the wells and cells received fresh medium with or without 0.2 mg / mL and 0.015 mg / mL of insoluble and soluble β-glucan preparation, respectively, to the appropriate wells of the plate. The plates were again incubated for 12 h. Following incubation the cell supernatants were

collected and preserved at -80 °C for subsequent experiments (**Appendix I: Method: 10.0).**

3.3.5.4 Nitric oxide (NO) measurement in activated macrophage cells

After incubation of the glucan with the J774A.1 cells for 24 h, the synthesis and release of NO by cells was determined by assaying culture supernatant for nitrite content **(Appendix I: Method: 10.0**). Briefly, 50µL supernatant was reacted for 10 min at room temperature with an equal volume of Griess reagent (1 % sulfanilamide, 0.1 % naphthylethylene-diamine dihydrochloride, 2.5 % phosphoric acid). The optical density was measured at 550 nm using varioskan micro-titer plate reader (Thermo Scientific). The nitrite content was quantified by comparison with a standard curve generated with sodium nitrite in the range of 0 - 100 µM (Appendix I: Method: 10.0).

3.3.5.5 Enzyme linked immunosorbent assay (ELISA)

Concentrations of cytokines in the supernatant were measured using ELISA kits obtained from R&D Systems (Abingdon, UK) as per the manufacturer's instructions (Appendix: Method: 10.0). Briefly, 96-well microtiter plates (Nunc) were coated with purified monoclonal capture antibodies for pro-inflammatory IL-12 and anti-inflammatory IL-10 quantifications. The plates were then incubated for 12 h at 4 °C. Recombinant standards and samples were added to the wells for 2 h. The well contents were then incubated for 2 h with detection antibodies and subsequently conjugated to horseradish peroxidase and allowed to bind for 1h at room temperature. The wells were then incubated with TMB substrate solution for 30 min, and the reaction stopped with 2 N H_2SO_4. Absorbance was then measured at 450 nm and 630 nm (reference wavelength) using a varioskan micro-titer plate reader (Thermo Scientific) and levels in each sample were calculated from the standard curve (Appendix I: Method: 10.0).

3.3.6 Human whole blood assay (HWBA) system

Approximately 8 mL peripheral blood was collected by venapuncture in sterile sodium citrate anti-coagulant buffered tube. Within 30 minutes of collection, whole blood was serially diluted with L-glutamine-supplemented RPMI 1640 medium. In triplicates, the blood was incubated in 24-well plates for 24 h under conditions of 37 °C, 5 % CO_2 and 96 % relative humidity with RPMI 1640

medium alone (control) or different cell wall preparations (PβG, S30R, S10R, S3R) adjusted to the final concentration of 50 µg β-glucan/mL. The cultured bloods were collected in sterile, endotoxin-free polypropylene tubes which were centrifuged for 10 minutes at 900 g and cell-free supernatants were collected, transferred to polypropylene vials and stored at -80 °C until cytokine analysis.

3.3.6.1 Cytokine analyses

Levels of TNF-α, IL-6, IL-8, MCP-1, TGF-β, and IL-10 in the supernatant were measured by ELISA kits obtained from R&D Systems (Abingdon, UK) using recombinant human cytokines provided by the manufacturer as calibration standards.

3.3.7 Statistical analysis

The results were analysed by SPSS software for mean and standard deviation (SPSS for Windows, Version 18.0, SPSS Inc., Chicago, IL). For cell proliferation, cytokine and NO release, SPSS one way ANOVA was used. A value of $p < 0.05$ was considered statistically significant.

3.4. Results and discussion

3.4.1 Composition of spent *Kluyveromyces maxirunus* yeast

The chemical composition of spent *Kluyveromyces maxirunus* yeast, biomass was determined and shown in Table 3.1.

Table 3.1. Chemical composition of crude spent *Kluyveromyces maxirunus* yeast.

Constituents	**% w/w, dry basis[1]**
Total Carbohydrate	34.56 ± 1.34
β Glucan	20.97 ± 1.29
α-Glucan	0.85 ± 0.09
Mannan	9.76 ± 0.99
Chitin (as glucosamine)	1.21 ± 0.24
Total Nitrogen	6.93 ± 1.05
Crude Protein	43.16 ± 1.22
RNA	7.52 ± 0.80
Ash	13.12 ± 0.98
Major minerals	**% w/w, dry basis**
Potassium	3.23 ± 0.16
Phosphorus	1.95 ± 0.05
Sodium	1.51 ± 0.04
Calcium	0.53 ± 0.001
Magnesium	0.14 ± 0.001
Iron	0.04 ± 0.001
Minor minerals	**mg / kg**
Iron	48.10 ± 0.28
Zinc	40.10 ± 0.12
Copper	4.03 ± 0.008
Molybdenum	< 0.25
Selenium	< 0.25

[1]All values were means ± SD of triplicates analyses.

On a dry matter basis biomass consisted of 6.93 ± 1.05 % total nitrogen, which was mainly composed of protein, peptides, free amino acids and RNA. The crude protein (Total Nitrogen x 6.25) content of yeast biomass was found to be 43.16 ± 1.22 %, which is at par with values reported for primary and whey

grown *Kluyeromyces maxirunus* yeast (Orban *et al.,* 1994, Otero *et al.,* 1996, Vasallo *et al.,* 2001).

The total carbohydrate (as glucose equivalent) was estimated as 34.56 ± 1.34 %, which was mainly composed of β-glucan (20.97 ± 1.29 %) α-glucan (0.85 ± 0.09 %), mannose (9.76 ± 0.99 %) and chitin (1.21 ± 0.24 %).

The RNA content was 7.02 ± 0.80 % of biomass on a dry matter basis which was in agreement with values reported by Lukondeh *et al.,* 2003.

Ash content of the dried cells of *Kluyveromyces marxianus* spent yeast was found to be 13.12 ± 0.98 % (Table 3.1).This amount is higher than that reported for primary grown yeast (Orban *et al.,* 1994) but was similar to yeast grown on whey (Moresi *et al.,* 1995). One of the reasons for the high ash content is due to the higher mineral content in whey permeates. Concentration of individual elements in yeast biomass varies from species to species (Halasz *et al.,* 1991a) and in the present context the potassium, phosphorus, sodium and calcium concentrations were found to be the major part of *Kluyveromyces marxianus* yeast waste as shown in Table 3.1. This profile is similar to the reported values for other strains of yeast (El-Samgragy, 1988, Halasz *et al.,* 1991a).

3.4.2 Phosphorylated yeast protein concentrates from spent *Kluyveromyces maxirunus* yeast

The compositions and yield of the yeast protein concentrate, with and without treatment with 3 % w/v STMP, prior to isoelectric precipitation of the extracted yeast protein are shown in Table 3.2. The protein concentrate obtained after phosphorylation treatment was characterised by its higher contents of protein and much lower contents of RNA when compared with the control yeast protein concentrate (**YPC**). This indicates that the phosphorylation procedure increased extractability of protein by 10 % with a 55 % reduction in nucleic acid content. This value is in good agreement with the nucleic acid content of the yeast proteins isolated by the phosphorylation of yeast homogenate (Giec *et al.,* 1989).

Table 3.2 Chemical composition (% w/w, dry basis), solid yield of yeast biomass and different yeast protein concentrate preparations.

	Protein	Total Carbohydrate	RNA	Ash	**Yield
Yeast Biomass	39.92 ± 1.22	24.53 ± 1.34	7.52 ± 0.80	13.12 ± 0.98	100
Yeast extract	42.32 ± 1.06	23.70 ± 0.12	12.55 ± 0.91	9.05 ± 0.36	46
Yeast Protein Concentrate (**YPC**)	68.16 ± 2.36	8.72 ± 0.19	16.35 ± 3.19	7.73 ± 2.36	16
Phosphorylated Yeast Protein Concentrate (**PYC**)	78.21 ± 1.16	7.10 ± 2.36	7.92 ± 1.10	17.94 ± 3.01	18
Supernatant after precipitation of **PYC**	20.48 ± 0.86	10.50 ± 0.39	8.60 ± 0.26	53.36 ± 2.96	29

**Compared with dry weight of yeast biomass. All values were means ± SD of triplicates analyses.

The reduction in nucleic acid content of protein concentrate can be attributed to the separation of proteins from nucleic acids due to destabilization of the nucleoprotein complex. This results in a reduction of nucleophilic properties of amino acids functional group of yeast protein due to introduction of orthophosphoric radicals into the amino acid residues of yeast protein (Halasz *et al.*, 1991).

Ash content in phosphorylated yeast protein concentrate (**PYC**) was over two times higher than yeast biomass and **YPC**. This could be attributed to the addition of STMP in the phosphorylation reaction.

The total amino acid distribution of yeast biomass (Figure 3.2) confirmed that among nonessential amino acids glutamic acid (9.75 g/100 g protein) and aspartic acid (8.05 g/100 g protein) were found in the highest amounts followed by alanine (5.79 g/100 g protein), glycine (4.18 g/100 g protein), arginine (3.59 g/100 g protein), while serine (3.22 g/100 g protein), histidine (2.89 g/100 g protein) and proline (2.13 g/100 g protein), were present in smaller amounts.

In the case of essential amino acids the most limiting amino acids in yeast biomass was found to be leucine, lysine threonine and methionine while for yeast protein concentrates they were the sulfur containing amino acids.

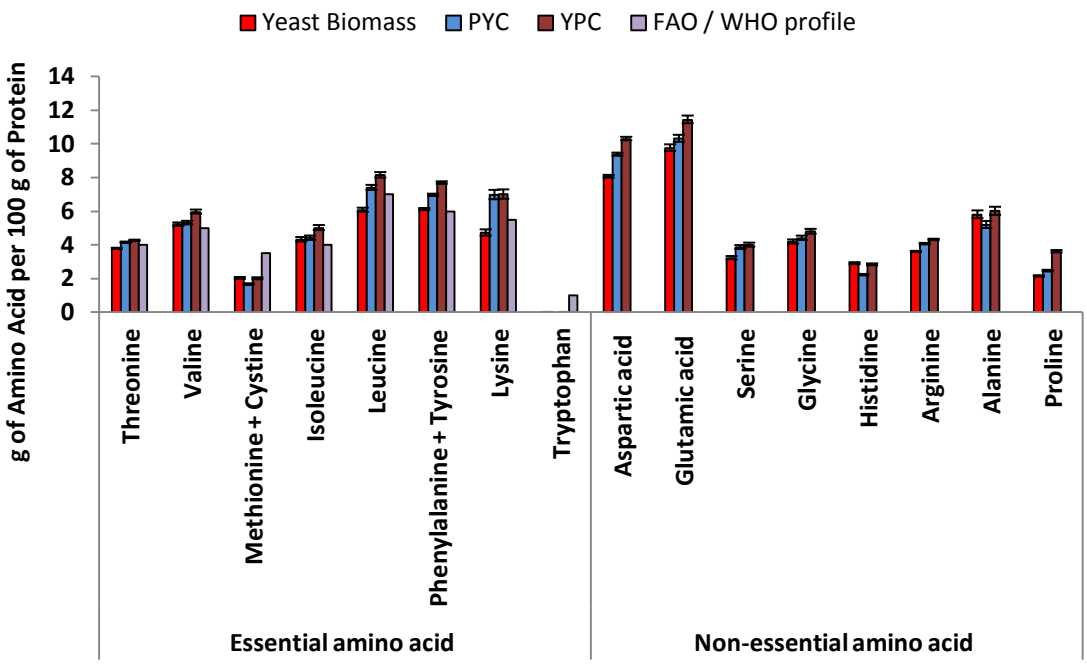


Figure 3.2 Amino acid profile and essential amino acid comparisons of spent yeast and yeast protein concentrate preparations. All values were means ± SD of duplicate analyses.

Moreover, except for sulphur containing amino acids, all essential amino acids were higher in yeast protein concentrate compared with yeast biomass. This difference can be due to the selective precipitation of proteins in the yeast protein concentrates.

It is worth to note that no significant difference was observed between essential amino acid values of PYP and YPC. This suggests that phosphorylation of yeast protein does not alter the essential amino acid concentration. This also offers a method to produce yeast protein concentrate with significantly less nucleic acid contamination with better functionality and application (Damodaran and Kinsella, 1984, Huang and Kinsella, 1986a, Giec *et al.*, 1989, Pacheco and Sgarbieri, 1998, Yamada and Sgarbieri, 2005).

3.4.3 β-glucan enriched cell wall preparations obtained from multi-step fractionation method of yeast cell wall residue.

In the present study an attempt was made to design a non-degrading process which produced different cell wall preparations enriched in β-glucan. These are

summarised in Figure 3.1 and Table 3.3a - 3.3b with their composition and yield on a dry matter basis.

It was observed that induced autolysis is an efficient method for extraction of protein from yeast biomass as 72 % of protein was removed. This was significantly higher than that obtained by the mechanical disintegration method where only 11 % of protein was removed. The autolytic removal of protein from biomass in the present study was higher than the value reported by Liu *et al.*, 2008 that can be attributed to the use of papain in this induced autolysis process.

When cell wall residue obtained by mechanical disintegration of yeast biomass was subjected to hot water treatment as per the Liu *et al.*, 2008 method with some modification (121 °C for 4 h at pH 9.0). This resulted in removal of 10 % of total protein with reduction of approximately 50 % of mannose content. On the other hand almost all insoluble glucan was retained with increase in β-glucan content from 11.28 ± 0.13 to 16.01 ± 0.19 %. Similar treatment to autolysed cell wall obtained at the end of autolysis resulted in the removal of around 58 % of total protein along with 90 % reduction of mannose content.

Nevertheless, 12 - 27 % and 12 - 50 % of initial mannose and protein, respectively, were still retained in hot water treated insoluble material. This is possibly due to strongly linked residual mannan with insoluble β-glucan via the amino acids serine or threonine of yeast protein (Kath and Kulicke, 1999). Therefore, the cell wall preparation obtained after hot water treatment was followed by an extended 24 h of alkaline protease treatment in a process designed to destroy the matrix of the manno-proteins complex and facilitate the removal of mannans and proteins from the insoluble cell wall residues. As shown in Table 3.3a and 3.3b the protease treatment step resulted in 15 - 25 % and 65 - 78 % reduction of protein and mannose content, respectively.

As shown in Table 3.3a and 3.3b, the final composition of glucan enriched cell wall preparation obtained after induced autolysis was of superior quality with 63.35 ± 0.60 % total carbohydrate, 4.44 ± 0.67 % protein, 58.08 ± 0.16 % of β-glucan, and 0.26 ± 0.09 % mannans. The yield of product on the basis of dry

weight of cell was 21 %, which was higher than that reported elsewhere and with a 91 % of purity on the basis of total carbohydrate content (Freimund *et al.,* 2003, Thammakiti *et al.,* 2004, Liu *et al.,* 2008).

In contrast the glucan enriched cell wall preparation obtained after mechanical disintegration had a lower purity of 70 % based on total carbohydrate content and with higher residual protein content. Therefore, purity of the β-glucan preparations depend on the quality of starting yeast cell wall residue materials as concluded by Freimund *et al.,* 2003 and Liu *et al.,* 2008.

3.4.4 Mannan enriched cell wall preparation obtained from multi-step fractionation method of yeast cell wall residue.

The supernatant obtained after removal of insoluble materials at the end of hot water and protease treatment mainly consist of mannoprotein. Therefore, as per Lukondeh *et al.,* 2003 method aqueous ethanol precipitation of mannoprotein was employed to obtain a colourless and amorphous product.

The yield of mannan enriched cell wall preparation obtained in the present study was 7 - 13 %, with a characteristic higher level of protein (16 - 40 %) and total carbohydrate of 26 - 28 %, consisting mainly of β-glucan (3 - 18 %) and mannan (2 - 3 %). This considerable higher value of glucan can be attributed to co-precipitation of glucan conjugated with mannoprotein (Freimund *et al.,* 2003).

Table 3.3a Chemical composition (% w/w, dry basis), and solids yield of yeast biomass and different cell preparations using a multistep fractionation method.

Treatments	Constituents	Protein	Total Carbohydrate	β-Glucan	α-Glucan	Mannan	Chitin (glucosamine)	RNA	Ash	**Yield
Yeast biomass		40.05 ± 0.72a	32.05 ± 0.67	19.46 ± 0.16	1.48 ± 0.09	9.06 ± 0.06	1.04 ± 0.01	7.82 ± 0.11	11.53 ± 0.17	100
Yeast Cell wall (YCW)		35.51 ± 1.05	39.56 ± 0.67	11.28 ± 0.13	3.02 ± 0.19	4.93 ± 0.08	0.69 ± 0.01	5.36 ± 0.09	8.35 ± 0.14	54
Hot water treated YCW		31.82 ± 0.60	34.59 ± 0.65	16.01 ± 0.19	3.34 ± 0.23	2.46 ± 0.04	0.83 ± 0.01	5.49 ± 0.10	7.30 ± 0.13	33
Glucan enriched YCW Preparation		23.65 ± 0.17	46.27 ± 0.60	32.76 ± 0.33	0.39 ± 0.01	0.86 ± 0.01	0.69 ± 0.03	3.26 ± 0.04	5.29 ± 0.06	20
Mannan enriched YCW		16.54 ± 0.19	28.82 ± 0.48	3.70 ± 0.01	0.64 ± 0.11	2.09 ± 0.31	0.98 ± 0.01	7.52 ± 0.12	4.06 ± 0.06	13
Secondary Yeast Extract		22.99 ± 0.82	17.26 ± 0.25	0.03 ± 0.01	0.21 ± 0.01	2.81 ± 0.02	0.78 ± 0.04	1.98 ± 0.02	12.15 ± 1.31	32

Table 3.3b Chemical composition (%w/w, dry basis) and solid yield of yeast biomass and different cell preparations using a multi-step fractionation method.

Treatments	Constituents	Protein	Total Carbohydrate	β-Glucan	α-Glucan	Mannan	Chitin (glucosamine)	RNA	Ash	**Yield
Yeast biomass		46.26 ± 0.89	37.05 ± 0.92	22.48 ± 0.31	0.25 ± 0.89	10.46 ± 0.29	3.00 ± 0.13	7.22 ± 0.34	5.09 ± 0.95	100
Autolysed Cell wall (AYCW)		12.80 ± 0.91	60.73 ± 1.51	41.00 ± 0.29	0.63 ± 0.17	18.74 ± 0.31	0.74 ± 0.01	4.64 ± 0.07	7.71 ± 1.10	45
Hot water treated AYCW		5.26 ± 0.71	61.97 ± 0.63	59.65 ± 0.23	0.78 ± 0.58	1.22 ± 0.53	0.72 ± 0.03	2.93 ± 0.06	14.77 ± 0.99	38
Glucan enriched AYCW Preparation		4.44 ± 0.67	63.35 ± 0.60	58.08 ± 0.16	0.14 ± 0.93	0.26 ± 0.09	0.79 ± 0.01	1.91 ± 0.12	11.20 ± 1.98	22
Mannan enriched AYCW		40.60 ± 0.51	26.82 ± 0.79	18.87 ± 0.98	0.51 ± 0.80	3.07 ± 0.23	0.81 ± 0.01	7.73 ± 0.03	12.75 ± 0.19	7
Secondary Yeast Extract		15.18 ± 0.79	63.79 ± 0.46	57.39 ± 0.53	3.94 ± 0.71	1.40 ± 0.91	0.89 ± 0.04	6.57 ± 0.08	15.00 ± 0.98	13

**Compared with dry weight of original spent yeast. All values were means ± SD of triplicate analyses

3.4.5 Secondary yeast extract preparations obtained from multi-step fractionation method of yeast cell wall residue.

Upon low pressure drying of considerable amount of supernatant that was obtained after alcoholic and acid precipitation of mannan protein constituents resulted in brownish coloured product. The yield of this product was around 13 - 21 % with protein and total carbohydrate content of 15 - 23 % and 17 - 63 %, respectively. Moreover, this product had a characteristic savoury odour and flavour and was therefore referred as secondary yeast extract.

3.4.6 Acid insoluble, alkali insoluble and water soluble β-glucan preparations obtained from multi-step fractionation of yeast cell wall residue.

The compositions of crude β-glucan (CβG), acid insoluble, alkali insoluble β-glucan (PβG) and water soluble β-glucan preparations are outlined in Table 3.4. The enzymatically hydrolysed soluble fraction with nominal molecular weight cut off of more than 30 kDa was found to be turbid in nature and contain traces of protein impurities. The soluble cell wall preparations S10R, S3R were visually clear solutions with higher content of β-glucan with no traces of protein impurities.

Table 3.4 Chemical compositions (% w/w, dry basis) of different β-glucan preparations from spent *Kluyveromyces marxianus* yeast.

Descriptions	β-Glucan	α-Glucan	Mannose	Crude Protein
Crude ß-glucan preparation (CβG)	32.76 ± 0.33	0.39 ± 0.01	0.86 ± 0.01	23.65 ± 0.17
Acid insoluble, Alkali Insoluble β-glucan Preparation (PβG)	36.65 ± 1.91	3.24 ± 0.21	0.12 ± 0.93	0.76 ± 0.69
S30R (NMWD >30 kDa)	51.10 ± 3.37	0.54 ± 0.11	0.11 ± 0.07	0.15 ± 0.01
S10R (NMWD ~30-10 kDa)	42.54 ± 2.02	0.65 ± 0.03	ND	ND
S3R (NMWD ~10-3 kDa)	60.15 ± 3.11	0.15 ± 0.06	ND	ND

NMWD: Nominal molecular weight distribution, ND: not detected. The results are reported as means ± standard deviation (n = 3).

The effects of various glucans on different macrophage cell lines have been reported. In the present study in order to evaluate immunomodulatory characteristics of the glucan preparations from *Kluyveromyces marxianus*, an

evaluation of the cell viability at different concentrations of each cell wall preparation was performed. The macrophages J774A.1 were incubated with different concentrations of cell wall preparations (Figure. 3.3 and Figure. 3.4). While performing the cell viability experiments, it was observed that the soluble and particulate β-glucan preparations with concentrations of 0.02 and 0.2 mg/mL respectively had no effect on cell proliferation. The cell viability was found to be more than 95 % (Figure. 3.3 and Figure. 3.4). This result is consistent with previous reports which outline that β-glucan preparations showed almost no stimulation effect on cell proliferation and the cell viability of macrophage RAW 264.7 and fibroblast cells *in vitro* (Mori *et al.*, 1997, Son *et al.*, 2007).

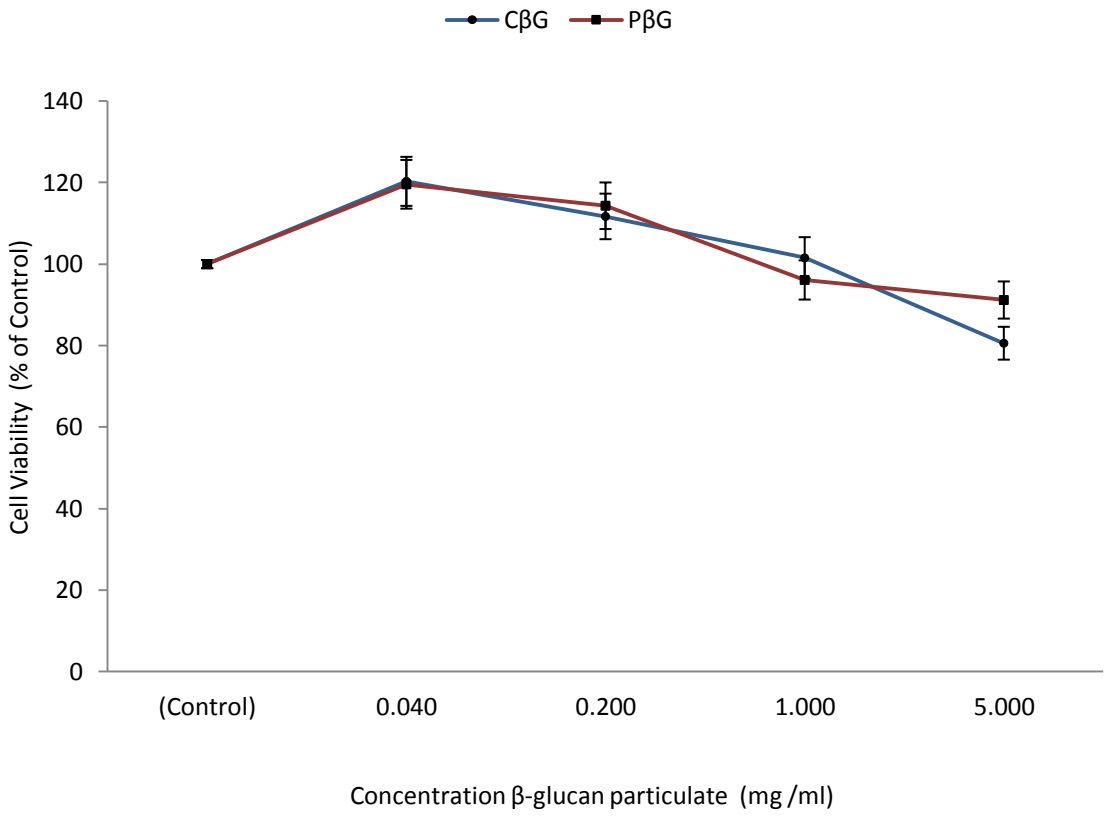


Figure 3.3 Effects of β-glucan particulate concentration (mg/mL) on viability of macrophage J774A.1. The results are reported as means ± standard deviation (n = 3).

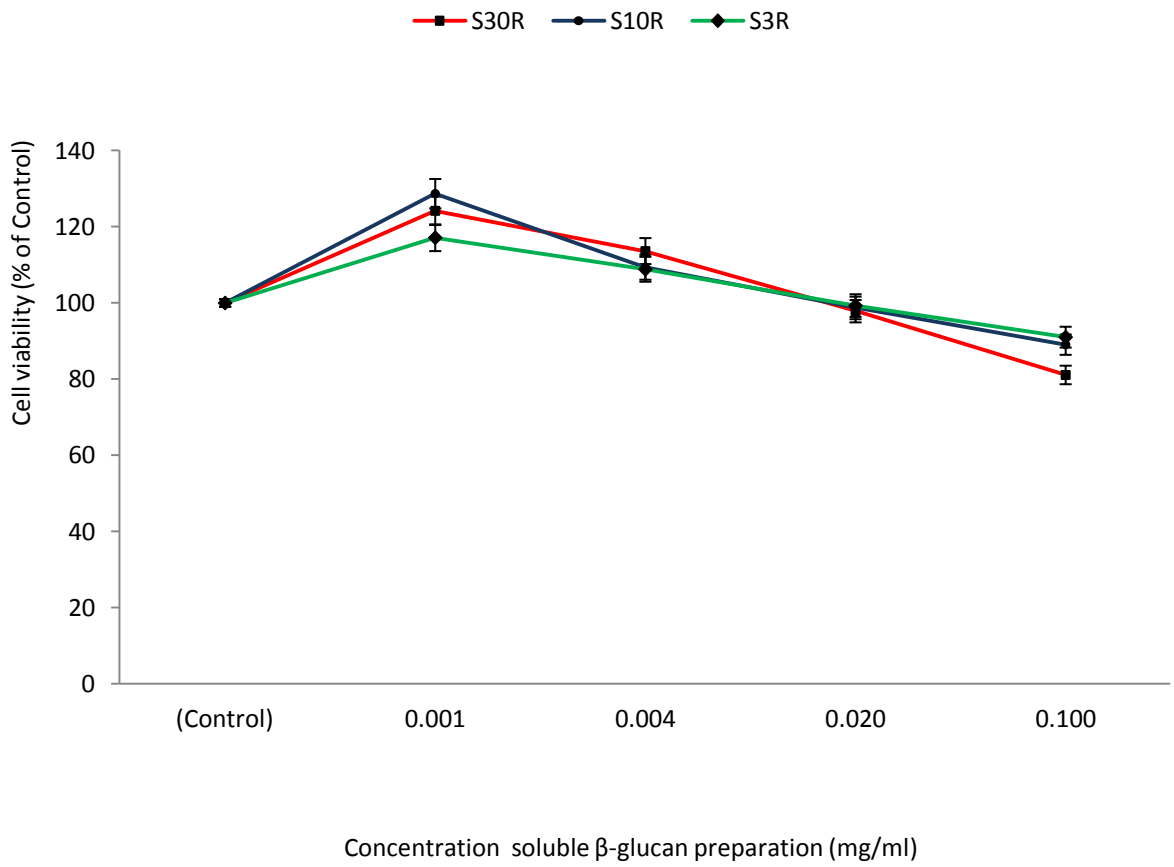


Figure 3.4 Effects of soluble β-glucan preparations (mg/mL) on viability of macrophage J774A.1. The results are reported as means ± standard deviation (n = 3).

3.4.7 Effect of β-glucan preparation on the production of nitric oxide, IL-12 and IL-10 by classically activated J774A.1 mouse macrophages.

To further investigate the effect of β-glucan treatment on the nitric oxide and cytokine release by macrophages, J774A.1 cells were incubated with 0.20 and 0.02 mg/mL of β-glucan particulate and soluble β-glucan preparations. In the present study macrophages J744A.1 were classically activated with IFN-γ and LPS treatment prior to β-glucan addition into the culture. IFN-γ is a potent macrophage activator which synergizes with LPS to enhance proinflammatory mediated production of macrophages (Miossec and Ziff, 1986, Gifford and Lohmann-Matthes, 1987, Hamilton *et al.*, 1989, Lorsbach *et al.*, 1993, Held *et al.*, 1999). The combined effect of IFN-γ and LPS on activation is more physiologically relevant than LPS alone, as IFN- γ is secreted by NK cells and T cells in response to LPS (Varma *et al.*, 2001).

It was demonstrated that the β-glucan addition to activated J774A.1 cells resulted in stimulation of nitric oxide production and proinflammatory IL-12 cytokine production. This was not the case in the same experiment using non-activated J774A.1 cells (Figure. 3.5). Similar observations were reported where *in vitro* cultures of peritoneal macrophages and RAW 264.7 cells were not able to produce nitric oxide unless they receive prior treatment with IFN-γ and β-glucan simultaneously, suggesting that the β-glucan-induced production of the proinflammatory mediator is IFN-γ dependent (Hashimoto *et al.*, 1997). Additionally, it was also reported that treatment of alveolar macrophages exposed to IFN-γ either before or at the same time as β-glucan, markedly augmented pro-inflammatory cytokine secretion along with enhanced production of nitric oxide (Sakurai *et al.*, 1996).

In the present study, it was also observed that the activated J744A.1 treated with specific β-glucan preparations (PβG and S30R) resulted in a 5 fold increase in the secretion of proinflammatory cytokine IL-12. This finding is consistent with a previous report suggesting that IFN-γ does not induce pro-inflammatory cytokines in the absence of another proinflammatory signal (Gifford and Lohmann-Matthes, 1987). Moreover, the production of nitric oxide and IL-12 in activated cells treated with PβG (acid and alkali insoluble β-glucan) particulate was significantly ($p < 0.05$) higher than cells treated with CβG (crude β glucan) particulate. The above observation indicates that the acid and alkali extraction of crude β-glucan resulted in the enhancement of immunomodulatory potency of cell wall preparation. Also, it is important to note that treatment of activated J744A.1 cells with soluble β-glucan preparations like S30R (at 0.02 mg/mL) were able to generate higher amounts of NO and IL-12 than activated cells treated with 10 fold higher concentration of particulate preparations (PβG).

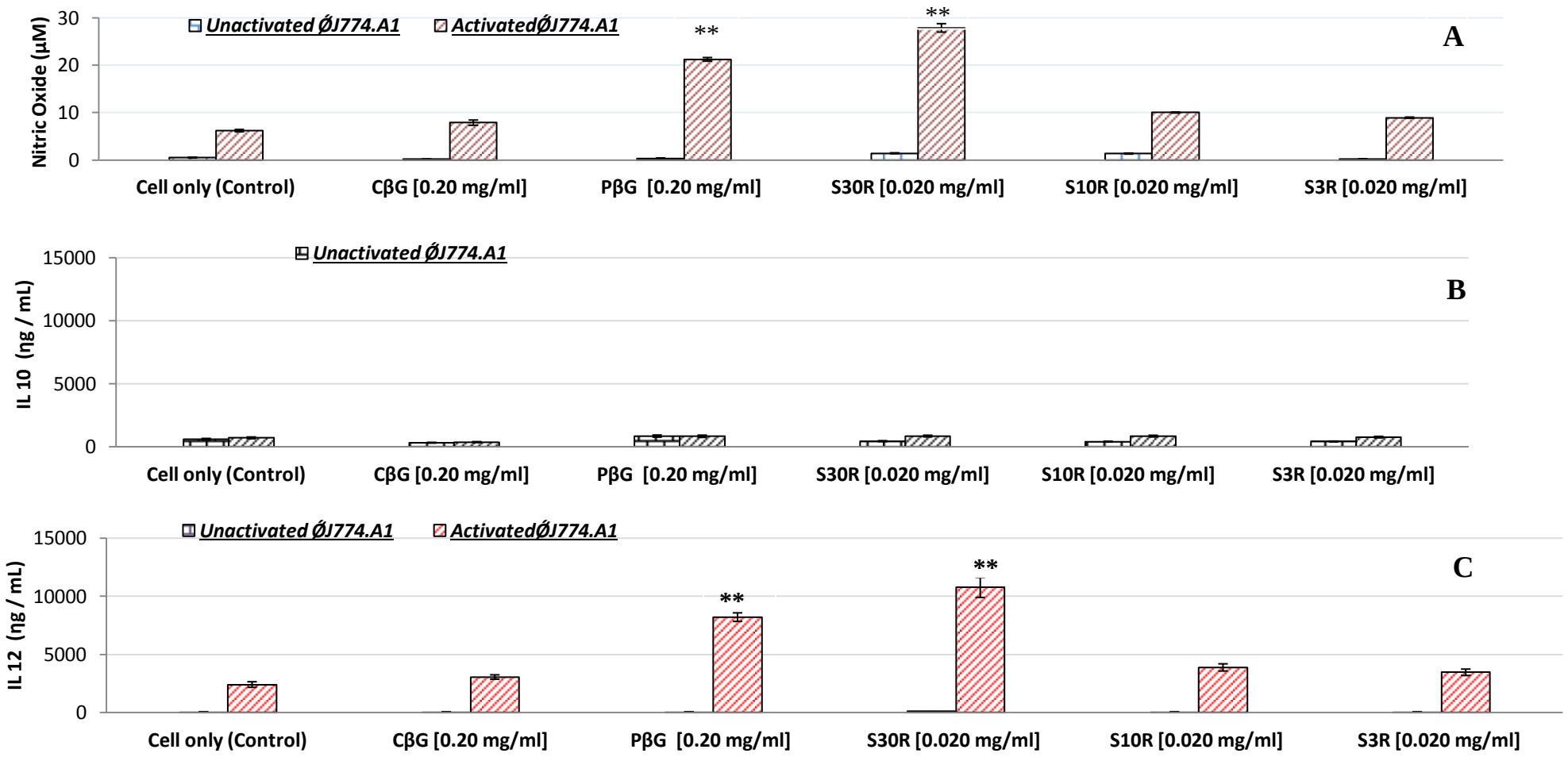


Figure 3.5 Generation of nitric oxide (A), IL-12 (B) and IL-10 (C) by cultured *in vitro* macrophage J744A.1 cells (Ø) in response different β-glucan preparations extracted from spent *Kluyveromyces marxianus.* β-glucan preparation (PβG, S30R) had induced the generation of significantly ($p < 0.05$) higher amounts of nitric oxide and pro-inflammatory IL-12 cytokine in activate macrophage with respect to control. In contrast all β-glucan preparations were not able to induced the production anti-inflammatory IL-10 in activated macrophage cell J744A.1 The results were reported as means ± standard deviation (n = 3). The data were analyzed by one way ANOVA, and the values marked with asterisks are significantly ($p < 0.05$) different from the non-treated control.

This suggests that soluble β-glucan is a more potent stimulator of proinflammatory response elements than the particulate preparations. The reason for this effect in not entirely clear but this may be due to low molecular weight. Such effects has been seen in previous reports (Thornton *et al.,* 1996, Brown and Gordon, 2001, Brown *et al.,* 2002, Kataoka *et al.,* 2002, Shao *et al.,* 2004) which proposed that it might be easier for low molecular weight material with a simplified structure to make the connection to the receptor of the signal transmission than material with a complex helix structure, or to be easily phagocytosed and cause the intracellular signal transmission and thus activate macrophages.

The treatment of activated and non-activated J774A.1 cells with all β-glucan preparations induced the release of small and non-significant amount of the anti-inflammatory IL-10 cytokine (Figure. 3.5). This further supports the pro-inflammatory role of β-glucan preparations from *Kluyveromyces marxianus* which can play important immunomodulatory role in cell system. The induction of nitric oxide in cell system may play an important role in a variety of pathophysiological functions, including neuronal transmission, vascular relaxation, immune modulation, and cytotoxicity against tumor cells (Lowenstein *et al.,* 1994, Chung *et al.,* 2001). Additionally activated macrophages and endothelial cells have reported ability to kill tumor cells via NO dependent pathways, in which i-NOS expression played a key role (Xu *et al.,* 2002).

The data obtained from the present study with J774A.1 cell line imply that β-glucan preparations *Kluyveromyces marxianus* exhibited the biological activity that stimulate macrophages *in vitro* and may offer an alternative potential source of natural pro-inflammatory immunomodulating substances. Furthermore, the method outlined in present study to produced soluble β-glucan preparations from *Kluyveromyces marxianus* were found to have more potent proinflammatory activity when compare to β-glucan particulates.

3.4.8 Effect of β-glucan preparations on production of cytokine in human whole blood assay.

As shown in Figure 3.6 in human whole blood assay (HWBA) *ex vivo* condition, the β-glucan preparations induced the release of small, but statistically significant amounts of pro-inflammatory TNF-α, IL-6, IL-8 and MCP-1 with respect to control. However, β-glucan (PβG, S30R) had no significant effect on secretion of anti-inflammatory IL-10 and TGF-β with respect to control. This suggests that β-glucan preparations from *Kluyveromyces marxianus* mainly exhibit pro-inflammatory activity and can play important immunomodulatory role in cell system by regulation of cytokines level as discussed in following points.

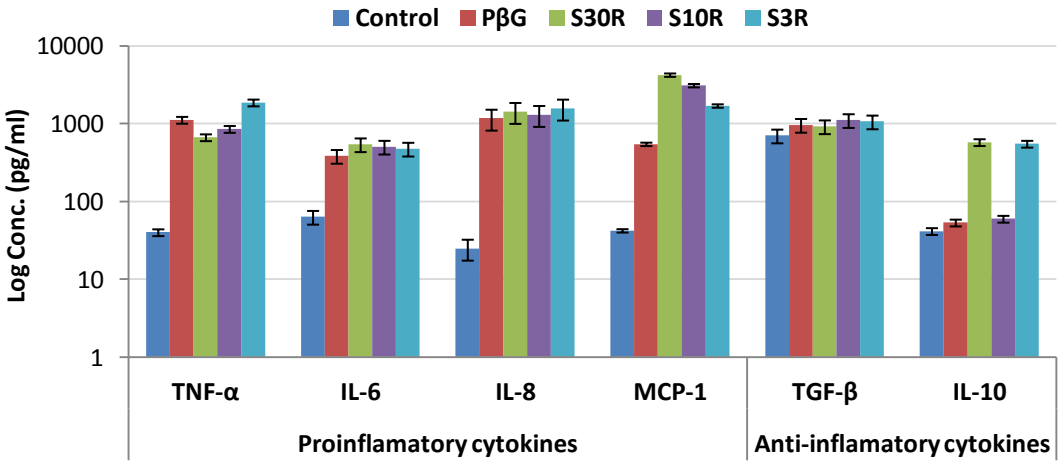


Figure 3.6 The β-glucan induced cytokine production in human whole blood. Human whole blood was incubated with 50 µg/mL β-glucan (PβG, S30R, S10R and S3R) or media (control) for 24 in incubator at 37 °C, CO_2 (5 %), 96 % RH. The results are reported as means ± standard deviation (n = 3).

- TNF-α is known as an important host protection cytokine that destroys tumor cells and also induces inflammation (Urban *et al.*, 1986, Decker *et al.*, 1987). In present study, it was observed that production of TNF-α was induced by all β-glucan preparations.

- MCP-1 and IL-8, potent chemo-attractants for monocytes across endothelial cells and epithelial cells layers (Randolph and Furie, 1995, Gunn *et al.*, 1997,

Maus *et al.*, 2001) was induced by the soluble β-glucan preparations (S30R, S10R and S3R).

- IL-6 with an activity associated with disseminated intravascular coagulation cascade (van der Poll *et al.*, 1994) and inflammatory responses (Heinrich *et al.*, 1998, Osterud and Bjorklid, 2001). In present study it was observed that production of IL-6 was significantly ($p < 0.05$) induced by all type of β-glucan preparations in comparison to control. These results may be explained by the ability of glucan preparations from *Kluyveromyces marxianus* to bind and crosslink the part of complement receptor 3 (CD11b/CD18) responses (Thornton *et al.*, 1996a) thereby activating the cell pattern recognition receptors that senses the activities where foreign cells are phagocytose.

- TGF-β that acts on polymorphonuclear leukocytes and endothelial cells to block the effects of pro-inflammatory cytokines and plays important role in wound healing and angiogenesis (Blobe *et al.*, 2000). It was found that production of TGF-β was not induced by all types of β-glucan preparations in comparison to control. This signifies that the β-glucan preparations from *Kluyveromyces marxianus* exhibit the anti-inflammatory activity.

- IL-10 cytokine produced by activated macrophages and T helper type 2 cells (Th2), which is predominantly an inhibitory cytokine that play role in inducing hypo-reactivity or anergy (dampening of immune responses) in trauma patients (Marie *et al.*, 2000, Cavaillon *et al.*, 2001). It was found that the secretion of IL-10 was significantly induced by soluble β-glucan preparations, S30R and S3R with respect to control. This action was assumed to be mediated in part through activation of Toll like receptor 2 and dectin-1–dependent activation of extracellular-signal-regulated kinases / mitogen-activated protein kinase (ERK MAPK) pathway which promotes IL-10 production (Dillon *et al.*, 2006).

The data obtained from the present study with the *ex vivo* human whole blood assay showed that β-glucan preparations from *Kluyveromyces marxianus* elevated the secretion of pro-inflammatory cytokines TNF-α, IL-6, IL-8, MCP-1

and T helper 1 cell (Th1) cytokines when compared with anti-inflammatory cytokines IL-10 and TGF-β.

The cytokine profile obtained from the HWBA revealed that β-glucan preparations derived from *Kluyveromyces marxianus* enhanced the protective T helper 1 cell (Th1) mediated innate immune responses (Jarek Baran *et al.,* 2007) that are responsible for killing intracellular parasites and enable autoimmune responses (Berger, 2000).

Similar results were also reported where β-glucan preparations induced the secretion of Th1 cytokine IL-12 in murine model (Suzuki *et al.,* 2001) and switched the non-protective Th2 immune response to a protective Th1 cell-mediated immune response (Jarek Baran *et al.,* 2007). This is very important for the treatments of immune-suppressed populations with predominant Th1 type immune response, i.e. patients suffering from trauma, organ transplant, by-pass surgery and sports persons (Bocsi *et al.,* 2006, Spruijt *et al.,* 2010, Woods *et al.,* 2000, Nieman, 2008, Kudsk, 2006).

Therefore, β-glucan preparations from *Kluyveromyces marxianus* yeast may be used as supplement for immunonutrition (Wichers, 2009, Calder, 2007, Nieman, 2008, Kudsk, 2006).

3.5 Conclusion

This study has showed that spent *Kluyveromyces marxianus* yeast obtained as a by-product from the dairy / brewing industry is a rich source of protein (43 %), β-glucan (20.97 %), mannose (9.76 %) and RNA (7.02 %) on a dry matter basis. The lab scale chemo-enzymatic treatment established in this study to process yeast biomass and cell wall residues is mild and environmental friendly with yeast biomass entirely utilized to produce phosphorylated yeast protein and different cell wall preparations.

Phosphorylation of yeast protein led to a reduction of nucleic acid content (55 %) of yeast protein concentrates with an appreciable increase in protein extraction (10 %) from yeast extract.

Simple chemo-enzymatic treatments (autolysis, hot water treatment, protease treatment, acid and alkali extraction and glucanase treatment) of cell wall residue presented in the study can be utilised by industry to recover protein enriched secondary yeast extracts, crude β-glucan particulate and soluble β-glucan preparation from cell wall residue.

Further optimisation and scale up studies in addition to market and sustainability analysis will be required before the industrial exploitation of the proposed integrated approach. The present study also showed that β-glucan preparations (particulate and soluble glucan) from spent *Kluyveromyces marxianus* yeast can recognise and interact with cells of the innate immune system of mammalian cell line and humans whole blood.

It was also found that β-glucan preparations from *Kluyveromyces marxianus* enhanced the production of Th1 cytokines IL-12 and TNF-α, IL-6, IL-8 under *in vitro* and *ex vivo* conditions. This indicated that cell wall preparations from *Kluyveromyces marxianus* may enhance the protective Th1 mediated innate immune responses in both *in vitro* mammalian cell line and *ex vivo* human whole blood condition. Nevertheless, the mechanisms behind this activity, the cellular receptors involved in these interactions and retention these activity in final product application were not addressed in the present study. This may form the basis of future collaborative studies which would be required in order to commence the claim making process.

Chapter 4.0

Angiotensin-I converting enzyme inhibitory activity of yeast protein hydrolysates derived from spent *Kluyveromyces marxianus* yeast

4.1 Introduction

Peptide contents that contribute to the sensory attributes of yeast extract (Dziezak, 1987, Breddam and Beenfeldt, 1991, Nagodawithana, 1992) may be multifunctional in nature and also possess bioactive properties of potential health benefiting interest. One such activity of specific interest to this study is the angiotensin-I converting enzyme (ACE) inhibitory activity. In recent years several studies have been conducted on the production and isolation of ACE inhibitory peptides from animal and plant-derived food proteins (FitzGerald *et al.,* 2004, Vercruysse *et al.,* 2005, Murray *et al.,* 2005, Matsui and Matsumoto, 2006, Miguel and Aleixandre, 2006, Guang and Phillips, 2009, Jakala and Vapaatalo, 2010). In recent years only a few studies have been reported which outline the ACE inhibitory activity of yeast-derived peptides (Table 1.5). Therefore, objective of this study was to generate bioactive peptide enrich extracts by proteinolytic / peptidylytic hydrolysis of phosphorylated yeast protein from spent yeast *Kluyveromyces marxianus* using selected enzymatic preparation (Figure 4.1, Figure 5.2). In brief, the digestive enzyme pepsin and pancreatin were selected to generate yeast protein hydrolysates containing a pool of peptides resembling those generated during the digestion of yeast protein in the gastrointestinal tract which may be absorbed by digestive epithelial cells along the small intestine. Similarly, papain which is mainly used in the production of commercial yeast extracts was used to produce pools of peptides resembling those generated during the yeast extract production. The optimum temperature, pH and % E/S ratio for protein hydrolysis experiments were selected on basis of the specific range of enzyme used (Table 4.1) and literature data (Adler-Nissen, 1986, Moresi *et al.,* 1995, Walsh *et al.,* 2004, Megaas *et al.,* 2004). Of all the yeast protein digests, created pepsin digest having the highest *in vitro* ACE inhibitory activity was selected for activity guided fractionation into peptide pool using size exclusion chromatography and two step RP-HPLC techniques. Subsequently, the most active fraction with highest ACE inhibitory potency and purity were then assessed for their peptidic contents using a Q-Exactive high performance benchtop quadrupole Orbitrap mass spectrometer system connected to a Dionex Ultimate 3000 chromatography system (Michalski *et al.,* 2011, Di Bernardini *et al.,* 2011).

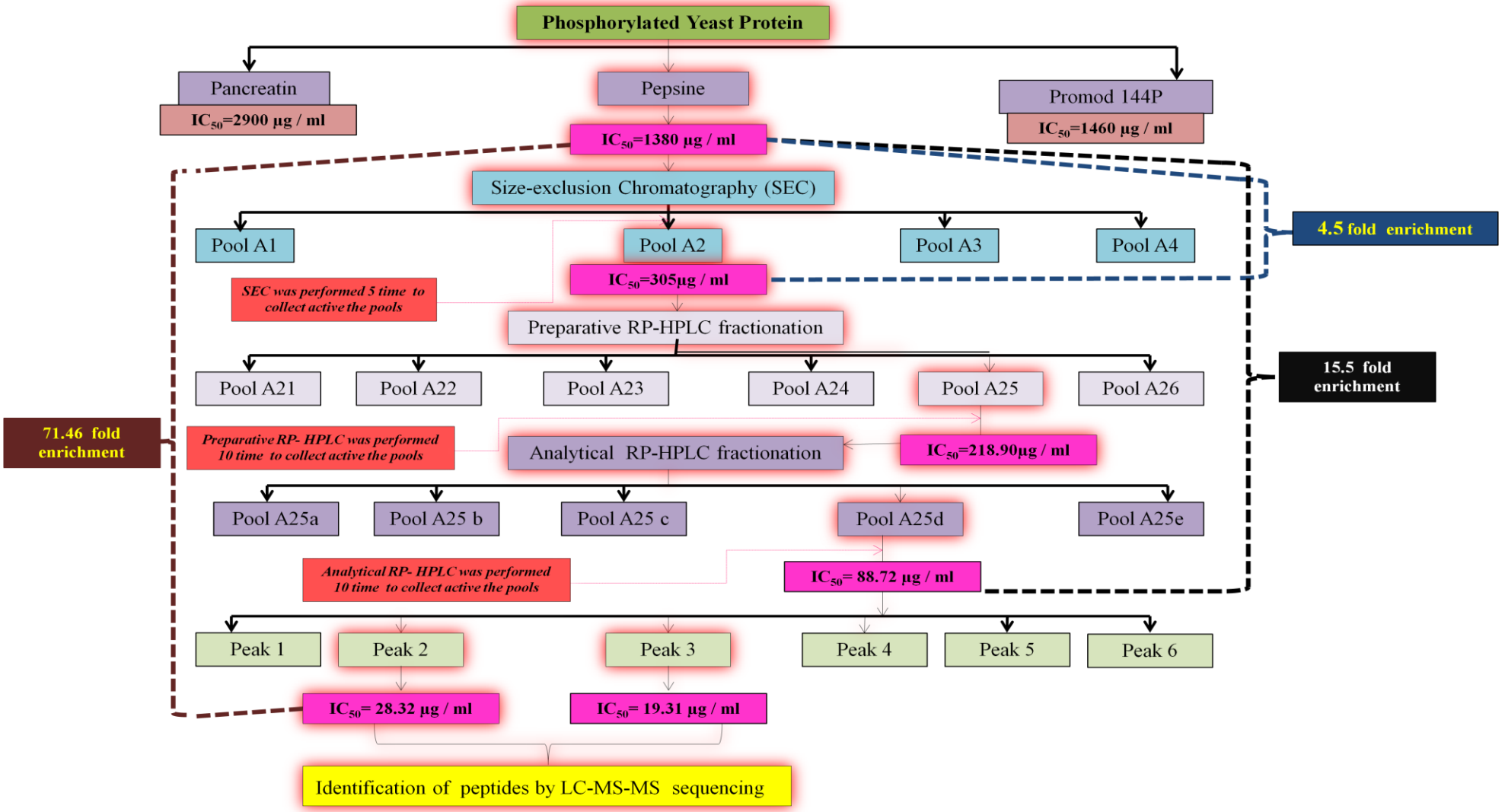


Figure 4.1 Schematic diagram of the main steps carried out in present study to isolate ACE inhibitory peptides from phosphorylated yeast protein obtained from spent *Kluyveromyces marxianus*.

Table 4.1 Specificity of each enzyme preparation use in present study.

Enzyme /Enzyme preparation	Specificity of enzyme
Porcine pepsin	Exhibits preferential cleavage for hydrophobic, preferably aromatic, residues in P1 and P1' positions. Increased susceptibility to hydrolysis occurs if there is a sulfur-containing amino acid close to the peptide bond, which has an aromatic amino acid. It will also preferentially cleave at the carboxyl side of phenylalanine and leucine and to a lesser extent at the carboxyl side of glutamic acid residues. It will not cleave at valine, alanine, or glycine linkages
Pancreatin	**Composed of :** **Trypsin** cleaves peptide chains mainly at the carboxyl side of the amino acids lysine or arginine, except when either is followed by proline. **Chymotrypsin** is responsible for cleaving peptide bonds following a bulky hydrophobic amino acid residue. Preferred residues include phenylalanine, tryptophan, and tyrosine, which fit into a snug hydrophobic pocket. **Elastase** is responsible for cleaving peptide bonds following a *small neutral* amino acid residue, such as Alanine, glycine, and valine.
Papain	Digest most protein substrates more extensively than the pancreatic proteases. Papain exhibits broad specificity, cleaving peptide bonds of basic amino acids, leucine, or glycine. It also hydrolyzes esters and amides. Papain exhibits a preference for an amino acid bearing a large hydrophobic side chain at the P2 position. It does not accept Val at the P1' position.

4.2 Materials and methods

4.2.1 Materials

Spent yeast biomass of *Kluyveromyces marxianus* was obtained as described in Section 2.2.1. Sodium trimetaphosphate (STMP), Captopril, rabbit lung acetone powder, N-[3-(2-Furyl)-acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG), gastric porcine pepsin and porcine pancreatin were purchased from Sigma Aldrich, Ireland. Papain (in the form of commercial crude preparation Promod 144P) was kindly provided by Biocatalyst, UK and all other chemicals used were of analytical grade.

4.2.2 Methodology

4.2.2a Preparation of yeast protein hydrolysates

A phosphorylated yeast protein was prepared as described in Section 3.2.1 and diluted with deionised water to yield a 4 % (v/v) protein solution. Prior to commencing the hydrolysis reaction, the pH of each protein solution was adjusted to the optimum pH of the proteolytic enzyme preparation using either 2 M NaOH or HCl. The pH adjusted solution was then stirred for 30 minutes at the desired reaction temperature for equilibration purposes. The protein suspensions were hydrolysed using various enzymes: substrate ratios (Table 4.2) for a period up to 240 minutes.

Table 4.2 Parameters for enzymatic hydrolysis of yeast protein.

Enzyme	Pepsin	Promod 144P	Pancreatin
pH*	2	5.5	7.5
Temperature (ºC)*	37	50	37
E:S*	1:40	1:20	1:20

*The pH and temperature conditions of hydrolysis were followed as per manufactures advised of these commercial enzyme preparations.

*E:S= Enzyme to substrate ratio were identified in preliminary trials to establish a rate of activity that permitted a measurable rate of hydrolysis over the period of hydrolysis.

During the hydrolysis reaction, samples were withdrawn from the proteolytic mixture at specified time intervals and were heated at 80 °C for 20 minutes in water bath to inactivate the enzyme. Heated hydrolysates were cooled and centrifuged at 10,000 rpm for 15 minutes at 4 °C. The clear supernatant was then

freeze dried and reconstituted to 50 mg/mL and stored at -20 ℃ prior to analysis. All hydrolysis reactions were carried out in duplicate. The work-flow after enzymatic hydrolysis of phosphorylated yeast protein is shown in Figure 4.1

4.2.3 Analytical methods

The dry solid content of the yeast extracts were determined following 24 h incubation at 105 ºC (Appendix I: Method 1.0). Protein content was determined using micro-Kjeldahl method as per International Dairy Federation method (I.D.F., 1993, Appendix I: Method 2.0). The α-amino nitrogen content of the protein hydrolysate was determined using the modified cadmium chloride ninhydrin method (Doi *et al.*, 1981, Appendix I: Methods 3.0) using leucine as a standard. The free α-amino nitrogen content and peptide content of protein hydrolysates were determined with a conventional stannous chloride ninhydrin method (Doi *et al.*, 1981, Appendix I: Methods 3.0). Peptides were quantified by calculating the difference between the results obtained with the conventional ninhydrin method and the modified cadmium chloride method. All the above analyses were done in triplicate. Total amino nitrogen content of protein was determined in duplicate after hydrolysis of samples at 110 ℃ for 24 h with 6 M HCl prior to the amino acid analysis. The individual amino acids were analysed in duplicate in an amino acid analyser (Biochrom 30, Pharmacia Biochrom Ltd) by using ion-exchange chromatography (Stenberg *et al.*, 2002).

4.2.4 Assay for ACE inhibitory activity

ACE inhibitory activity was measured using the furanacryloyl-L-phenylalanylglycylglycine (FAPGG) assay (Shalaby *et al.*, 2006) with some modification as per Murray *et al.*, 2004.

In brief, ACE inhibitory values were obtained for hydrolysate samples by incorporating a 10 µL solution of the test hydrolysate (~ 50 mg/mL) in the standard assay (14.7 mU/mL ACE, 0.78 mM FAPGG). The control sample contained water instead of inhibitor solution. The ACE activity was expressed as slope of the decrease in absorbance at 340 nm (ΔA). The % ACE inhibition was calculated from the ratio of the slope in the presence of inhibitor to the slope obtained in absence of inhibitor, according to the formula: % ACE inhibition =

[1–(ΔAinhibitor / ΔAcontrol)] x 100. Captopril was used as a positive control inhibitory substance (Appendix I: Method 4.0). Analyses were performed in quintuplet for all samples.

The selected protein hydrolysate with highest ACE inhibitory activity was subsequently tested for their IC_{50} value. The IC_{50} values were determined from plots of % ACE inhibition versus inhibitor (Captopril and protein hydrolysate as internal quality control standard) concentration present in the assay. Uninhibited activity was the activity obtained in the absence of an inhibitory substance in the reaction mixture. All reported IC_{50} values are the mean of independent duplicates, tested in triplicate.

4.2.5 Definitions

The degree of hydrolysis (DH) is defined as the ratio of the number of peptide bonds cleaved (number of free amino groups formed during proteolysis) expressed as hydrolysis equivalents (*h*), in relation to the total number of peptide bonds before hydrolysis (h_{total}). The *h* values for protein hydrolysates were determined using the modified cadmium chloride ninhydrin method (Doi *et al.*, 1981, Appendix I: Methods 3.0). The h_{total} is equivalent to the amino acid composition of the protein and was calculated from amino acid analyses by summing the individual amino acid content per gram of protein in the case of phosphorylated yeast protein (Adler-Nissen, 1979). **ACE inhibition Index** is the percentage ACE inhibition observed for given concentration of peptide added to test reaction compare to control. ACE inhibitory potency (IC_{50}) is defined as amount of peptide that mediated a 50 % inhibition of ACE activity.

4.2.6 Purification of ACE inhibitory peptides from yeast protein hydrolysates

4.2.6.1 Gel filtration chromatography

Gel filtration chromatography is used for the separation of biomolecules with different molecular weight. It is commonly used for desalting protein solutions, protein separations and removal of low molecular weight substances from colloids. It has also been applied to the group separation of protein hydrolysates and biological extracts (Gelotte, 1960, Whitaker, 1963). In this study gel filtration was applied to separate protein and low molecular weight peptides in yeast protein hydrolysate. For this, yeast hydrolysates with highest ACE inhibitory potency obtained after incubation of phosphorylated yeast protein

with pepsin (4 h) were filtered through 0.2 µm PVDF syringe filters to remove particulates and microbes. The filtered protein hydrolysates (5 mL, 45 mg/mL) were loaded to a Sephadex 25 gel filtration column (1 cm x 25 cm) at a flow rate of 60 mL/h in 100 mM of volatile ammonium acetate buffer, pH 8.33. During gel filtration eluting fractions were pooled into consecutive 5 minutes pools. These pools were freeze dried to concentrate and to remove buffer, afterward resuspended in distilled water prior to ACE analyses. Based on the ACE inhibitory activity of eluted fraction the most potent pool was identified and targeted for purification using reverse phase C_{18} high-performance liquid chromatography (RP-HPLC). Fractions selected for further purification of peptides were pooled and lyophilized before high-performance C_{18} RP-HPLC. Peptide molecular masses were determined by reference to a calibration curve created by running molecular mass markers on the Sephadex G-25 under identical running conditions to test samples. Molecular mass standards were bovine serum albumin (66 kDa), myoglobin (16.8 kDa), cytochrome C (11.7 kDa), vitamin B_{12} (1.35 kDa) and FAGGP (0.399 kDa). Peptide and protein elution was monitored at 214 nm and 280 nm, respectively.

4.2.6.2 Two-step reversed phase high pressure liquid chromatography

The pool with the highest ACE inhibitory activity obtained after gel filtration chromatography separation (Figure 4.1) was further fractionated using preparative reversed phase high-performance liquid chromatography on an XBridge^{TM} BEH300 Prep C_{18} (5 µm, 10 mm x 150 mm, Waters Ireland Ltd). Eluent A was prepared as Milli-Q water containing 0.1 % trifluoroacetic acid and eluent B acetonitrile containing 0.1 % trifluoroacetic acid. The chromatographic column was conditioned with 100 % eluent B at flow rate of 4 mL / min at 30 °C. The injection volume of 200 µL (10 mg/mL protein sample) was applied to C_{18} column. Concentrations of eluent B were set to increase from 0 - 30 % (v/v) in 0 - 30 minutes, 30 - 40 % (v/v) for 40 - 50 minutes, in 100 % in 50 - 60 minutes. Elution was monitored at 214 nm for peptide detection. Six fractions were collected for assay of ACE inhibitory activity. These pools were freeze dried to remove solvent and assessed for ACE inhibition index. The fraction with highest ACE inhibitory index or potency (pool A25, Figure 4.1) was injected in an analytical HPLC reversed phase column (XBridge^{TM} BEH300

C_{18} 3.5µm 4.6 mm x 150 mm column) for the analysis of ACE inhibitory activity in individual peaks. The injection volume was 50 µL and the sample protein concentration was 5 mg/mL. Elution was achieved by a non-linear gradient of acetonitrile in water (0 - 30 % in 40 minutes, 30 - 100 % in 40 - 50 minutes and 100 % in 50-60 minutes) containing 0.1 % trifluoroacetic acid at a flow rate of 0.25 mL/min at 30 °C. Elution was monitored at 214 nm and six fractions were pooled, concentrated and lyophilized for ACE inhibitory test.

4.2.6.3 Peptides identification using LC-MS-MS and *in silico* analysis of peptides sequences for ACE inhibitory active amino acid motif

The two fractions (Peak 2 and Peak 3, Figure 4.1) with highest ACE inhibitory potency obtained from RP-HPLC purification were applied to a Thermo Scientific Q-Exactive mass spectrometer connected to a Dionex Ultimate 3000 (RSLC nano) chromatography system. Pepsin derived peptides were resuspended in 0.1 % formic acid. Each sample was loaded thrice onto Biobasic Picotip Emitter (120 mm length, 75 µm ID) packed with Reprocil Pur C_{18} (1.9 µm) reverse phase media and was separated by an increasing acetonitrile gradient over 20 minutes at a flow rate of 250 nL/min. The mass spectrometer was operated in positive ion mode with a capillary temperature of 220 °C and a potential of 1800 V applied to the frit. All data was acquired with the mass spectrometer operating in automatic data dependent switching mode. A high resolution (70,000) MS scan (350 - 1600 Da) was performed using the Q Exactive to select the 15 most intense ions prior to MS/MS analysis using higher energy collisionally activated dissociation (HCD). The automated spectral processing, peak list generation and database search and De Novo were performed using the software PEAKS Studio 5.3. In the present study the Uniprot protein database (htt://www.uniprot.org, uniprot-taxonomy 4911) was also used to identify the potential peptides cleaved from *Kluyveromyces marxianus* protein using pepsin (pH 1.3) and to back identify the source protein. Each peptide was targeted for protein identification as per the software manufacture's guideline. Only peptides whose scored corresponded to a false discovery rate (FDR) of ≤ 1 % were accepted from the Peaks database search. False discovery rate (FDR) is a probabilistic tool that indicates whether the identified peptide-spectrum matches are correct. The peaks de novo results were

filtered using an average local confidence (ALC) of ≥ 65 %, a total local confidence of (TLC) of ≥ 6 and peptide score (-10 lgP) of ≥ 15. ALC and TLC specify confidence that the correct amino acid residue in each position has been identified. Moreover, peptide score (-10 lgP) is a measure of significance of a peptide-spectrum match in database search. The score is -10 times the common logarithm of the P-value.

Amino acid sequences, the peptide position within source protein, peptide charge states and the retention times of peptides were obtained using the PEAK studio version 5.3 software programs of the system.

Using the peptide cutting program *ExPASy peptide cutter* (http://ca.expasy.org/tools/peptidecutter/) with enzyme pepsin at pH 1.3, yeast protein from the Uniprot protein database were mapped in terms of potential proteolytic cleavage sites and were compared with peptide sequenced in present study. Moreover, using BIOPEP (http://www.uwm.edu.pl/biochemia/), all the peptides sequenced in present study were subjected to *in silico* analyses to ascertain the presence of active amino acid motifs with potent ACE inhibitory activity (Iwaniak *et al.*, 2005). Also the % ratio presence of ACE potent amino acid residue (**Y, W, F, L, P, I, V, K** and **R**) to peptide length was calculated for peptides sequenced in present study (Murray *et al.*, 2005, Hernández-Ledesma *et al.*, 2011). Similarly, calculation was also done for presence of ACE potent hydrophobic (**F, L, P, I** and **V**) and aromatic (**Y, W, F**) amino acid residue. These obtained % ratio were plotted against each peptides identified in fraction A25d-P2 and A25d-P3 in the form of profile wheel (B&D).

4.3 Results and discussion

4.3.1 Generation of yeast protein hydrolysates

Phosphorylated yeast protein extracts were hydrolyzed with a range of proteolytic enzyme preparations i.e. pepsin, pancreatin and Promod144P. These enzymes were preferred on the basis of their specificity and their nature (Table 4.1 & 4.2). Pepsin is the main proteolytic enzyme generated in the human stomach during food digestion. It is the first proteolytic enzyme encountered during gastrointestinal digestion. The pancreatin preparation derived from

porcine pancreatic extracts contains mixed proteolytic activities such as trypsin, chymotrypsin and elastase and is part of pancreatic juice. Vast arrays of bioactive peptides are generated following pepsin and pancreatin hydrolysis (FitzGerald *et al.*, 2004, Vercruysse *et al.*, 2005, Matsui and Matsumoto, 2006, Miguel and Aleixandre, 2006, Guang and Phillips, 2009, Jakala and Vapaatalo, 2010). Papain (Promod 144P) is a plant proteolytic enzyme mainly used during the production of yeast extracts. The general reasons for application of this enzymatic step were the solubilisation of spent yeast biomass and generate bioactive peptide.

The time courses for hydrolysis of phosphorylated yeast protein by pepsin, pancreatin and papain are displayed in Figure 4.2. In all cases of hydrolysis, it was evident that the DH % increased with increasing incubation time of the yeast extract with the enzyme preparations. However, the enzyme preparations differed in their ability to hydrolyse the same yeast extract substrate. This is an expected result which is due to limited availability of cleavage sites.

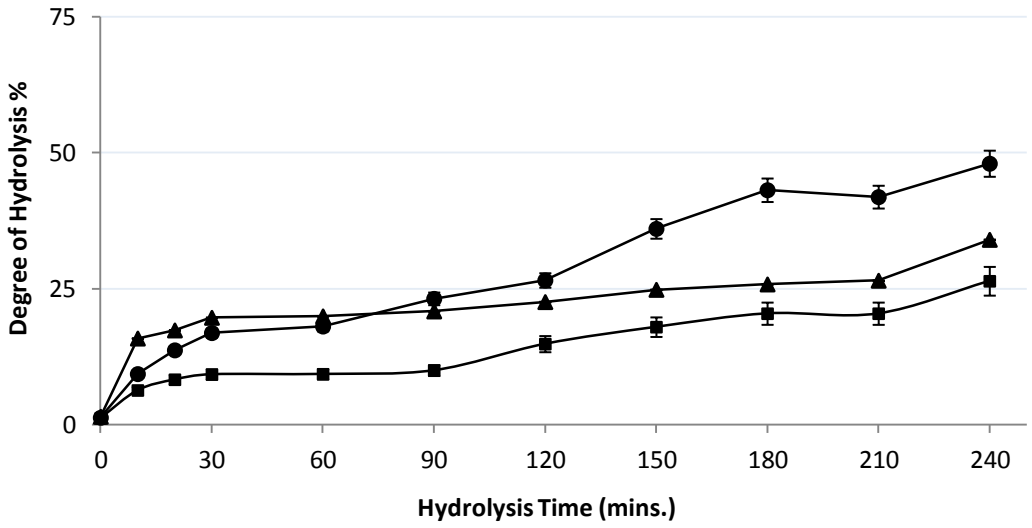


Figure 4.2 Effect of hydrolysis time on the % degree of hydrolysis of yeast protein hydrolysate by pepsin (■), pancreatin (●) and Promod 144P (▲), All values are means ± SD of triplicates analyses of two independent trials.

It was observed that the ability of the enzyme preparations to hydrolyse the yeast extract decreased in the order of pancreatin, Promod 144P and pepsin. At the end of 240 minutes pepsin incubation time the DH % corresponded to 26 %.

Since DH % is defined as the ratio of the number of peptide bonds cleaved, it means that 26 % of available peptide bonds were cleaved. In contrast the pancreatic reaction continued to progress to DH % values of 48 % over the 240 minutes time period. This is an expected result as the purer pepsin preparation most likely cleaved all available specific cleavage sites available in this time period.

As outlined earlier, the pancreatic extract contained several different enzyme activities (trypsin, chymotrypsin and elastase) that led to extensive hydrolysis of yeast protein. Table 4.1 outlines the peptide sequences cleaved by each enzyme preparation. Thus, variations in extent of hydrolysis (% DH) of yeast protein between different enzymes were attributed to the specificity of the different enzyme preparations. It was also observed that more than 50 % of the generated peptides were generated in first 30 minutes of the hydrolysis reaction (Figure 4.3). The two main reasons for a drop in the hydrolytic reaction in the latter 210 minutes are associated with enzyme denaturation and a reduction in available cleavage sites.

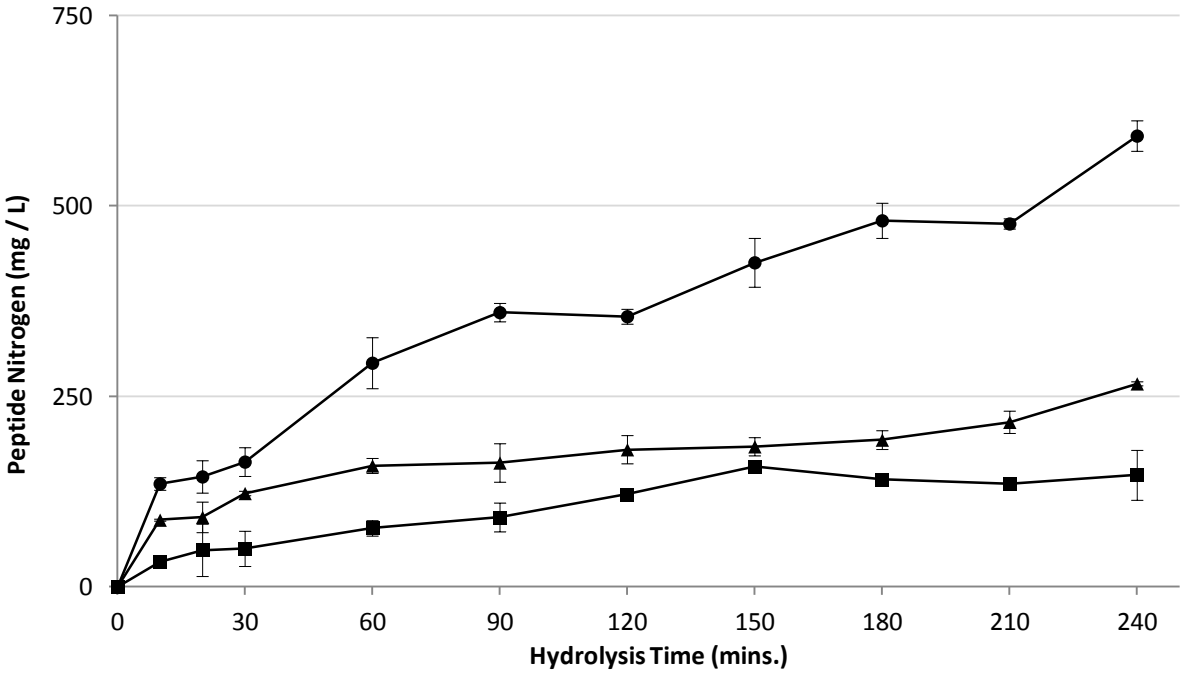


Figure 4.3 Effect of hydrolysis time on the peptide content of yeast protein hydrolysate by pepsin (■), pancreatin (●) and Promod 144P (▲), All values are means ± SD of triplicates analyses of two independent trials.

4.3.2 ACE inhibitory activity of yeast protein hydrolysates

As shown in Figure 4.4 phosphorylated yeast protein extract without any enzymatic pre-treatment (0 h) displayed an ACE inhibitory activity of approximately 15 % with less than one % DH value. This result indicates that partial hydrolysis of phosphorylated yeast protein suspension may have occurred during sample preparation by action of indigenous proteolytic enzymes of yeast cells (Achstetter and Wolf, 1985, Roy M. K. *et al.*, 2000, Ramírez-Zavala *et al.*, 2004a, Ramírez-Zavala *et al.*, 2004b).

The most potent ACE inhibitory activity (62 %) was realised for Promod 144P after 1.5 h corresponding to a % DH of 20. Similarly, the ACE inhibitory activity of hydrolysates produced by pepsin increased to 61 % after 4 h of hydrolysis, corresponding to 26 % DH. Thereafter the ACE inhibitory activity plateaued most likely due to the utilisation of all available cleavage sites. The ACE inhibitory activity of pancreatin derived hydrolysates increase up to 37 % DH into the first 30 minutes of incubation and more or less plateaued thereafter.

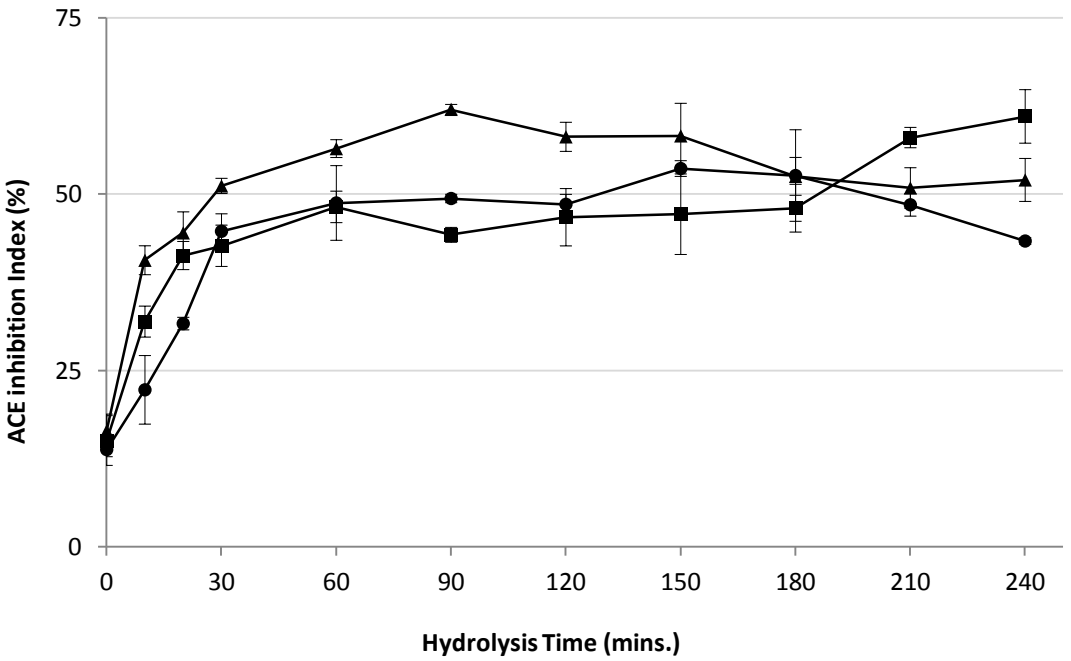


Figure 4.4 Effect of hydrolysis time on the ACE inhibitory activity (%) of yeast protein hydrolysates by pepsin (■), pancreatin (●) and Promod 144P (▲), All values are means of quintuplet analyses. ACE inhibition Index is the value for the percentage ACE inhibition observed based when 500 µg of peptide is added to test reaction compare to control.

However ACE inhibitory index never reached more than 54 % corresponding to % DH of 36, although % DH and peptide content of protein hydrolysates increases until the end of hydrolysis (Figure 4.2 and 4.3). These results suggest that despite the peptide content increases during the hydrolysis of yeast protein, not all of them contribute equally to ACE inhibitory activity.

Yeast protein hydrolysates generated during hydrolysis by action of papain, pepsin and pancreatin exhibited IC_{50} values of 1.46 ± 0.4, 1.38 ± 0.05 and 2.39 ± 0.11 mg/mL of peptide in assay, respectively (Figure 4.5).

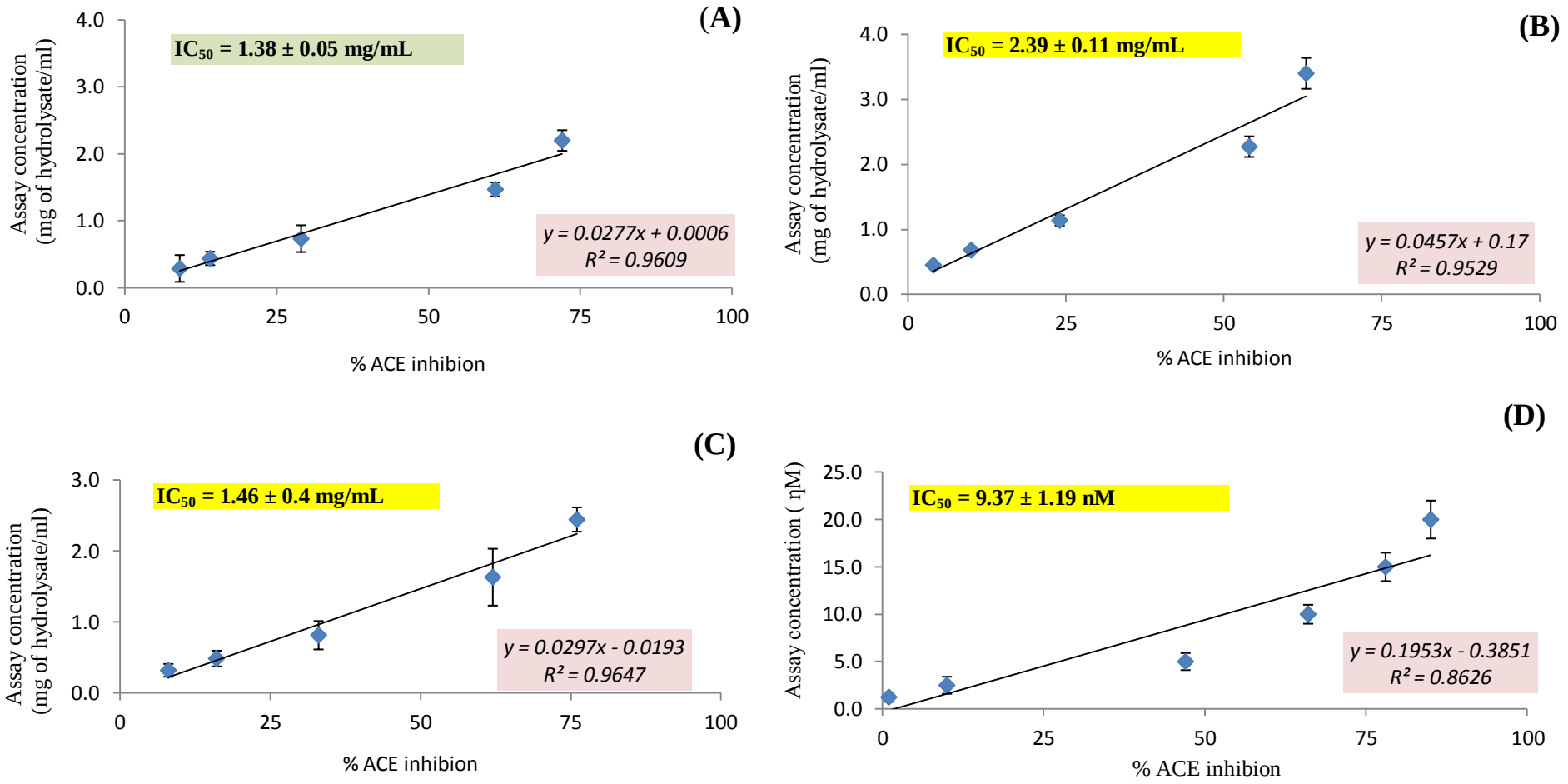


Figure 4.5 The effect of different enzyme treatment on IC_{50} (mg of hydrolysate/mL) value of yeast protein hydrolysate (A): pepsin hydrolysate obtained at end of 4 h, (B): pancreatin hydrolysate obtained at end of 2.5 h, (C): Promod 144P hydrolysate obtained at end of 1.5 h) and Captopril (D) in assay condition with 14.7 mU/mL ACE, 0.78 mM FAPGG in assay solution. All values are means ± SD of quintuplet analyses. IC_{50} is defined as amount of peptide measured in mg hydrolysate/mL that mediated a 50% inhibition of ACE activity.

Although these values are much larger (less potent) than IC_{50} value of captopril 9.37 ± 1.19 nM this is not unexpected as this drug is synthetically designed to inhibit ACE and potential hypotensive activity. The results indicate that hydrolysates from yeast protein have ACE inhibitory activity. Especially, pepsin generated hydrolysates obtained at end of 4 h of hydrolysis were shown to have more potent ACE inhibitory activity than the other hydrolysates obtained after papain and pancreatin hydrolysis of yeast protein. Therefore, pepsin hydrolysate obtained after 4 h hydrolysis was selected for further purification to isolate and identify ACE inhibitory peptides.

4.3.3 Gel filtration chromatography of protein hydrolysate

In this study the most potent hydrolysate (IC_{50}: 1.38 ± 0.05 mg/mL) obtained after incubation of phosphorylated yeast protein with pepsin (4 h) was applied to a gel filtration column (1 cm x 25 cm) at a flow rate of 60 mL/h in 100 mM of volatile ammonium acetate buffer, pH 8.33. The size exclusion profile protein hydrolysate indicates that peptides pools with gradually decreasing molecular masses were eluted later stages of chromatogram (Figure 4.6).

Also different elution volumes constitute pools of peptides with characteristic molecular weights for example, elution volumes between 65 and 75 mL (pool A3) included free amino acids and peptides with molecular masses up to 400 Da. It was observed that the highest ACE inhibitory activity was found in the fractions containing peptides of higher molecular masses which eluted around 50 mL.

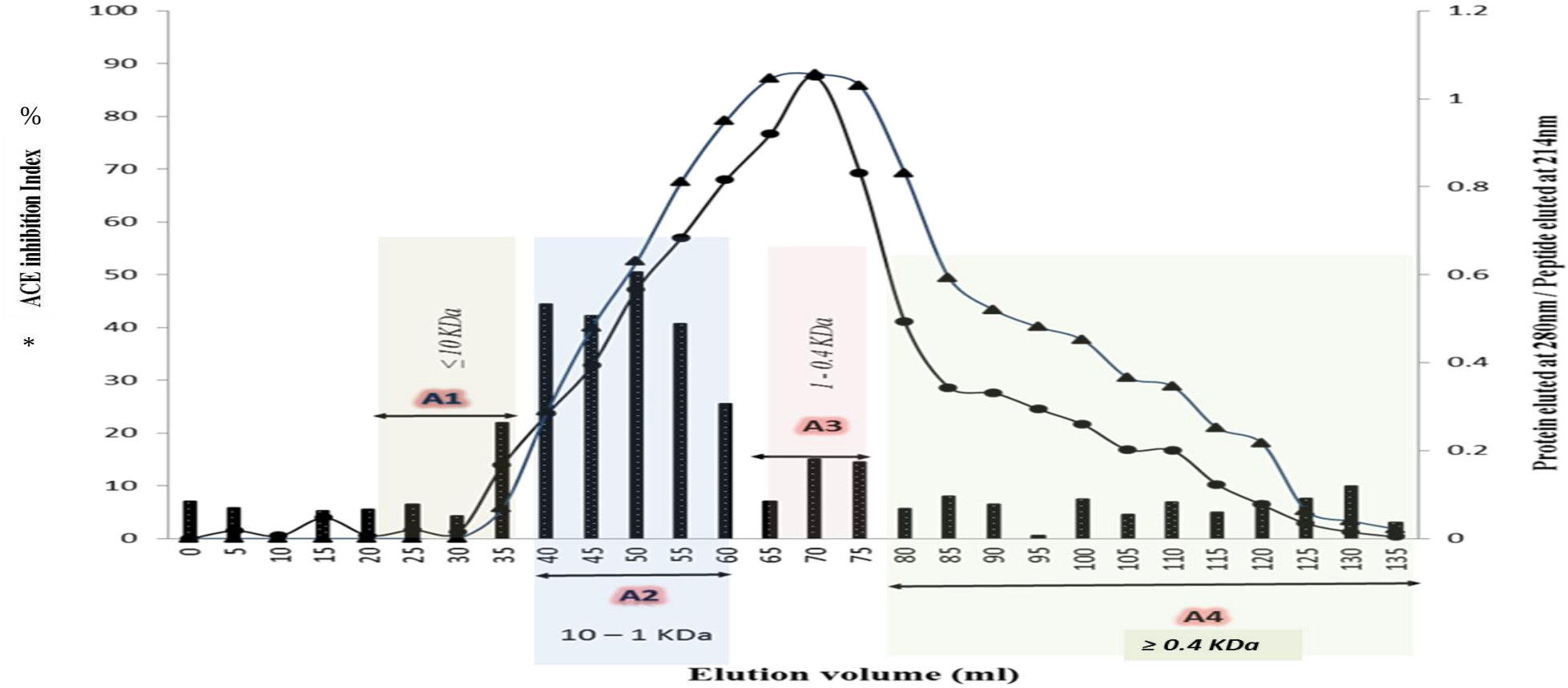


Figure 4.6 Elution profile of a yeast protein hydrolysate obtained after incubation of phosphorylated yeast protein with pepsin (4 h) in a Sephadex G-25 gel filtration column. The protein (●) and peptide (▲) content of elution during gel filtration was monitored at 280 and 214 nm.

***ACE inhibition Index (%) is the value for the percentage ACE inhibition observed based when 500 µg of peptide is added to test reaction compare to control.**

Therefore, peptides corresponding to elution volumes between 40 and 60 mL (pool A2) were pooled and concentrated by freeze drying. This pool A2 was found to possess the ACE inhibitory peptide fraction with an IC_{50} value of 0.30 ± 0.01 mg/mL in final assay volume. This shows that the application of gel filtration technique had successfully enriched the ACE potency to peptide fraction by 4.6 fold. Enrichment resulted from removal of non-ACE inhibitory peptides from the pool. Therefore, this pool with highest ACE potency was subjected to RP-HPLC chromatography for further purification.

4.3.4 Two stage reversed phase HPLC of fractionation of ACE inhibitory potent peptide fraction from gel filtration chromatography

The active pool A2 obtained from gel filtration was further separated by preparative RP-HPLC using the XBridge^{TM} BEH300 Prep C_{18} column. As shown in Figure 4.7, the sample was separated over a 60 minute gradient program. It can be seen from the chromatogram that baseline is rising and falling throughout the gradients suggesting numerous peptides eluting across the gradient. Therefore, the eluent were pooled sequentially in batches corresponding to 10 minute elution periods. So pool A21 (0 -10 min.), pool A22 (10 - 20 min.), pool A23 (20 – 30 min.) etc. (Figure 4.7 and Table 4.3). The ACE inhibition indices of freeze dried concentrated pools were assessed using the same protein concentration for each pool. The ACE inhibition indices were shown in Table 4.3.

Table 4.3 ACE inhibition index of different pools obtained from preparative RP HPLC.

Time interval (min.)	Pool	*ACE inhibition Index	ACE inhibitory potency (IC_{50}) (µg/mL)
0 to 10	**A21**	2.10 ± 0.31	3501.14 ± 28.57
10 to 20	**A22**	13.09 ± 0.27	561.72 ± 4.13
20 to 30	**A23**	14.27 ± 0.07	529.91 ± 4.07
30 to 40	**A24**	19.71 ± 0.53	373.05 ± 2.11
40 to 50	**A25**	***33.59 ± 0.76***	***218.63 ± 1.09***
50 to 60	**A26**	8.07 ± 0.44	911.14 ± 5.21

Results were the means ± SD of triplicates analyses. *ACE inhibition Index is based when 25 µg of peptide is added to test reaction compare to control.

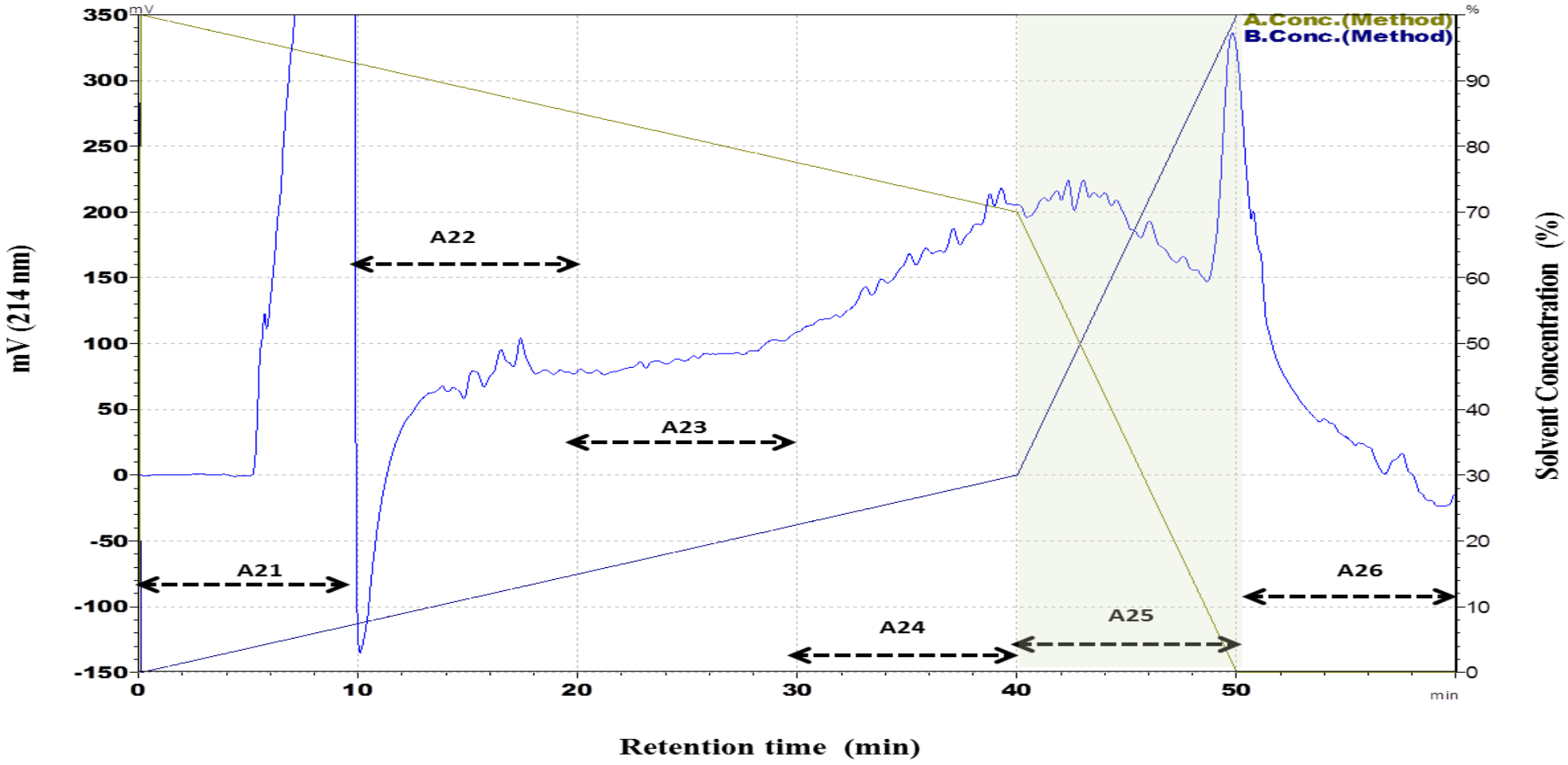


Figure 4.7 Preparative RP-HPLC profile of pool (A2) obtained from gel filtration separated on an XBridgeTM BEH300 Prep C18 column where line represents the gradient of solvent-B (0.1 % trifluoroacetic acid in acetonitrile) and solvent-A (0.1% trifluoroacetic acid in acetonitrile in Milli-Q water).

The pool collected from 40 to 50 minute (A25) displayed the highest ACE inhibitory index and was further analysed to determine its IC_{50} value as 218.63 ± 1.09 µg/mL. This indicated that the application of preparative RP-HPLC had successfully enriched the pool by 6.2 fold based on ACE inhibition. This may be due to removal of peptides that eluted at later stages of separation from the reverse phase columns (corresponding to higher hydrophobicity). The eluted peptides were rich in hydrophobic amino acids, and were responsible for higher ACE inhibitory activity as reported previously for protein hydrolysates (Pedroche *et al.*, 2002, Megaas *et al.*, 2004).

Therefore, this fraction (A25) was further re-chromatographed on to an analytical XBridgeTM BEH300 C_{18} column (3.5 µm, 4.6 mm x 150 mm) using a 60 minute gradient program outlined earlier in Section 4.2.5.2. A chromatogram for Fraction A25 is shown in Figure 4.8 and the ACE inhibition index of the fractions collected at defined time intervals are shown in Table 4.4. These results showed that the peptides present in different pools isolated from analytical RP-HPLC had varying ACE inhibitory activity.

Table 4.4 ACE inhibition index of different pools obtained from analytical RP-HPLC.

Time interval (min)	Pools	*ACE inhibition Index (%)	ACE inhibitory potency (IC_{50}, µg/mL)
20 to 30	**A25a***	13.74 ± 0.57	411.39 ± 3.76
30 to 35	**A25b***	15.91 ± 1.31	389.76 ± 7.13
35 to 40	**A25c***	**31.12 ± 4.93**	**187.98 ± 1.03**
40 to 45	**A25d***	**65.91 ± 5.73**	**88.72 ± 6.32**
45 to 50	**A25e***	19.39 ± 3.59	347.51 ± 9.21

Results were the means ± SD of triplicates analyses. *ACE inhibition Index observed when 20 µg of peptide is added to test reaction compare to control.

Also fraction (A25d) collected from 40 to 45 minute showed the strongest ACE inhibition (65.91 ± 5.73 %) and IC_{50} value of 88.72 ± 6.32 µg/mL. During this period, there were six independent peaks that may correspond to peptide pool with potent ACE inhibitory activity. Therefore, these six peaks after elution from the detector were collected separately.

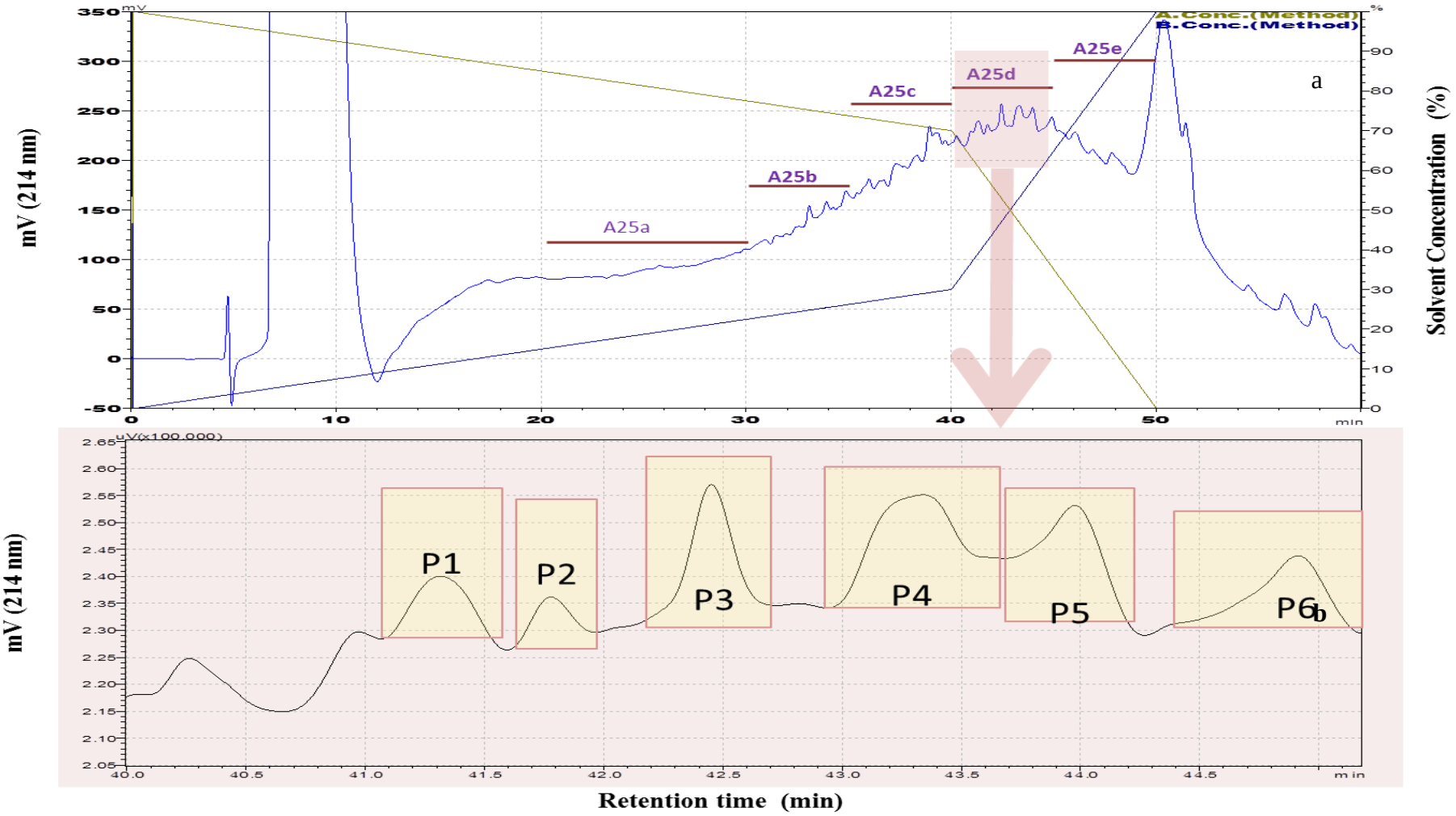


Figure 4.8 Analytical RP-HPLC profile of pool (A25) obtained from preparative RP-HPLC was separated on XBridge™ BEH300 C18 3.5µm 4.6 mm x150 mm column where line represents the gradient of solvent-B (0.1% trifluoroacetic acid in acetonitrile) and solvent-A (0.1 % trifluoroacetic acid in acetonitrile in Milli-Q water).

For this, fractionation was repeated 10 times (Figure 4.1), and collected fractions were pooled, freeze-dried and dissolved in 100 µL of Milli-Q water to a final concentration of 0.50 mg/mL. Their ACE inhibition index (%) were subsequently determined (Table 4.5). It was observed that peaks P3 and P2 showed highest ACE inhibition potency with IC_{50} values of 28.32 ± 1.57 and 19.31 ± 0.55 µg/mL, respectively. This showed that the application of two stages RP-HPLC successfully enriched the ACE inhibitory potency of peptide fractions by 15.5 fold. Therefore, the pools with highest ACE inhibitory potency, i.e., A25d-P2 and A25d-P3 were submitted for identification of peptides by LC-MS-MS method as describe in Section 4.2.6.3**.**

Table 4.5 ACE inhibition index of different peaks pools obtained from analytical RP-HPLC.

Peaks	Pools	*ACE inhibition Index (%)	ACE inhibitory potency (IC_{50}) (µg/mL)
P1	**A25d-P1**	19.19 ± 1.62	61.05 ± 3.21
P2	**A25d-P2**	**51.20 ± 9.87**	**28.32 ± 1.57**
P3	**A25d-P3**	**75.09 ± 2.75**	**19.31 ± 0.55**
P4	**A25d-P4**	11.27 ± 2.07	133.47 ± 2.79
P5	**A25d-P5**	13.72 ± 5.71	110.19 ± 5.26
P6	**A25d-P6**	**ND	

Results were the means ± SD of triplicates analyses.

***ACE inhibition Index observed when 5 µg of peptide is added to test reaction compare to control. **ND, Not Detected**

In relation to the extraction and enrichment of ACE inhibitory peptide from yeast extract, the profile outlined in Figure 4.1 clearly overviews this progress. As pepsin hydrolysates liberated latent ACE inhibitory activities, they were subsequently enriched by utilization of various chromatography steps. This enrichment is evident from the decreasing IC_{50} values recorded for pooled fractions. Altogether a maximum 71 fold increase in potency was recorded.

4.3.5 Peptide identification by LC-MS-MS and data analysis

The peptides present in the A25D-P2 and A25D-P3 were analysed using electrospray Q-Exactive Quadrupole Orbitrap coupled to a nano-ultra performance liquid chromatography system. The obtained chromatograms are shown in Figure 4.9 and 4.10.

The Tables 4.6 and 4.7 list characterise amino acid sequences, positions of identified peptides in the source protein, the observed masses to charge ratio (m/z), peptide charge states, retention time and the *in silico* generated amino acid (one letter code) sequence. The theoretical amino acid sequence was also obtained using Expert Protein Analysis System (ExPASy) peptide cutter tool with pepsin (pH 1.3) for assessment of sequence obtained by LC-MS-MS analysis.

It was found that 25 and 30 different peptides were identified in the potent ACE inhibitory pools A25d-P2 and A25d-P3, respectively from yeast source proteins of *Kluyveromyces marxianus* using the Uniprot database. The mean number of amino acid residue per peptide ranged between 5 and 15. It was found that the identified source proteins from which the peptides were liberated by pepsin were alcohol dehydrogenase-2, pyruvate decarboxylase, glyceraldehyde-3-phosphate-dehydrogenase-1, glyceraldehyde-3-phosphate-dehydrogenase-2, heat shock protein SSB, triosephosphate isomerase, 40S ribosomal protein S28 and 3-isopropylmalate dehydrogenase as shown in Table 4.6 and 4.7.

It was observed that the molecular weight of peptides identified in fraction A25d P-2 and A25d P-3 ranges between 788.44 and 2,353.72 Da, with their median value of 1,165.42 Da.

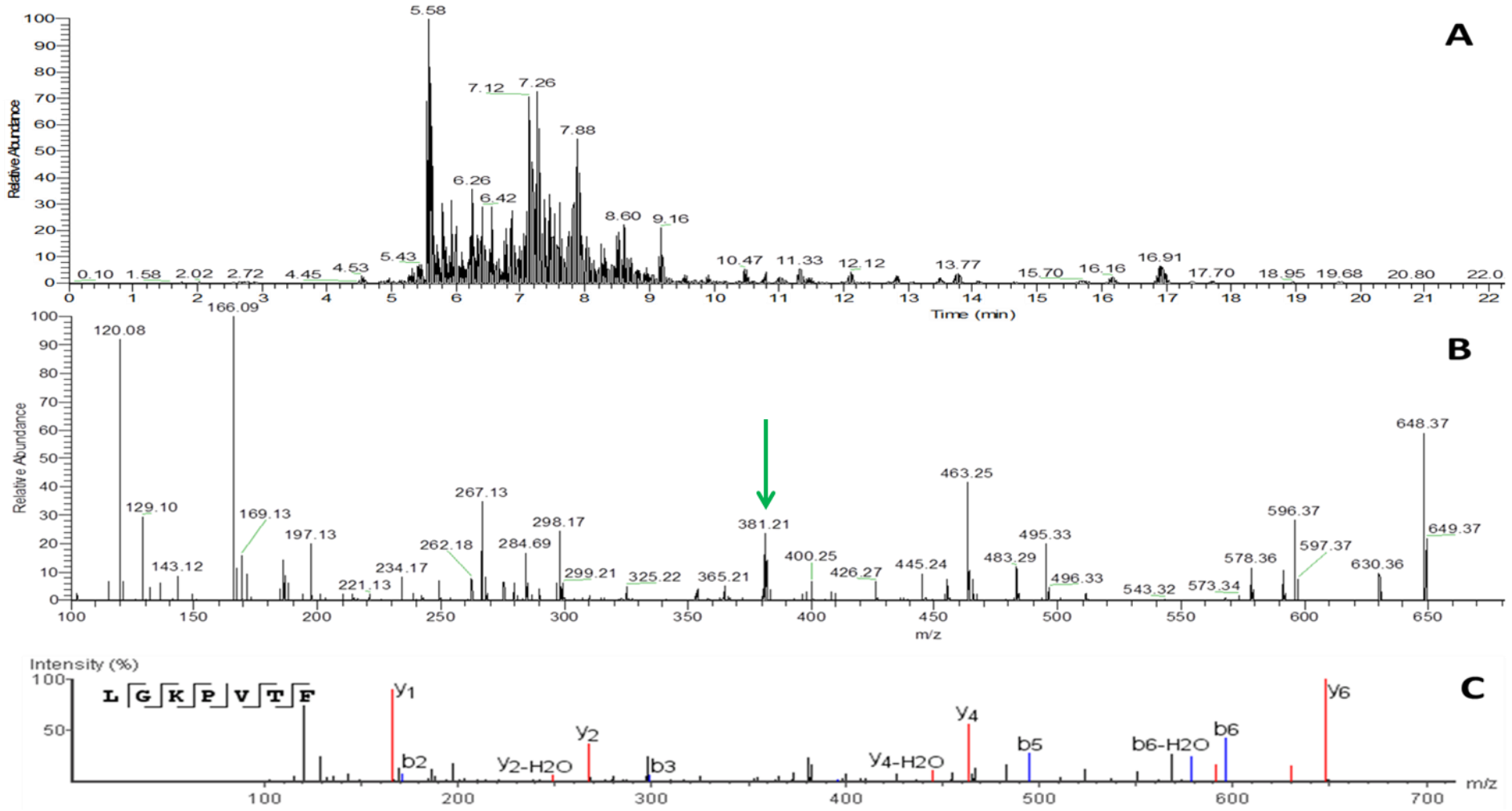


Figure 4.9 Identification of an ACE inhibitory peptide within peak pool A25d-P2. **(A)** Shows base peak chromatogram of peptide peak pool A25d- P2 obtained using RP-nano-UPLC of Q Exactive mass spectrometer, it shows the presences of numerous high intensity spectrums which confirm existence of low abundance peptides in pooled peak P2. **(B)** Demonstrates low intensity mass spectrum with RT: 10.06 min comprises of interesting precursor ions selected for fragmentation (indicated green arrow). **(C)** The collision induced fragmentation of m/z 381.2 is illustrated. For clarity, only b (N-terminal) and y (C-terminal) fragmented ions are labeled. On basis of fragment ions observed, peptide was sequenced using de novo algorithm using PEAK 5.2 software. With 100 % of average local confidence (ALC), doubly charged peptide **LGKPVTF** was identified and sequenced. The N-terminal amino acid was leucine and aromatic amino acid phenyl alanine at C terminus.

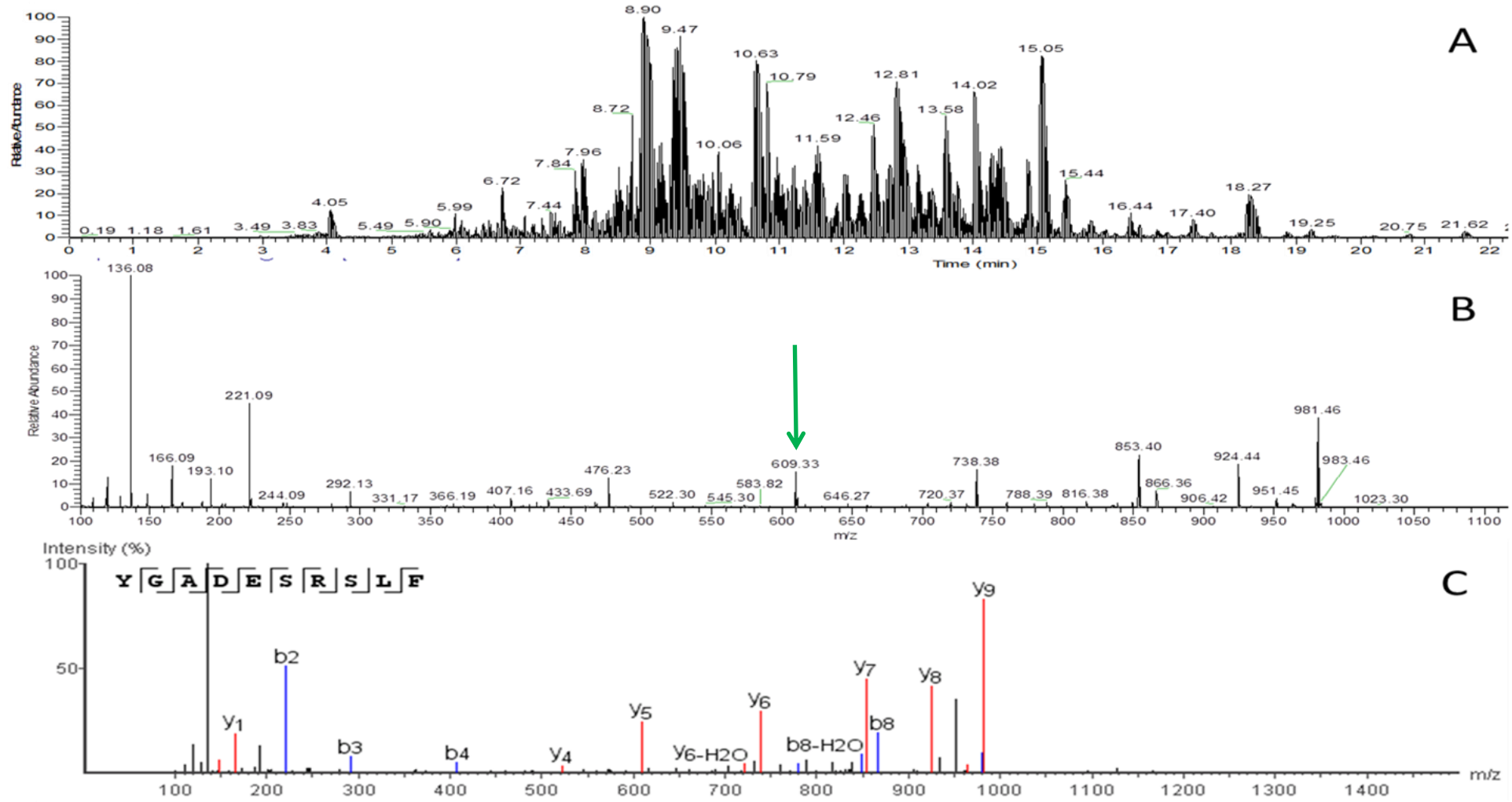


Figure 4.10 Identification of an ACE inhibitory peptide within peak pool A25d-P3. **(A)** Shows base peak chromatogram of peptide peak pool A25d- P3 obtained using RP-nano-UPLC of Q Exactive mass spectrometer, it shows the presences of numerous high intensity spectrums with retention between 9 and 15 min which confirm complexity of pooled peak P3 with existence of low abundance peptides in. **(B)** Demonstrates low intensity mass spectrum with RT: 10.87 min comprises of interesting precursor ions selected for fragmentation (indicated green arrow). **(C)** The collision induced fragmentation of m/z 609.33 is illustrated. For clarity, only b (N-terminal) and y (C-terminal) fragmented ions are labeled. On the basis of the fragment ions observed. One decapeptide was sequenced by de novo algorithm was using PEAK 5.2 software. The sequence was shown to be **YGADESRLSLF**.

Also for each protein, little variation was found between peptide sequences obtained from MS spectra and the *in silico* peptide sequences using ExPASy peptide cutter tool for pepsin at pH 1.3 (Table 4.6 and 4.7). Therefore, prominent mass spectrometry spectra that do not match with a significant peptide hit in the ExPASy were then theoretically sequenced with the *de novo* algorithm of PEAK 5.3 proteomic software tool which permits identification of the peptides from variable protein mixtures such as spent yeast protein.

In the present study only high confidence rated peptide sequences obtained by the *de novo* algorithm were selected with average local confidence (ALC) of 99 %. The ALC confirms the percentage of correct amino acids in the *de novo* sequence. Table 4.8 lists the amino acid sequences of the peptide present in A25d P-2 and A25d P-3 obtained by *de novo* algorithm with PEAK 5.3 software. It was observed that in total 24 peptides were present in fractions A25d P-2 and A25d P-3 with median molecular weight of about 1,111.69 Da.

4.3.6 Basic ACE structure and inhibition

ACE is a zinc-containing peptidyl dipeptide hydrolase. The active site of ACE is known to consist of three parts: a carboxylate binding functionality such as the guanidinium group of arginine, a pocket that exhibited a preference for hydrophobic side chain of C-terminal amino acid residues and zinc ion. The zinc ion coordinates to the carbonyl of the penultimate peptide bond of the substrate, whereby the carbonyl group becomes polarized and is subjected to a nucleophilic attack (Strittmatter and Snyder, 1986). Furthermore, the kinetic studies had reported that competitive inhibition or mixed type inhibition was observed depending on the type of ACE inhibitory peptides (Udenigwe *et al.*, 2009, Ahhmed and Muguruma, 2010).

A common sequence for ACE inhibition among the ACE inhibitory peptides has not been identified and reported. However, in general the most potent ACE inhibitory peptides reported mostly contain tryptophan (W), phenylalanine (F) and proline (P) at the C-terminus. It has also been suggested that the presence of the amino acid leucine (L) at N-terminus also contributed significantly to an increased ACE-inhibitory activity (Ruiz *et al.*, 2004). Furthermore, other branched chain aliphatic amino acids such as isoleucine (I) and valine (V) were

also found predominantly in high ACE inhibitory potent peptides (Hernández-Ledesma *et al.*, 2011).

Table 4.6 Details of the probable peptides identified from RP-HPLC pool A25d-Peak 2 generated from pepsin hydrolysate by LC-MS-MS, linked to search Engine PEAKS 5.3 with Swiss Uniprot data base. The sequence of various peptides identified and their position in yeast source proteins (SwissProt accession number) with the observed masses to charge ratio (m/z), peptide charge states (Z), retention time (RT) and the in silico generated amino acid (one letter code) sequence are shown.

Parent protein and accession number (UniProtKB/Swiss-Prot)	**Position**	**m/z**	**z**	**PEAK DB sequence[2]**	**RT**	***In silico* sequences**
Alcohol dehydrogenase 2 (Q9P4C2)	196-210	491.2621	3 (+)	RVLGIDGGEGKEEL	10.861	GIDGGEGKEEL
	187-196	543.2754	2 (+)	AVQYAKAMGF	12.903	AVQYAKAMGF
	211-219	492.2635	2 (+)	FKSLGGEVF	13.594	GGEVF
	211-219	418.729	2 (+)	KSLGGEVF	10.706	GGEVF
	20-34	597.6608	3 (+)	YYKDIPVPKPKSNEL	10.611	YYKDIPVPKPKSNEL
Pyruvate decarboxylase (P33149)	187-201	777.8837	2 (+)	SLKPNDPEAENEVL	11.122	KPNDPEAENEVL
	208-217	555.8477	2 (+)	IKDAKNPVIL	9.276	IKDAKNPVIL
	208-217	370.9009	3 (+)	IKDAKNPVIL	9.322	IKDAKNPVIL
	386-393	813.3608	1 (+)	TETGTSAF	8.679	TETGTSAF
	206-217	451.6098	3 (+)	ELIKDAKNPVIL	12.467	IKDAKNPVIL
	293-304	609.3292	2 (+)	DAAAGIQLSPKF	14.417	DAAAGIQL
	284-292	495.7479	2 (+)	LGDTHSTIF	11.688	GDTHSTIF
	66-75	363.5654	3 (+)	IINGKKVAVF	9.867	IINGKKVAVF
Glyceraldehyde-3-phosphate dehydrogenase 2 (Q01077)	284-292	488.7403	2 (+)	LGDTHSSIF	11.596	GDTHSSIF
	66-75	566.3607	2 (+)	IIDGKKVLVF	13.249	IIDGKKVL
	66-75	377.9092	3 (+)	IIDGKKVLVF	13.266	IIDGKKVL
Triosephosphate isomerase (Q70JN8)	231-240	502.7733	2 (+)	VGGASLKPEF	11.389	VGGASL
	116-125	507.8133	2 (+)	ALDSGVKVIL	14.339	DSGVKVIL
	241-248	458.2666	2 (+)	VDIINSRV	10.667	VDIINSRV
	141-151	641.877	2 (+)	EVVQRQLQAVL	13.406	EVVQRQL
Heat shock protein SSB (P41770)	603-613	416.9103	3 (+)	KRAVTKAMSTR	3.931	KRAVTKAMSTR
	41-52	531.2848	2 (+)	VAFTPEERL	12.17	TPEERL
	47-52	372.697	2 (+)	TPEERL	6.826	TPEERL
40S ribosomal protein S28 (P33286)	57-66	638.8224	2 (+)	MESEREARRL	5.974	MESEREARRL
	57-66	426.2179	3 (+)	MESEREARRL	5.969	MESEREARRL

[2] **Each peptide targeted for protein identification as per software manufactures guideline only peptide score that corresponded to false discovery rate (FDR) of ≤ 1% were accepted from the Peaks database search. False discovery rate (FDR) is probabilistic tool that indicates the identified peptide-spectrum matches are correct.**

*Note: On the basis reported of relationship between peptide structure and it ACE inhibitory activity, potential peptides, active amino motif in sequences and presence of **F**, **W**, **L**, **V** and **P** amino acid residues at C-terminus of peptides were coloured in red* (Murray *et al.*, 2005, Hernández-Ledesma *et al.*, 2011).

Table 4.7 Details of the probable peptides identified from RP-HPLC pool A25d-Peak 3 generated from pepsin hydrolysate by LC-MS-MS, linked to search Engine PEAKS 5.3 with Swiss Uniprot data base. The sequence of various peptides identified and their position in yeast source proteins (SwissProt accession number) with the observed masses to charge ratio (m/z), peptide charge states (Z), retention time (RT) and the in silico generated amino acid (one letter code) sequence are shown.

Parent protein and accession number (UniProtKB/Swiss-Prot)	Position	m/z	z	PEAK DB sequence[2]	RT	*In silico* sequences
Alcohol dehydrogenase 2 (Q9P4C2)	20-35	952.5275	2 (+)	YYKDIPVPKPKSNELL	11.688	YYKDIPVPKPKSNEL
	20-34	895.9897	2 (+)	YYKDIPVPKPKSNEL	9.932	YYKDIPVPKPKSNEL
	197-210	736.3913	2 (+)	RVLGIDGGEGKEEL	9.969	GIDGGEGKEEL
Pyruvate decarboxylase (P33149)	541-555	805.9323	2 (+)	PVMDAPSNLVKQAQL	15.683	VKQAQL
	556-564	481.2512	2 (+)	TASINAKQE	4.164	TASINAKQE
	521-535	569.9849	3 (+)	TTDKKFQENSKIRL	7.226	QENSKIRL
	172-180	471.2866	2 (+)	VDLKVPASL	13.651	KVPASL
	206-217	676.9109	2 (+)	ELIKDAKNPVIL	11.912	IKDAKNPVIL
	208-217	555.8475	2 (+)	IKDAKNPVIL	8.411	IKDAKNPVIL
	188-201	777.8833	2 (+)	SLKPNDPEAENEVL	10.512	KPNDPEAENEVL
Glyceraldehyde-3-phosphate dehydrogenase 1 (P84998)	293-304	609.3312	2 (+)	DAAAGIQLSPKF	14.842	SPKF
	66-75	544.8445	2 (+)	IINGKKVAVF	8.762	IINGKKVAVF
	286-292	495.7468	2 (+)	LGDTHSTIF	10.745	GDTHSTIF
Glyceraldehyde-3-phosphate dehydrogenase 2 (Q01077)	293-304	609.3118	2 (+)	DAAAGIQLSPQF	17.783	SPQF
	66-75	566.3598	2 (+)	IIDGKKVLVF	12.689	IIDGKKVL
Triosephosphate isomerase (Q70JN8)	231-240	502.774	2 (+)	VGGASLKPEF	10.514	KPEF
	221-230	554.2881	2 (+)	KDKADVDGFL	10.008	KDKADVDGF
	241-248	458.2667	2 (+)	VDIINSRV	9.868	VDIINSRV
	118-125	415.7527	2 (+)	DSGVKVIL	11.971	DSGVKVIL
	132-140	523.2983	2 (+)	EEKQKGITL	7.081	EEKQKGITL
	141-151	641.8772	2 (+)	EVVQRQLQAVL	13.166	EVVQRQL
Heat shock protein SSB (P41770)	603-613	416.9111	3 (+)	KRAVTKAMSTR	2.83	KRAVTKAMSTR
	464-478	760.4117	2 (+)	DLKGVPPMPAGEPVL	17.538	KGVPPMPAGEPVL
	44-52	531.2851	2 (+)	VAFTPEERL	11.355	TPEERL
	563-578	566.6841	3 (+)	SAKIKRNAKAKVEAAL	5.807	SAKIKRNAKAKVEAAL
	241-248	525.7664	2 (+)	LEHFKTEF	9.254	KTEF
40S ribosomal protein S28 (P33286)	33-54	827.7977	3 (+)	LEDTTRTIVRNVKGPVREGDIL	11.738	EDTTRTIVRNVKGPVREGDIL
3-isopropylmalate dehydrogenase (P41766)	113-127	840.9265	2 (+)	DLSPLKPEYAKGTDF	13.307	KPEYAKGTDF
	26-37	666.8677	2 (+)	NAISEARPSIKF	10.964	NAISEARPSIKF
	172-185	743.4147	2 (+)	ALQHNPPLPIWSL	20.623	PIWSL

[2] **Each peptide targeted for protein identification as per software manufactures guideline only peptide score that corresponded to false discovery rate (FDR) of ≤ 1% were accepted from the Peaks database search. False discovery rate (FDR) is probabilistic tool that indicates the identified peptide-spectrum matches are correct.**

Note: On the basis reported of relationship between peptide structure and its ACE inhibitory activity, potential peptides, active amino motif in sequences and presence of ***F****,* ***W****,* ***L****,* ***V*** *and* ***P*** *amino acid residues at C-terminus of peptides were coloured in red* (Murray *et al.*, 2005, Hernández-Ledesma *et al.*, 2011)

Table 4.8. Details of the probable peptides identified from two pools obtained by RP-HPLC, generated from pepsin hydrolysate using theoretical de novo algorithm of PEAKS 5.3 software tool. The sequence of various peptides identified, observed masses to charge ratio (m/z), peptide charge states (Z), retention time (RT) and % average local confidence (ALC) are shown.

Fraction	De novo sequences obtained from PEAK 5.2 software tool[1]	m/z*	Z[1]	RT[2]	ALC (%)
FractionA25d-P-2	**LGKPVTF**	381.2308	2 (+)	10.069	100
	VVAKAEKL	429.2748	2 (+)	6.045	99
	DEKLNVNSKTKDL	752.4024	2 (+)	7.44	99
	AHVFNNHESL	584.2797	2 (+)	6.621	99
	GVVKVNL	364.7366	3 (+)	10.673	99
	AKAEGKEKPGF	581.3145	2 (+)	5.352	99
	DEKLNVNSKTKDL	501.9379	3 (+)	7.415	99
	EKLNVNSKTKDL	694.8893	2 (+)	6.721	99
Fraction A25d-P-3	LLGKPVTF	437.7739	2 (+)	11.968	99
	YGADESRSLF	609.3312	2 (+)	10.877	99
	VAKFHLDKL	535.8209	2 (+)	8.884	99
	LSKLLGKPVTF	401.5887	3 (+)	11.972	99
	KAVLEGKYDHLPENAF	915.973	2 (+)	11.459	99
	EGLGAKLVEL	514.8027	2 (+)	12.417	99
	LKEHNVKPDLL	653.3795	2 (+)	7.506	99
	YAKNKDALAQGHDLF	845.9317	2 (+)	8.779	99
	VVEEKTLF	482.771	2 (+)	10.173	99
	LAYGLDKKGSSEHNVL	865.9568	2 (+)	8.545	99
	LGVEVHPLAF	541.3058	2 (+)	17.031	99
	PTYVKSHPSLF	425.895	3 (+)	9.358	99
	FSELTKTTPEKSLL	797.4399	2 (+)	11.184	99
	AVAKFHLDKL	381.2285	3 (+)	9.329	99
	VAKFHLDKL	357.5493	3 (+)	8.88	99
	FKSMLEANKF	607.8162	2 (+)	10.301	99

[1] The de novo sequences results obtained by Peaks Studio 3 were filtered using an average local confidence (ALC) of ≥ 65 %, a total local confidence of (TLC) of ≥ 6 and peptide score (-10lgP) of ≥ 15. ALC and TCL specify confidence that the correct amino acid residue in each position has been identified.

*Note: On the basis reported of relationship between peptide structure and its ACE inhibitory activity, potential peptides, active amino motif in sequences and presence of **F**, **W**, **L**, **V** and **P** amino acid residues at C-terminus of peptides were coloured in red* (Murray *et al.*, 2005, Hernández-Ledesma *et al.*, 2011).

In addition, this structure–activity data has suggested that the positive charge amino acids such as lysine (K) and arginine (R) with respective ε-amino and guanidine group as the C-terminal residue may contribute to the inhibitory potency (Ondetti *et al.,* 1977, Ariyoshi, 1993).

For long chain peptides, presence of amino acid sequences rich in hydrophobic amino acids constitutes an active motif. The type of amino acid present in the vicinity of the active motif influences and contributes to the overall ACE inhibitory activity (Terashima *et al.,* 2011).

In the present study on the basis of relationship between peptide structure and the ACE inhibitory activity, four peptide sequences were identified in A25d-P2 and A25d-P3. These were FKSLGGEVF (*f*211 – 219) of alcohol dehydrogenase 2, IKDAKNPVIL (*f*208 – 217), VDLKVPASLL (*f*172 – 187) of pyruvate decarboxylase and IIDGKKVLVF (*f*66 - 75) of glyceraldehyde-3-phosphate dehydrogenase 2 (Table 4.6 and 4.7). These contain a high content of amino acids known to contribute to ACE inhibition (Figure 4.11 Hernández-Ledesma *et al.,* 2011). Likewise, amino acid residues that are responsible for ACE inhibitory activity of peptides were also found in *de novo* sequences such LGKPVTF, LLGKPVTF, LSKLLGKPVTF and GVVKVNL which were identified in fraction A25d-P2 and A25d-3.

In a recent study by Terashima *et al.,* 2011 the chicken myosin derived decapeptide VTVNPYKWLP (IC_{50} : 5.5 μM) had an active amino acid motif of KW which was identified as important for ACE inhibitory activity of the decapeptide. Moreover, the amino acids neighbouring to the KW may effect the inhibitory potency of the peptide. Particularly, the presence of a proline residue at the C-terminal end strongly enhanced ACE inhibition. In the same study, it was also observed that the IC_{50} value of KWLP (5.5 μM) was same as that of ACE inhibitory peptide (VTVNPYKWLP).Whereas, the IC_{50} value of KW was higher as 7.8 μM.

Thus it may be possible that the overall potent ACE inhibitory activity of a large peptide identified in present study is due to the presence of characteristic small amino acid motifs in entire peptide length. This was further supported by report that longer peptides generated during enzymatic hydrolysis undergo further

hydrolysis *in vivo* that might yield small peptide fragments that have higher ACE inhibitory activity than the respective parent peptides (Fujita *et al.,* 2000).

Therefore when all the above mentioned peptides sequences of the present study were probed for active amino acid motifs that are known to be associated with potent ACE inhibitory activity using the Biopep database (Iwaniak *et al.,* 2005), it was found that large peptide sequence obtained in present study consist of several small dipeptides and tripeptides motif such as DA, DG, EV, GE, GG, GK, GKP, GV, KP, LG, TF, VF, VK, VKV, VP and VV (Murray *et al.,* 2005, Hernández-Ledesma *et al.,* 2011, Biopep data base, http://www.uwm.edu.pl/biochemia/).

In the present study, the presence of amino acid residues which were reported to be present in many potent ACE inhibitory peptides were profiled for all probable peptide sequences identified by LC-MS-MS for fraction A25d-P1 and A25d-P2 (Figure 4.11). It was observed that most of the peptides identified in both pools contained more than 50 % of hydrophobic amino acid residues with presence of active ACE inhibitory amino acid motifs. Profile wheel A & C for peptides identified in fraction A25d-P2 suggests that on average 55 % and 15 % of total peptide chain length of peptides were made up of hydrophobic amino acid and aromatic acid residues, respectively. Similarly on an average total peptide chain length in fraction A25d-P3 was made up of 63 % and 24 % of hydrophobic amino acid and aromatic amino acid residues, respectively.

Overall, profile wheel demonstrates that pool A25d-P3 contains more total potent amino acid residue than pool A25d-P2. This was reflected in their ACE inhibitory potency (Table 4.5)

----Total potent amino acid residue, ---- Hydrophobic amino acid residue, ---- Aromatic amino acid residue

(All values were express as % of peptide chain length)

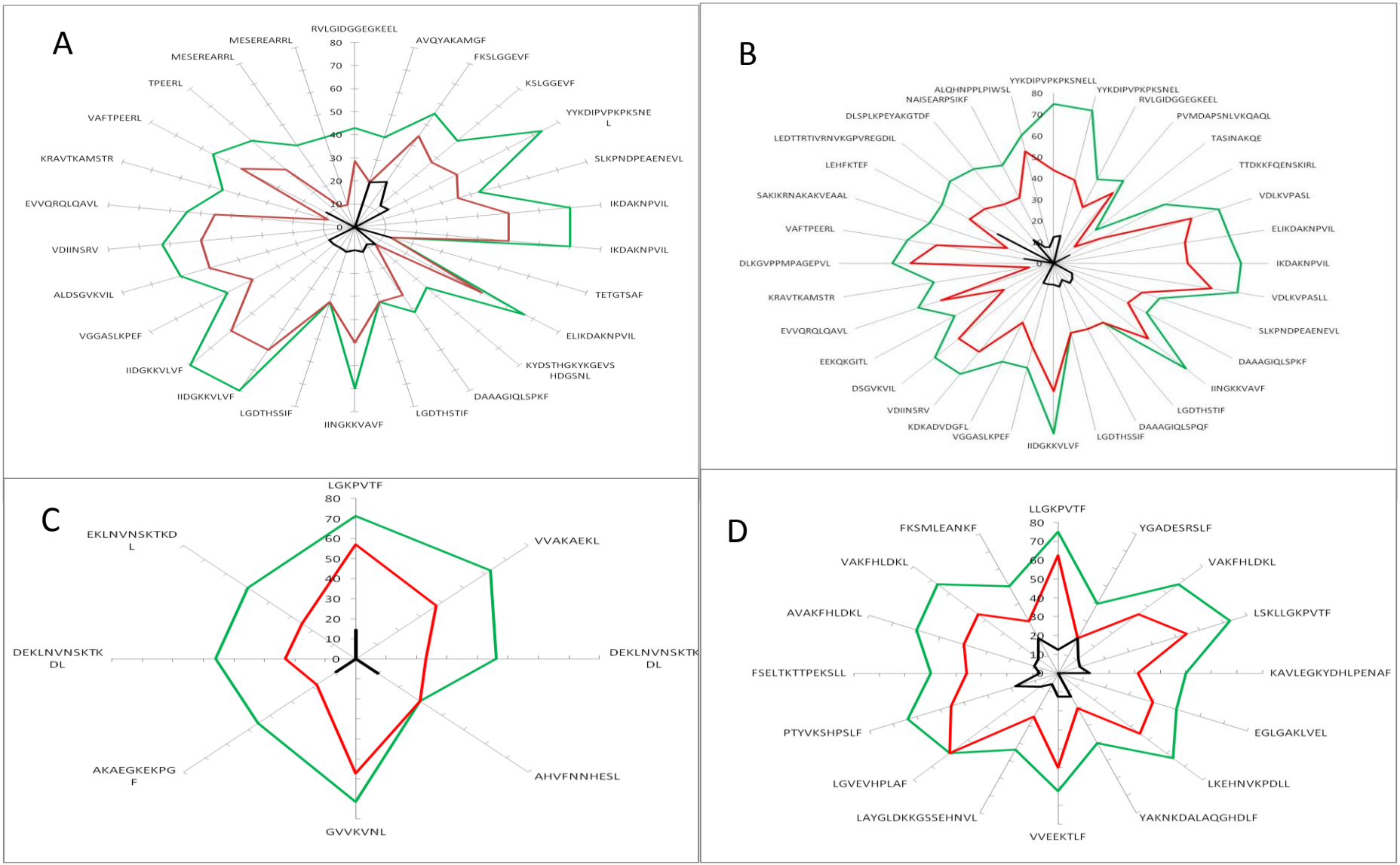


Figure 4.11 Profile wheel of type of amino acid residue present in all probable peptide sequences identified by LC-MS-MS for fraction A25d-P2 (A&C) and A25d-P3 (B&D).

Of all the peptides identified, the heptapeptide identified by LCMS with sequence of LGKPVTF and having lowest molecular weight of 762.46 Da, was found to have optimum structure and attributes to be a potent ACE inhibitory peptide with presence of branched chain amino acid Leu at N-terminus and aromatic amino acid Phe at C-terminal. Also more than half of amino acid residues of LGKPVTF, Phe, Leu, Pro, Ile and Val were hydrophobic (Figure 4.11). These compositional features of heptapeptide suggested that it may possess potent ACE inhibitory activity and requires further study to establish its potency (IC_{50}) and confirm the speculated structural credibility.

While molecular mass data obtained from the mass spectrophotometry analysis has identified the presence of a heptapeptide and a decapeptide within ACE inhibitory fractions with structural motifs akin to known ACE inhibitory peptides, further confirmatory analyses are required. Such analyses will require the reapplication of standard synthetic peptides LGKPVTE and YGADESRSLF to the LCMS system. Additionally the IC_{50} of these peptides on a molar basis would also need to be assessed by the standard ACE assay applied within this project.

4.4 Conclusion

The results obtained in this work show that ACE inhibitory protein hydrolysates can be produced by treating spent yeast protein (*Kluyveromyces marxianus*) with pepsin, pancreatin and papain. The pepsin digest obtained at the end of 4 h of hydrolysis showed a highest ACE potency with IC_{50} value of 1374 ± 5.00 µg/mL. Moreover, purification of pepsin digested yeast protein hydrolysates with gel filtration, two stage reversed phase HPLC had successfully enriched hydrolysate by up to 71 fold to yield very potent ACE inhibitory peptide mixture with IC_{50} value of 19.31 ± 0.55 and 28.32 ± 1.57 µg/mL. This inhibitory activity was comparable to values reported for a variety of ACE inhibitory food protein hydrolysates (Murray *et al.,* 2005).

The peptide content of these potent fractions were further characterized by electrospray LC-MS-MS that resulted into large number of peptides with peptide

sequence shown to have amino acid residues and active amino acid motifs that are previously reported in ACE inhibitory peptide (Murray *et al.,* 2005).

Among all peptide sequences obtained by LC-MS-MS sequencing, a novel heptapeptide LGKPVTF with structural feature to have potent ACE inhibitory activity has been identified. However, further work will be of interest for its synthesis to established its potency (IC_{50}) and confirm the speculated structural credibility. This peptide has been associated with glycine dehydrogenase. This cell wall enzyme is present in relatively large amount in *Kluyveromyces marxianus* species.

Human trials would need to be performed to establish the *in vivo* antihypertensive activity of the hydrolysates obtained in present study. Accordingly their application into food and feed system would be established.

Due to increasing concerns about the safety of synthetic antihypertensive drugs, yeast protein hydrolysates obtained in present study represent a novel source of natural ACE inhibitory hydrolysates with their potential use in food products.

Chapter 5

General conclusions and future perspective

5.1 General conclusion

There exists a sustainable availability of around 5500 - 6000 tonnes of spent Kluyveromyces marxianus yeast per annum from Irish Dairy Industry. This spent dairy yeast biomass was analysed for the composition to estimate its value to be used as a starting material for research. ***This work ascertain that spent Kluyveromyces marxianus yeast have 43.16 % crude protein, 34.56 % total carbohydrate, 20.97 % β-glucan, 9.76 % mannan, 7.02 % RNA, 1.21 % chitin and 13.12 % ash content on a dry matter basis***. Despite the fact that, this spent *Kluyveromyces marxianus* yeast contains valuable constituents, it is currently used as low value unprocessed animal feed after heat inactivation. This is a missed opportunity to innovate, add value and enhance the commercial potential of this large and sustainable Irish waste stream.

Therefore a lab based autolytic and chemo-enzymatic extraction methods were developed to produce value added yeast extract, protein hydrolysates, and different glucan preparations with characterised functional and biological activity and potential significant commercial value. The overall objective based conclusions from this thesis are:

5.2 Study to develop a process to generate yeast extracts with unique sensory attributes

As discussed in chapter 2.0, this study was done to determine and described the sensory and chemical attributes of the yeast extract that were arose following different autolytic treatments at different pH and temperatures over a defined period of time. This was assumed that pH and temperature of autolysis may influence the generation of flavour precursors like peptides, amino acids, sugars and nucleotides in yeast extract. These flavour precursors, during autolysis may undergo Maillard reaction to yield flavoured yeast extract with quantifiable chemical and sensory attributes. For this study with help of external sensory panel, the odour, flavour, and taste descriptors for yeast extract were developed. Thereafter, yeast extract with highest acceptability score were analysed for sensory attributes and amino acid composition.

It was found that the spent Kluyveromyces marxianus yeast autolysis process developed at 50 ˚C, pH 7.0 for 72 h produces yeast extract with significantly

higher (p < 0.05) savoury attributes with 80 % product yield. This is deemed to be suitable for food applications (Figure 5.1).

A yeast extract with a less acceptable sensory attributes was generated and identified to be ideal for microbial media applications. This possessed higher content of free amino nitrogen (6.12 % w/w) and was produced by autolysing spent Kluyveromyces marxianus yeast at 40 ˚C, pH 5.5 for 72 h (Figure 5.1).

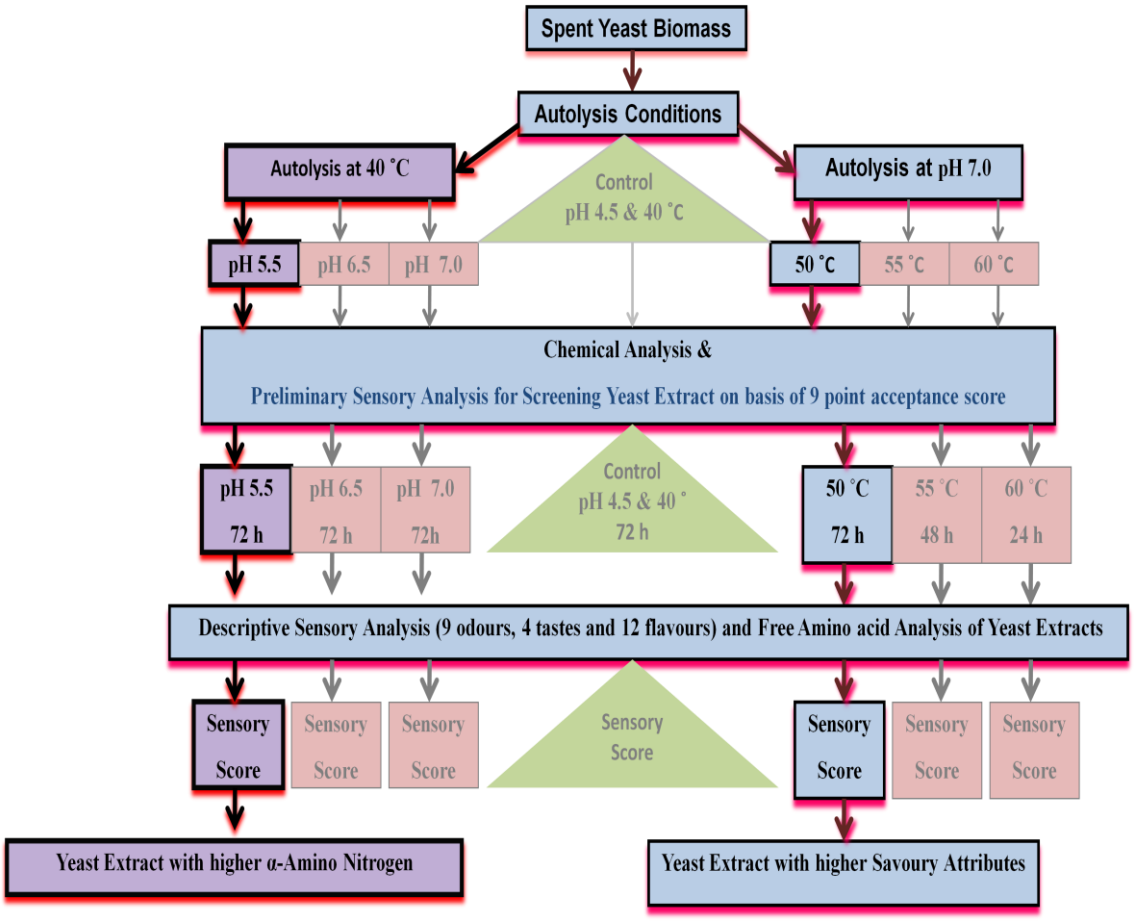


Figure 5.1 Overview of optimum experimental conditions for production of yeast extract with significant higher savoury attributes and higher α-amino nitrogen content.

Most of the prior studies were primarily focused on chemical characterisation of yeast extract. It was found that yeast extract obtained after autolysing spent yeast had the highest level of α amino nitrogen at pH 5.5 (Champagne *et al.*, 1999) and 50˚C (Tanguler and Erten, 2008). Moreover, pH and temperature of autolysis also influenced the general composition of RNA degradation products in yeast extract (Zhao, J and Fleet, G, 2005). However, there is no information

available on the influence of different pH and temperature conditions of autolysis on sensory descriptive score and acceptability of yeast extract produced.

The application of descriptive sensory analysis in current study to monitor the effect of pH and temperature was a novel approach. This provides the insight into the effect of autolysis conditions on sensory attributes of yeast extract as a result of difference in chemical compositions of yeast extract produced. For example, shifting the pH of autolysis from 5.5 towards 7.0 may enable the production of yeast extract with limited free amino nitrogen and more total solids with higher sensory acceptability. On the other hand, a temperature shift towards 50 °C during incubation may result in yeast extract with higher acidic and basic free amino acid with significantly higher savoury attributes yeast extract as desired for savoury application.

Moreover, this study also confirms one of the hypothesis of the study that various pH values and temperatures of autolysis have an affect on the yield and sensory attributes of yeast extract produced.

5.2.1 Future outlook and practical perspective

As a continuation of this work the proposed lab based process outlined in the current study would need further research to produce flavored yeast extract with desirable sensory attributes in lesser time.

This work has demonstrated that specific interactions between pH and temperature of autolysis exists that influences the chemical composition and sensory attributes of yeast extracts. This may be exploited to produce tailor made yeast extracts by choosing specific autolysis conditions.

5.3 Study to develop a process to generate a glucan enriched product(s) with characterized immunomodulatory activities

As discussed in Chapter 3.0, the cell wall residues obtained at end of the autolysis and protein extraction process were subsequently subjected to a hot water and protease treatment. The extracted protein from the cell wall residues

were subsequently acid precipitated to yield mannoprotein sediments and non precipitate secondary yeast extract.

The β-glucan enriched product obtained at the end of the hot water and protease treatment were subjected to a multi-step acid and alkali treatment to produce alkali insoluble, acid insoluble β-glucan particulates (PβG). Moreover, this PβG was made into different soluble β-glucan preparations by enzymatic treatment.

The hot water and protease treatment was aimed to extract maximum protein out of cell wall residue. The multi-step acid and alkali treatment was done to remove lipids and protein traces from β-glucan enriched products. The subsequent enzymatic treatment to acid and alkali insoluble β-glucan was done to increase solubility of β-glucan preparations into cell culture media and enhance their biological activity (Williams *et al*., 1991, Demleitner *et al*., 1992, Williams *et al*., 1992).

It was found that the autolysis treatment efficiently extracted 72 % of the protein from yeast biomass. Additional hot water treatment to the cell wall residue obtained after autolysis of spent yeast removed 58 % of the protein with an additional 90 % reduction of mannose content. Later protease treatment to the hot water treated cell wall residues again reduces the protein content by 15 %.

Also, one of the valuable by-products obtained after hot water and protease treatments of the cell wall residue was mannoprotein (7 % w/w) and secondary yeast extract (13 % w/w).

The β-glucan enriched preparation obtained from autolysed spent yeast residue contained 58.08 ± 0.16 % of β-glucan, with only 4.44 ± 0.67 % protein.

The yield of β-glucan product on the basis of dry weight of yeast biomass was 21 %, with a 91 % of purity on the basis of total carbohydrate content.

The alkali and acid insoluble β-glucan particulate preparations (PβG) obtained at the end of the step wise alkali and acidic extraction treatment resulted in significant enhancement in the *in vitro* immunomodulatory potency.

It was also found that treatment of activated J744A.1 cells with soluble β-glucan preparations obtained after glucanase treatment of PβG, produced higher

amounts of NO and IL-12 than cell treated with PβG. This suggested that soluble β-glucan was a more potent stimulator of proinflammatory response elements than the particulate preparations.

From human whole blood assay results, it was found that all particulate and soluble preparation of alkali and acid insoluble β-glucan induced the release of small, but statistically significant amounts of pro-inflammatory TNF-α, IL-6, IL-8 and MCP-1 cytokinines in comparison with control.

The previous studies were mainly focused on β-glucan preparation from spent *Saccharomyces cerevisiae* yeast and found that multi step autolysis, hot water and protease treatment produced similar products with at par yield, purity and proinflammatory activity resulting in a responses induction.

This is the first study to report on the immunomodulatory activity of cell wall β-glucan preparations from spent Kluyveromyces marxianus yeast. This work has shown that the cell wall preparations from *Kluyveromyces marxianus* were recognized as foreign by cells of the innate immune system of a mammalian cell line and human whole blood.

The lab scale enzymatic process outlined in the present research is novel and produces soluble cell wall preparation that were found to enhance the production of Th1 cytokines IL-12 and TNF-α, IL-6, IL-8 under in vitro and ex vivo conditions. This indicated that cell wall preparations from *Kluyveromyces marxianus* may enhance the protective Th1 mediated innate immune responses in both *in vitro* mammalian cell line and *ex vivo* human whole blood condition. Therefore, indications are that β-glucan preparations from *Kluyveromyces marxianus* yeast may be used as supplement for Immunonutrition. ***Moreover, this study also validate the hypothesis of the study that the in vitro mammalian cell culture and ex vivo human whole blood models can be used to characterise cell wall preparations from Kluyveromyces marxianus.***

5.3.1 Future outlook and practical perspective

Chemo-enzymatic processes such as autolysis, hot water treatments, protease digestion, acid and alkali extraction and glucanase treatments of cell wall residues presented in this study may be utilised by industry to recover protein enriched secondary yeast extracts, crude β-glucan particulate, soluble β-glucan preparation from cell wall residue with commercial potential.

However, further detailed optimisation and scale up studies would be required before the proposed integrated approach can be exploited industrially.

Further studies could entail on elaboration of the mechanisms behind the proinflammatory activity of soluble β-glucan preparation from *Kluyveromyces marxianus*.

5.4 Study to purify and characterise the anti-hypertensive peptide(s) in a novel prepared yeast protein hydrolysate

As discussed in chapter 4.0, yeast protein extract obtained after mechanical disintegration was subjected to a phosphorylation treatment. The phosphorylated yeast protein was enzymatically hydrolysed with pepsin, pancreatin and papain at their respective recommended optimal temperature, pH and % E/S ratios. The protein hydrolysates were assayed for their *in vitro* ACE inhibitory activity. The protein hydrolysate with highest ACE inhibitory activity was selected for activity guided fractionation to obtain active ACE inhibitory peptide pools using size exclusion chromatography and a two step RP-HPLC technique. Subsequently, the most active fractions with highest ACE inhibitory potency and purity were then assessed for their peptidic contents using a Q-Exactive high performance benchtop quadrupole Orbitrap mass spectrometer system connected to a Dionex Ultimate 3000 chromatography system (Michalski *et al*., 2011, Di Bernardini *et al.,* 2011).

Phosphorylation of the yeast protein was aimed to reduce the nucleic acid content in extracted yeast proteins. The enzymatic hydrolysis of yeast proteins with pepsin / pancreatin and papain was performed to generate pools of peptides resembling those generated during the digestion of yeast protein in the GI track

and yeast extract production, respectively. The activity guided fractionation of selected protein hydrolysates were performed to obtain a step wise enrichment of ACE inhibitory peptide pools with potent ACE inhibitory activities. Thereafter, the potent peptide pool of interest was subjected to LC-MS-MS for characterisation.

Phosphorylation of the yeast protein led to a reduction of the nucleic acid content (55 %) of yeast protein concentrates with an appreciable increase in protein extraction (10 %) from yeast extract observed.

The results obtained in this work demonstrate that ACE inhibitory protein hydrolysates can be produced by treating spent yeast protein (*Kluyveromyces marxianus*) with pepsin, pancreatin and papain. The pepsin digest obtained at the end of 4 h hydrolysis displayed the highest ACE inhibitory potency with an IC_{50} value of 1374 ± 5.00 µg/ mL.

ACE inhibitory activity guided purification of the pepsin digest by gel filtration, two stage reversed phase HPLC had successfully enriched the hydrolysate to several folds. Enrichment resulted from removal of non-ACE inhibitory peptides from the pool to increase the ACE inhibitory potency as low as 19.31 ± 0.55 µg/ mL in case of *gel filtration* and 28.32 ± 1.57 µg/ mL in case of *two stage reversed phase HPLC,* which is shown in Figure 5.2. Electrospray LC-MS-MS characterisation of these active peptide pools also confirms the presence of large number of peptides in very minute proportions. Only a few studies have reported the ACE inhibitory activity of *Saccharomyces cerevisiae* derived peptides in wine and alkaline protease hydrolysates of brewer's yeast (Table 1.5, Section 1.6.2c). However, ***this is the first study to report on spent Kluyveromyces marxianus yeast protein as a source of ACE inhibitory protein hydrolysate peptides when generated by proteolytic enzymes***.

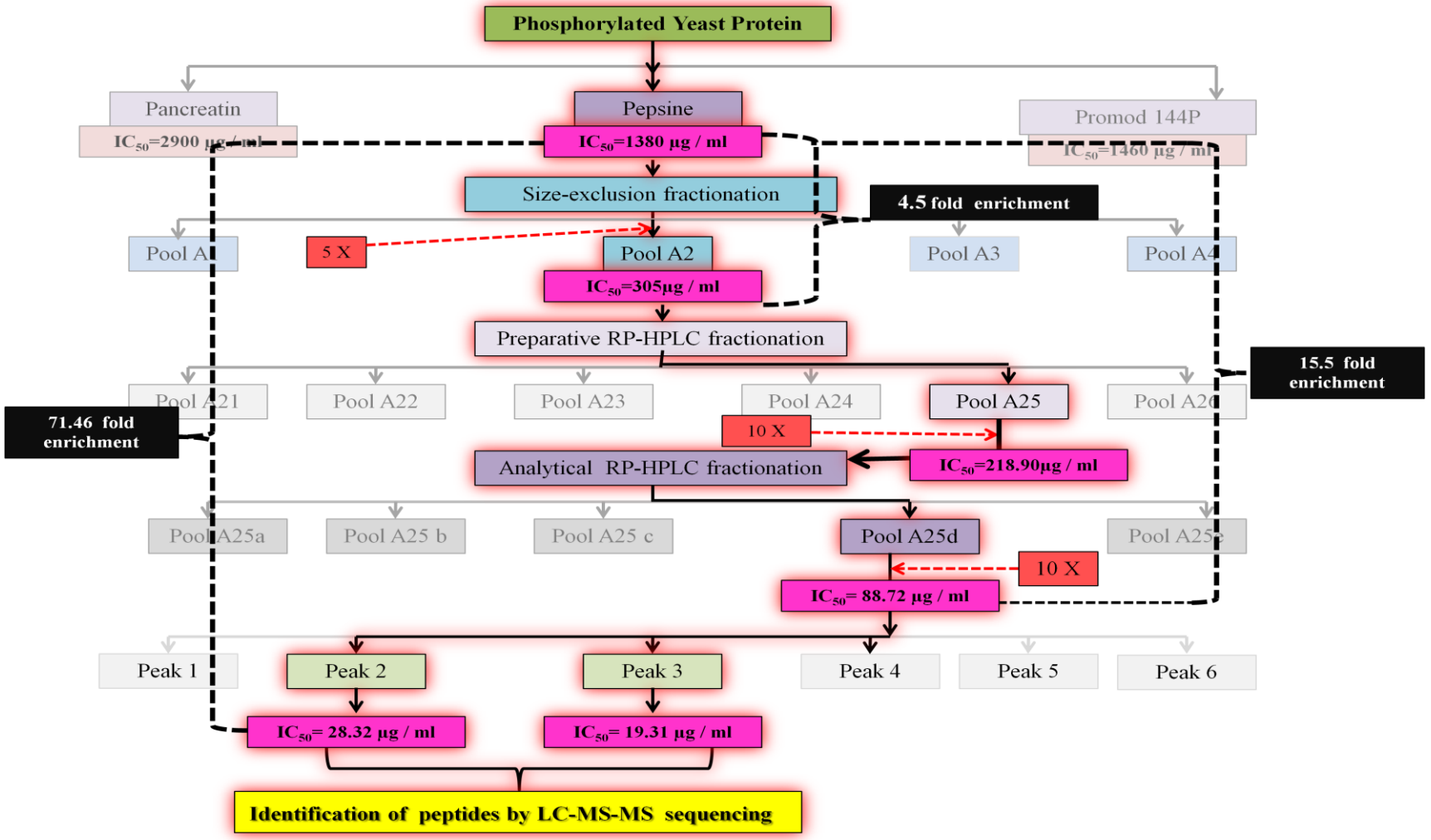


Figure 5.2 Main steps for enrichment of ACE inhibitory active peptide by bioactivity guided purification of pepsin digest of spent *Kluyveromyces marxianus* protein by gel filtration, two stage reversed phase HPLC and subsequently peptide characterization by LC-MS-MS sequencing.

In the present study a proposed purification scheme of the protein hydrolysate was capable of enriching peptide pools ACE inhibitory activities by ***71.46 folds.*** The ACE inhibitory potency of enriched peptide pools were comparable to values reported for a variety of ACE inhibitory protein hydrolysates (Murray *et al.,* 2005). This study has also identified the presence of a number of peptide sequences in ACE inhibitory potent pools that were known to have active amino acid motifs which were previously reported in ACE inhibitory peptide sequences (Murray *et al.,* 2005).

This study also confirms the hypothesis that bioactivity guided fractionation can purify yeast protein hydrolysate with elevated ACE inhibitory activity. Although, peptide purification scheme of present study was not able to isolate individual ACE inhibitory peptide(s). This can be attributed to complexity of yeast hydrolysate as it is made of variable protein mixture such as spent yeast protein.

5.4.1 Future outlook & practical perspective

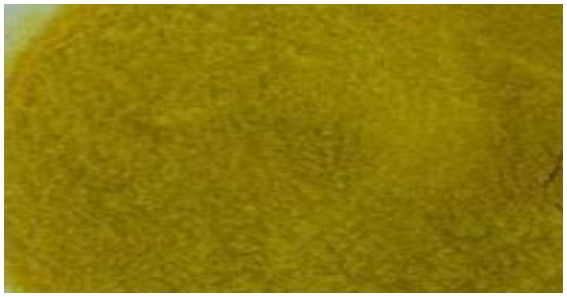
Synthetic ACE inhibitory drugs are potent prescription compounds prescribed to individuals suffering from hypertension at elevated levels. Many individuals are borderline high in blood pressure and do not need expensive drugs but more so like life style changes. Research has shown as summarised in Fitzgerald *et al*., 2004, that dietary consumption of ACE inhibitory peptides in food products offers a potential alternative non-prescription approach to life style changes to treat hypertension. The hydrolysates/extract generated in this research offers the potential to enhance the functionality of food products and enhance the health of an individual in multiple ways including potential ACE inhibition and immunomodulation.

Among the peptide sequences identified by LC-MS-MS a novel heptapeptide LGKPVTF with structural motifs akin to potent ACE inhibitory peptides. Further work is required using synthetic LGKPVTF to obtain the ACE inhibitory IC_{50} in micro molar units also to confirm RP-HPLC elution and detection findings.

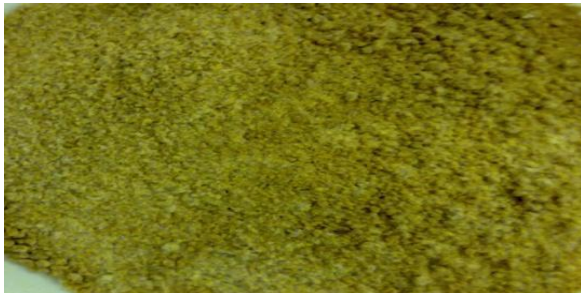
Human trials would also require to ascertain the efficacy of the hydrolysate and peptides antihypertensive activity of the hydrolysates obtained in the present study. Accordingly their application into food and feed system would be established. This could form the basis of a future collaborative study.

5.5 Summary conclusion

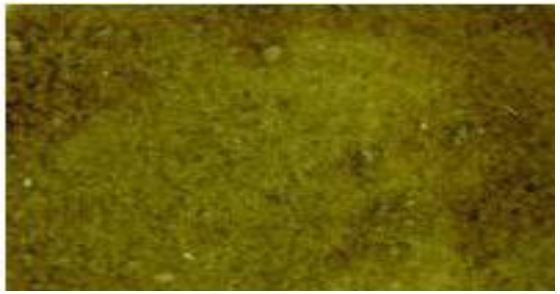
The results of this study opens up possibilities for better utilization of this sustainable Irish food industry waste streams for recovery of valuable functional and bioactive extracts (Figure 5.3). Such an approach is one step closer towards an amelioration of the economy and makes Irish food industry greener.



Lyophilised yeast extract produced at pH 7.0, 50 °C



Vacuum dried β Glucan enrich product



Vacuum dried Mannoproteins enrich product

Secondary yeast extract recovered from Yeast Cell Wall

Liquid suspension of yeast protein enzymatic hydrolysate

Figure 5.3 Value added model products derived from spent *Kluyveromyces marxianus* yeast.

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Appendix I

Analytical and cell culture methodologies

1.0 Total solids determination

➲ **Materials and reagents**

i) Lab-line instruments oven, ii) Ceramic dishes, iii) Analytical Balance, with least count ~ 0.0001 g, iv) Tong and heat resistant gloves, v) Dessicator.

➲ **Method**

Ceramic dishes were washed and put into the oven for 15 minutes to dry and remove any excess moisture. The dishes were placed into a dessicator until further use. Each dish was then weighed and 1 mL of analyte was then weighed into each dish. The weight of the dish and sample together were recorded. Immediately after, the dishes were placed into an oven for 24 h at 105 °C. The dishes with the dried sample were placed in dessicator for cooling at room temperature for 30 minutes and weighed; and the results were recorded.

➲ **Calculation of result of total solids**

$$\textbf{\% Total solids} = \frac{\text{(Weight dish (g) + dried residue (g)) – Weight dish (g)}}{\text{Weight of sample (g)}} \times 100$$

2.0 Determination of total nitrogen content by micro-Kjeldahl method

➲ **Materials and reagents**

i) Kjeldahl copper catalyst tablets (Kjeltabs), consisting of 5 g potassium sulphate and the equivalent of 0.1 g copper (II) sulphate, ii) Boric acid solution, dissolve 40 g of boric acid (H_3BO_3) in 1 L of hot water and allow cooling it, iii) Hydrochloric acid, standard volumetric solution (0.1 N), iv) Standard nitrogen sample or ammonium sulphate: Minimum assay 99.9 % on dried material, v) Sulphuric acid, low nitrogen content, at least 98 % (m/m), density at 20 °C ($\rho 20$) 1.84 g/mL, vi) Sodium hydroxide solution, low in nitrogen, containing at least 50 g of sodium hydroxide per 100 g, vii) Analytical balance, with least count ~

0.0001 g, viii) Digestion block, fitted with an adjustable temperature control and device for measuring block temperature, ix) Digestion tubes, suitable to be used with digestion block of capacity 250 mL, x) Exhaust manifold, suitable for use with the digestion tubes, xi) Graduated measuring cylinders of different capacity (25, 50, and 100 mL), xii) Dispensing pump, capacity 25 mL to deliver concentrated sulphuric acid, xiii) Weighting disc, xiv) pH meter, xv) 250 mL Erlenmeyer flasks, xvi) 50 mL manual burette, xvii) electrical stirrer and stir bars, xviii) appropriate safety supplies, including: cotton lab coat; safety glasses or goggles, xix) appropriate device for handling hot (> 100 °C) digestion tubes (large flask tongs or a "hot hands" holder was highly recommended), xx) Deionised water and all other analytical grade reagents.

➲ **Principle of total nitrogen analysis**

Total nitrogen of sample was measured as per International Dairy Federation method (I.D.F., 1993). The method may be separated into three categories, **digestion** (conversion of nitrogen in the sample to ammonia), **distillation** (separation of the ammonia from the digestate and collection for analysis), and **titration** (quantification of the ammonia and calculation of the initial protein concentration).

➲ **Sample portion**

Appropriately diluted sample was weighed and transferred into digestion tube. One tablet of Kjeltabs catalyst and 20 mL of sulphuric acid using appropriate bottle-top dispenser was added to the digestion tube.

➲ **Digestion**

The tubes were transferred to the digestion block and the assembly was set. The sample was digested at low initial temperature by setting temperature knob at level 4 for 30 minutes or until white fumes develop. Then the temperature of the digestion block was increased to 410 - 430 °C by setting temperature knob at level 9. Digestion of the sample was continued until the digest was clear. After the digest get cleared (with light blue-green colour) digestion was further continued for at least 1 h. During this period the sulphuric acid must be boiling. The total digestion time was between 1.75 and 2.5 h. At the end of digestion, the

digest was clear and free of undigested material. Remove the tube from block to cool.

➲ **Distillation**

Distillation unit was calibrated as per manufacturer instructions and set the operating parameter. Digestion tube was attached, containing the digest to the distillation unit. Erlenmeyer flasks containing 50 mL of boric acid solution was placed under the outlet of the condenser in such a way that the delivery tube was below the surface of the boric acid solution. Distillation was started and continued until at least 200 mL of distillate was collected. Flask was removed for titration.

➲ **Titration**

Burette was filled to the zero mark, or initial reading on the burette was recorded. Sample was stirred with stir bar and pH meter probe was immersed into sample. Titrant was added slowly to the sample. An effective technique was to adjust the burette so that a steady, slow drip of titrant occurs with continuous stirring. Sample was titrated till the pH of sample reached 4.6.

➲ **Calculation of results**

The general equation for the protein content was:

✓ **Percent nitrogen = PN = [(Vb – Vs) (N) (1.4007) / (W)]**

Vb = mL titrant for the blank(s)

Vs = mL titrant for the individual samples

N = Normality of the acid titrant (nominally 0.1)

1.4007 = a single factor that takes into account the molecular weight of nitrogen, the conversion of the milli equivalent result of V*N, and the conversion to %

W = the weight of sample in grams.

✓ **Percent protein = PN x F**

F = Nitrogen to protein (N: P) conversion factor, conversion factor for yeast protein was 6.25.

Percent protein on dry matter basis = (% protein / % total solid of analyte) x 100

3.0 Determination of α-amino acid nitrogen and peptide nitrogen content

➲ **Principle of α-amino acid nitrogen and peptide nitrogen analysis**

The α-amino acid nitrogen content of sample was determined on basis of modified cadmium chloride ninhydrin method where ninhydrin cadmium chloride reagent reacts with free amino group (Doi *et al.*, 1981). Similarly free amino acids plus peptides were determined with conventional ninhydrin method based on reaction of amino group with a ninhydrin stannous chloride reagent(Doi *et al.*, 1981). Then peptides were quantified by the difference between the results obtained with conventional ninhydrin method and modified cadmium chloride ninhydrin method. The method was further modified into microplate based method (Baer *et al.*, 1996; Doi *et al.*, 1981).

➲ **Material and reagents**

i) Acetate citrate buffer made of 0.5 mole acetic acid and 0.1 mole citric acid in 950 mL adjusted to pH 5.0 with 10 N NaOH and brought to 1000 mL with deionised water ii) Ninhydrin Stannous Chloride (NSC) reagent made of 200 mg of ninhydrin and 20 mg of $SnCl_2$ mixed together in 10 mL of methyl cellosolve, followed by the addition of 10 mL acetate citrate buffer. This mixture was prepared fresh each time iii) Diluent solution made of acetone and 0.1 M trisodium phosphate in water such that ratio proportion of 4:2:4 was maintained, iv) Ninhydrin Cadmium Chloride (NCC) reagent made of 800 mg of ninhydrin in 80 mL of 99.5 % ethanol and 10 mL of acetic acid. To it 1 g of $CdCl_2$ dissolved in 1 mL water was added, v) 96-well microplates, vi) 10 M NaOH, vii) VarioskanFlash 96-well plate reader, viii) Weighting disc, ix) Analytical balance with least count ~ 0.0001 g, x) Deionised water and all other reagents of analytical grade.

➲ **Procedure for determination of α-amino nitrogen by modified $CdCl_2$ – Ninhydrin method**

40 µL standard Leucine (0.1 mM to 0.25 mM) solution and appropriately diluted analyte was placed into a well of microtiter plate respectively. Then 80 µL of NCC reagent was added to each well using a multichannel pipette then mixing

was done by repeated pipetting and was incubated at 84 °C for 5 minutes by carefully floating the microtiter plate in water bath. Subsequently microtiter plate was transferred in ice water bath for cooling. Then 120 µL diluent was added in each reaction mixture. Afterwards, the microtiter plate was wiped dry to take absorbance of these standards and samples at 507 nm in VarioskanFlash 96-well plate reader. With the standards, a standard curve was plotted to determine the sample concentrations.

- **Procedure for determination of peptide nitrogen by conventional $SnCl_2$-Ninhydrin method**

40 µL standard Leucine (0.1 mM to 0.25 mM) solution and appropriately diluted analyte was placed into a well of microtiter plate respectively. Then 80 µL of NSC reagent was added to each well, using a multichannel pipette then mixing was done by repeated pipetting and was incubated at 90 ºC for 20 minutes by carefully floating the microtiter plate in water bath. Subsequently for cooling microtiter plate, it was transferred in ice water bath. Then 120 µL diluent was added in each reaction mixture. Afterwards, the microtiter plate was wiped dry to take the absorbance of these standards and samples at 570 nm in VarioskanFlash 96-well plate reader. With the standards, a standard curve was plotted to determine the sample concentrations as mM Leucine.

- **Calculation of results**

% Leucine (w/v) = [(Average OD value)/ (Slope of standard curve)] x DF x CF_1 x 100

% Nitrogen as Leucine (w/v) = % Leucine x CF_2

% Nitrogen as Leucine (w/w) = [(% Soluble Protein) / (%Total solid of analyte)] x 100

DF = Dilution Factor

Conversion Factor (CF_1) = 1 mM concentration of Leucine solution was 0.0131% (w/v) of Leucine

Conversion Factor (CF_2) = 131.1 gram of Leucine content 14 gram of nitrogen

4.0 Determination of Angiotensin converting enzyme (ACE) inhibitory activity

Phosphorylated yeast protein hydrolysate produced by action of different commercial proteinases preparation was subjected to microplate based ACE

inhibitory activity assay (Shalaby *et al.*, 2006) with some modification(Murray *et al.*, 2004).

➲ **Material and reagents**

i) Captopril, ii) N-(3-[2-furyl]acryloyl)-Lphenylalanylglycylglycine (FAPGG), iii) Rabbit lung acetone powder, iv) Sodium tetraborate decahydrate (borax), iv) Borate buffer made of 100 mM sodium borate with 5 % v/v of glycerol and pH adjusted to 8.3, v) Assay buffer made of 50 mM sodium borate buffer with 300 mM NaCl; pH adjusted to 8.3, vi) Substrate solution made of 1.6 mM FAPGG in assay buffer, vii) 96-well microtiter plate, viii) VarioskanFlash 96-well plate reader, ix) Weighting disc, x) Analytical Balance, with least count ~ 0.0001 g, xi) Deionised water and all other reagents of analytical grade.

➲ **Extraction and preparation of ACE from rabbit lung acetone powder**

The method of preparation of ACE extract from rabbit lung acetone powder was adopted form Murray *et al.*, 2004. Rabbit lung acetone powder (1 g) was mixed in 10 mL of sodium borate buffer using gentle magnetic stirring at 4 ºC overnight. The extract solution was then centrifuged at 15,000 rpm for 60 minutes in a Beckman Coulter centrifuge at 4 ºC and the clear wine red-coloured supernatant containing ACE activity was taken and aliquoted in eppendorf tubes to be stored at -20 ºC. These extracts generally contained˜ 2000 unit ACE activity per L and subsequently diluted to ACE activity of 250 ± 10 units per L for their use in assay.

➲ **Procedure of ACE and ACE inhibitory activity assay**

The assay was performed in 96 well microtiter plate with total assay volume of 170 µL. ACE assays containing 10 µL of ACE extract (250 units per L) and 10 µL of assay buffer and ACE inhibitory assay containing 10 µL of ACE extract (250 units per L) and 10 µL of inhibitor (diluted protein hydrolysate or different concentration of Captopril) were pre-incubated at 37 ºC for 10 minutes to establish the contact between inhibitor and ACE. To each well 150 µL of preheated (37 ºC for 15 minutes) substrate solution was added in less than one minute with the help of multichannel pipette to start the reaction. The microtiter plate was immediately transferred to VarioskanFlash 96 well plate reader

maintained at 37 ºC. The absorbance at 340 nm was recorded every 30 s for 30 minutes and the negative slope averaged over a linear interval of 15 minutes was taken as a measure of the ACE activity. The synthetic ACE inhibitory compound Captopril was used in assay as positive control.

➲ **Calculation of results**

% ACE inhibition was calculated from the ratio of the slope in the presence of inhibitor to the slope obtained in absence of inhibitor, according to the formula: % ACE inhibition = [1– (ΔAinhibitor ⁄ ΔAcontrol)] x 100.

IC_{50} was obtained by plotting graph of % ACE inhibition value versus inhibitory concentration of ACE inhibitory compounds.

5.0 Determination of total carbohydrate

The estimation of total carbohydrate of analyte was based on phenol sulphuric acid method (DuBois *et al.*, 1956) with some modification (Masuko *et al.*, 2005). All the analysis was done in triplicate.

➲ **Materials and reagents**

i) VarioskanFlash 96-well plate reader, ii) 96 well microtiter plates, iii) Grant W38 water-bath, iv) 80 % aqueous solution of phenol, v) 95.5 % of H_2SO_4, vi) Dispensing pump of capacity 10 mL to deliver concentrated sulphuric acid, vii) Glucose as standard (Analar), viii) Weighting disc, ix) Analytical Balance, with least count ~ 0.0001 g, x) Deionised water, and other reagents of analytical grade.

➲ **Sample preparation**

10 to 15 mg of dried sample was taken in eppendorf tube. Then 1 mL of 2 N H_2SO_4 was added and tube was screw caped. Then sample was kept for 3 h at room temperature with intermittent mixing for proper wetting of dried sample. Afterwards sample was heated at 95 ˚C for 5 minutes and cooled at room temperature. Subsequently sample mixture was stirred vigorously at room temperature until a homogenous solution was achieved. Then sample was diluted with deionise water if require for subsequent analysis.

➲ **Procedure for microplate based phenol sulphuric acid method**

50 µL appropriately diluted analyte and standard glucose (25 nM – 150 nM) solutions were all placed into each well of 96 well microtiter plate. To this 150 µL of concentrated sulphuric acid was added rapidly into liquid surface to cause maximum mixing with multichannel pipette and immediately 30 µL of aqueous phenol solution was also added similarly. Afterwards plate was incubated for 5 minutes at 90 ºC in a static water bath by floating the microtiter plate carefully. The plate was cooled to room temperature for 5 minutes in another water bath. Plate bottom were wiped dry to take the absorbance of standards and samples at 490 nm in VarioskanFlash 96-well plate reader. With the standards, a standard curve was plotted to determine the sample concentrations.

➲ **Calculation of results**

% Total carbohydrate (w/w) = [(Average OD value)/ (Slope of standard curve)] x DF x100

DF = Dilution Factor.

6.0 Measurement of β-Glucan content

Yeast β-glucan was measured using a Megazyme kit named Mushroom and Yeast Beta-Glucan, K-YBGL.

➲ **Principle of β-glucan analysis**

Mixture of 1,3:1,6-β-Glucan + 1,3-β-Glucan + α-Glucan + H_2O was subjected to acid hydrolysis in presence of conc. HCL. This was later nutralize by conc. NaOH. Portion of diluted aliquots was treated with exo-1,3-β-glucanase and β-glucosidase to convert soluble glucan to D glucose. When this D-glucose was treated with glucose oxidase/peroxidase mixture (GOPOD) gave the hydrogen peroxides and gluconate. Hydrogen peroxides react with p-hydroxybenzoic acid and 4-aminoantipyrine to give stable coloured compound quinoneimine. That in turn measure at OD of 510.

(conc. HCl, 30°C, 45 min)
(1) 1,3:1,6-β-Glucan + 1,3-β-glucan + α-glucan + H_2O → soluble glucan

(1.3 M HCl, 100°C, 2 h)
(2) Soluble glucan + H_2O → D-glucose + laminarisaccharides (trace)

(*exo*-1,3-β-glucanase + β-glucosidase)
(3) Laminarisaccharides + H_2O → D-glucose

(glucose oxidase)
(4) D-Glucose + H_2O + O_2 → D-gluconate + H_2O_2

(peroxidase)
(5) H_2O_2 + *p*-hydroxybenzoic acid + 4-aminoantipyrine → quinoneimine + H_2O

➲ **Materials and reagents**

i) Spectrophotometer, ii) Thermo electro-corporation Helios α, iii) ZX Classic Vortex mixer, iv) Grant W38 water-bath, v) Falcon 6/3,00 Centrifuge, vi) Sodium acetate buffer (200 mM, pH 5.0) made with 11.6 mL of glacial acetic acid (1.05 g/mL) to 900 mL of distilled water and pH adjusted to 5.0 using 4 M (16 g/100 mL) NaOH solution. Adjust the volume to 1 L, vii) Potassium Hydroxide (2 M) made with 112 g of KOH to 800 mL of distilled water and dissolved by stirring. Final volume adjusted to 1 L, viii) Hydrochloric acid (37 % v/v; ~ 10 M), ix) Blank made of 0.02 mL of sodium acetate buffer (200 mM, pH 5.0) and 3 mL glucose oxidase/peroxidase reagent, ix) D-glucose standard made of 0.1 mL D-glucose standard (1 mg/mL) and 0.1 mL of sodium acetate buffer (200 mM, pH 5.0), plus 3 mL glucose oxidase/peroxidase reagent.

➲ **Procedure**

Approximately 100 mg of weighed sample A, C and D (as per purpose of analysis) was added in test tube (20 x 125 mm size). The tube was tapped to ensure that the entire sample falls to the bottom of the tube. Then 1.5 mL of conc. HCL acid was added to each tube, capped and stirred vigorously on a vortex mixer. The tubes were placed in a water bath at 30 ˚C for 45 minutes and stirred on a vortex mixer every 15 minutes (to ensure complete dissolution of the β-glucan). Then 10 mL of water was added to each tube, capped and stirred on a vortex mixer. The caps were loosened and placed in a boiling water bath (~ 100 °C). After 5 minutes the caps were tightened and incubated for 2 h. The tubes were cooled to room temperature, caps were loosened carefully and 10 mL of 2 N KOH was added. Then quantitatively the content of each tube was

transferred to a 100 mL volumetric flask and the volume was adjusted using acetate buffer. The flask was then mixed thoroughly by inversion. An aliquot of each suspension was filtered through Whatman GF/A glass fibre filter paper, or centrifuged at 1,500 g for 10 minutes. Subsequently 0.1 mL aliquots (in duplicate) were transferred to glass test tubes (16 x 100 mm) and 0.1 mL Laminarinase solution was added and incubated at 40 °C for 60 minutes. Then 3 mL of GOPOD reagents was added to each tube and incubated at 40°C for 20 minutes. Afterwards the absorbance of all solutions was measured at 510 nm against the reagent blank. The reagent blank consists of 0.2 mL of acetate buffer, plus 3 mL GOPOD reagent. The D-glucose standard consists of 0.1 mL D-glucose standard (1 mg/mL), plus 0.1 mL sodium acetate buffer plus 3 mL GOPOD reagent.

➲ **Calculations**

Total glucan (% w/w) = ΔE x F x 100/0.1 x 1/1000 x 100/W x 162/180

= ΔE x F/W x 90

α-glucan (% w/w) = ΔE x F x 1000 x 1/1000 x 100/W x 162/180

= ΔE x F/W x 90

β-glucan (% w/w) =Total Glucan - α-glucan

Where:

ΔE = reaction absorbance – blank absorbance

F = a factor to convert absorbance to micrograms of D-glucose

= a factor to convert absorbance to micrograms of D-glucose

= (100 µg of D-glucose standard)/ (GOPOD absorbance for 100 µg of the D-glucose standard)

100/0.1 = volume correction factor; for total glucan (yeast), (0.1 mL out of 100 mL was analysed).

1/1000 = conversion from µg to milligrams.

100/W = conversion back to 100 mg of sample (e.g. as % w/w)

W = weight of sample analysed.

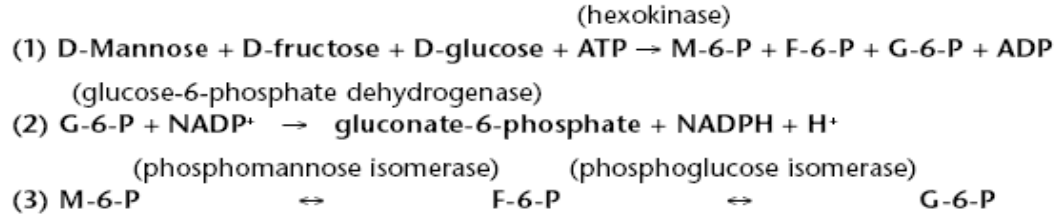
162/180 = a factor to convert from free D-glucose, as determined, to anhydroglucose, as occurs in β-glucan.

7.0 Measurement of mannan content

Mannan of yeast cell wall was measured using the kit "D-mannose, D-fructose and D-glucose, K-MANGL" by Megazyme.

➲ **Principle:**

D-Glucose, D-fructose and D-mannose are phosphorylated by the enzyme hexokinase (HK) and adenosine-5'-triphosphate (ATP) to glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P) and mannose-6-phosphate (M-6-P), respectively with the simultaneous formation of adenosine-5'-diphosphate (ADP). After conversion of D-fructose to F-6-P then it is converted to G-6-P by phosphoglucose isomerase (PGI).Similarly M-6-P is converted to F-6-P, which in turn converted to G-6-P in presence of phosphomannose isomerase (PMI) and phosphoglucose isomers (PGI) respectively. In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidised by NADP+ to gluconate-6-phosphate with the formation of reduced NADPH. The amount of NADPH formed in this reaction is stoichiometric with the amount of D-glucose. This is measured by the increase in absorbance at 340 nm.



➲ **Materials and reagents:**

i) Spectrophotometer, ii) Thermo electro corporation Helios α, iii) Vortex mixer, iv) Grant W38 Water-bath, v) Falcon 6/300 centrifuge, vi) Aqueous ethanol 80% (v/v), vii) Ice cold Sulphuric acid, viii) 60 % NaOH, ix) 2 M Triethanolamine (TEA) buffer (12 mL, 1 M, pH 7.6) plus magnesium chloride (100 mM) plus sodium azide (0.02 % w/v), x) Substrate solution (SS) made of 150 mg of $NADP^+$ and 440 mg of ATP in 12 mL distilled water, xi) Enzyme (E) solution made of 1.2 mL enzyme suspension containing Hexokinase (425 U/mL) plus glucose-6-phosphate dehydrogenase (212 U/mL), xii) Phosphoglucose isomerase suspension (1.2 mL, 1,000 U/mL), xiii) Phosphomannose isomerase suspension (1.2 mL, 1,000 U/mL),

xiv) D-Glucose, D-fructose plus D-mannose standard solution (5 mL, 0.1 mg/mL of each sugar).

➲ **Procedure**

Approximately 25 mg of sample was added in universal tubes with continuous tapping to ensure that the entire sample fell to the bottom of the tube. Then, 8 mL of aqueous ethanol (80 % v/v) was added to each tube and stirred vigorously on a vortex mixer, then incubated at ~ 80 ˚C for 15 minutes. After that, another 8 mL of aqueous ethanol was added and contents were allowed to cool down and later centrifuged at 1,500 g for 10 minutes. The supernatant was carefully decanted and discarded. The obtained pellets were again treated with 8 mL of aqueous ethanol with continuous stirring. Tubes were centrifuged at 1,500 g for 10 minutes, and supernatants were decanted. This step was repeated twice. A magnetic stirrer bar was added to tubes with palate and then placed in a test-tube rack in an ice water bath over a magnetic stirrer. 2 mL of ice-cold, sulphuric acid was added to tube and stirred for 1 h. Then 3 mL of water was added to each tube and placed in a boiling water bath at ~ 100 °C for 5 minutes without cap and then for 2 h with cap. Then tubes were allowed to cool down at room temperature. The content was transferred to 25 mL beaker and adjusted to approximately 10 mL with distilled water and the pH was adjusted to 7.6 with 2 M NaOH (using a pH meter). Then contents were quantitatively transferred again to a 25 mL volumetric flask using distilled water and final volume was adjusted to 25 mL. An aliquot of the suspension was centrifuged at 1,500 g for 10 minutes. At the same time to each cuvette 2 mL distilled water, 0.1 mL of sample aliquot plus 0.2 mL of TEA buffer and 0.2 mL of substrate solution were added. Similarly, for blank 2.1 mL distilled water + 0.2 mL of TEA buffer + 0.2 mL of substrate solution were added. Cuvettes were mixed by inversion and kept for 3 minutes for reaction. Then absorption was taken at 340 nm for blank: Ab1 and test solution: As1. After this 0.02 mL of enzyme solutions was added and mixed by inversion. The mixture was kept for 5 minutes for reaction. Then 0.02 mL of (PGI) solution was added and mixed by inversion and kept for 10 minutes for reaction. Afterwards, 0.02 mL of (PMI) soln. was added, mixed by inversion and kept for 25 minutes for reaction. OD was taken at 340 nm for blank: Ab2 and solution: As2.

➲ **Calculations**

The concentration of D-glucose, D-fructose and D-mannose was calculated as follows:

$c = (v \times MW \times \Delta A) / (e \times d \times v)$

Where:

V = final volume (mL)

MW = molecular weight of D-glucose, D-fructose or D-mannose (g/mol)

$\Delta A = \Delta$ absorbance

Adb= (Ab2)-(Ab1)

Ads= (As2)-(As1)

ΔA = (Ads)-(Adb)

e = extinction coefficient of NADPH at 340 nm, 6300 ($l \times mol^{-1} \times cm^{-1}$)

d = light path (cm)

v = sample volume (mL)

Because samples were originally solid, the content (g/100 g) was calculated from the amount weighed as follows:

Content of D-glucose

$= c_{\text{D-glucose}}$ (g/L) x 100 / Weight sample (g/L) _____ [g/100 g]

Content of D-fructose

$= c_{\text{D-fructose}}$ (g/L) x 100 / Weight sample (g/L) _____ [g/100 g]

Content of D-mannose

$= c_{\text{D-mannose}}$ (g/L) x 100 / Weight sample (g/L) _____[g/100g]

8.0 Determination of ribonucleic acid (RNA)

The RNA content of analyte was determined on basis colorimetric estimation of purine bound ribose sugar of RNA by Orcinol method. In this method RNA content of sample was extracted by sample incubation with 0.5 M perchloric acid at optimum condition. The method of extraction and estimation was adopted from Herbert *et al.*, 1971 with some modification(De Mey, 2006). All the analysis was done in duplicate.

➲ **Material and reagent**

i) Orcinol reagent made of 0.1 % Orcinol and 0.1 % $FeCl_3 \cdot 6H2O$ in concentrated HCl, ii) 0.5 M Trichloroacetic, iii) 0.5 M Perchloric acid, iv) VarioskanFlash 96-well plate reader, v) 96 well microtiter plates, vi) Grant W38 water-bath, vii) Eppendorf tubes with screw cap, viii) Micro centrifuge, ix) Standard Yeast RNA, x) Dispensing pump, capacity 10 mL to deliver concentrated HCl, xi) Weighting disc, xii) Analytical Balance, with least count ~ 0.0001 g, xiii) Deionised water, and all other reagents of analytical grade.

➲ **Sample preparation**

25 mg of dried sample was weighed in Eppendorf tubes with screw cap. Then sample was washed with 1 mL of 0.5 M trichloroacetic acid by incubating for 1 h in ice bath with frequent shaking to remove acid soluble constituents. The content was centrifuged at 15000 rpm for 15 minutes to sediment sample and was subsequently re-suspended in 1 mL of 0.5 M perchloric acid for 90 minutes at 37 °C with shaking. The sample mixture was again centrifuged at 15000 rpm for 15 minutes and supernatant that contains all RNA pool was further diluted in 0.5 M perchloric acid for further analysis.

➲ **Procedure for RNA estimation by Orcinol method**

0.5 mL appropriately diluted analyte and standard RNA (20 µg to 100 µg) was taken in eppendorf tubes and later 0.5 mL Orcinol reagent was added to it. Mixture in eppendorf tubes were incubated at 100 °C for 15 minutes and followed by immediate cooling under running water. Afterwards, the absorbance of standards and samples were determined at 650 nm in VarioskanFlash 96-well plate reader. With the standards, a standard curve was plotted to determine the analyte concentrations.

➲ **Calculation of results**

% RNA (w/w) = [(Average OD value)/ (Slope of standard curve)] x DF x100

DF = Dilution Factor.

9.0 Determination of N-acetyl glucosamine

The N-acetyl glucosamine content of analyte was determined on basis of reaction of glucosamine with acetylacetone to form a chromogenic compound with *p*-dimethylaminobenzaldehyde. Therefore, before analysis the glucosamine must be liberated from the sample by acid hydrolysis (Herbert *et al.*, 1971). The estimation of N-acetyl glucosamine was adopted from Morgan-Elson method (Popolo *et al.*, 1997).

➲ **Material and reagents**

i) Sodium bicarbonate reagent made of 1.5 N Na_2CO_3 in 4 % acetyl acetone, ii) Ehrlich reagent made of 1.6 g of p-dimethylaminobenzaldehyde in 30 ml of concentrated HCl and 30 mL of ethanol, iii) VarioskanFlash 96-well plate reader, iv) 96 well microtiter plates, v) Grant W38 water-bath, vi) Eppendorf tubes and test tube with screw cap, viii) Micro centrifuge, ix) Standard N-acetyl glucosamine, x) Dispensing pump, capacity 10 mL to deliver concentrated HCl, xi) Weighing disc, xii) Analytical Balance, with least count ~ 0.0001 g, xiii) Deionised water, and all other reagents of analytical grade.

➲ **Sample preparation**

25 mg of dried sample was weighed in Eppendorf tubes with screw cap. To this 6 N HCl was added and sample was hydrolysed at 100 ºC for 24 h. Then mixture was cooled to room temperature and content was centrifuged at 15000 rpm for 15 minutes to sediment residue. Then supernatant was diluted with deionised water for further analysis.

➲ **Procedure for N-acetyl glucosamine estimation by Morgan-Elson method**

1 mL appropriately diluted analyte and standard N-acetyl glucosamine (20 µg to 100 µg) solution was taken in test tube and later 0.5 mL sodium carbonate reagent was added to it. Mixture was incubated at 100 °C for 20 minutes and after cooling 3.5 mL of 96 % ethanol was added. Subsequently, 0.5 mL of Ehrlich reagent and mixture was incubated for 45 minutes at room temperature. Afterwards, the absorbance of these standards and samples were determined at 520 nm in VarioskanFlash 96-well plate reader. With the standards, a standard curve was plotted to determine the analyte concentrations.

➲ **Calculation of results**

% **N-acetyl glucosamine** (w/w) = [(Average OD value)/ (Slope of standard curve)] x DF x100

DF = Dilution Factor.

10.0 Cell culture procedures

➲ **Freezing of cells in Dimethy sulphoxide (DMSO)**

➢ **Materials**

i) 20 % Dimethyl sulfoxide (DMSO), ii) 60 % RPMI 1640 medium, iii) 20 % Foetal Calf Serum, and Mr Frosty.

➢ **Methods**

To prevent ice crystal damage to cells and for maintaining a viable state, cells were frozen in a mixture of foetal calf serum and DMSO. 20 % DMSO medium consisting of RPMI 1640 supplemented with 20 % DMSO, 20 % FCS was prepared and stored at 4 ^{0}C until required. Gentamicin was added to prevent bacterial contamination.

The cell suspension was diluted in culture medium (CCM) to concentration of 5 x 10^6 cells per mL and placed on crushed ice to cool. The freezing medium was added drop-wise to an equal volume of the cell suspension giving a final concentration of CCM and freezing medium. The cell suspension was dispensed into pre-cooled cryovials (pre-labelled) by placing them on crushed ice. All the procedures were carried out in a laminar flow hood. The cryovials were placed in a controlled rate freezer (MrFrosty) and then stored at -80 ^{0}C. The process done immediately as DMSO is too toxic for cells. The cells were gradually frozen in the fridge at a rate of approximately 1 ^{0}C per minute.

➲ **Thawing of stored cells**

➢ **Materials**

Complete culture medium

- **Methods**

Frozen cells were thawed carefully to prevent cell loss and preserve viability for functional assays.

A cryovial from the freezer was immediately thawed in a pre-warmed water bath at 37 ˚C, with constant mixing. When the mixture was almost thawed, the vial was wiped, and immersed in 70 % ethanol before being transferred to the safety cabinet. Thawing medium was gradually added to the cryovials whilst mixing gently. Thereafter, the cell suspension was transferred to a 15 mL Falcon tube containing thawing medium and the remaining medium added over the next five minutes while being mixed gently, allowing the DMSO to diffuse out of the cells. All the procedures were carried out in the bio-safety cabinet.

The tube was centrifuged at 400 g for 10 minutes, supernatant was discarded and pellet was resuspended in washing medium and centrifuged for another 10 minutes at 300 g. The supernatant was discarded again and cell pellet was resuspended in 1 mL of CCM and then counted.

➲ **Cell counting**

- **Materials**

Trypan blue, trypan blue solution was filtered (0.22 µm) and stored at 4 ˚C.

- **Methods**

Cell suspension was taken into a micro-centrifuge tube containing equal volume of trypan blue. The suspension was mixed and transferred into a clean Haemocytometer counting chamber for counting.The counting chamber is made up of nine squares (1 mm each).

The centre square of the counting chamber is further subdivided into 25 smaller squares and all other squares are further subdivided into 16 small squares (Figure 2.4). The number of cells was counted from the centre square as in the figure below. If the cell count in the centre square of the chamber is below 100, the four corner squares are usually used each having volume of 0.00625 mm^3 (micro litre).

Therefore, the number of cells counted in four of these squares is the number of cells in 0.025 micro litres. Cell counting was done for PBMC and mouse macrophage cells. Red blood cells (if in the suspension) or reticulocytes were ignored in the count for PBMC. The depth is 1/10 mm. Therefore where:

N = Number of cells counted in one square mm on the counting chamber

N x 10 = Number of cells in one mm^3.

N x 10 x 1000 x Dilution Factor = Number of cells in one ml

The following figures demonstrate the squares of the counting chamber and the yellow colour showed the usual counting area.

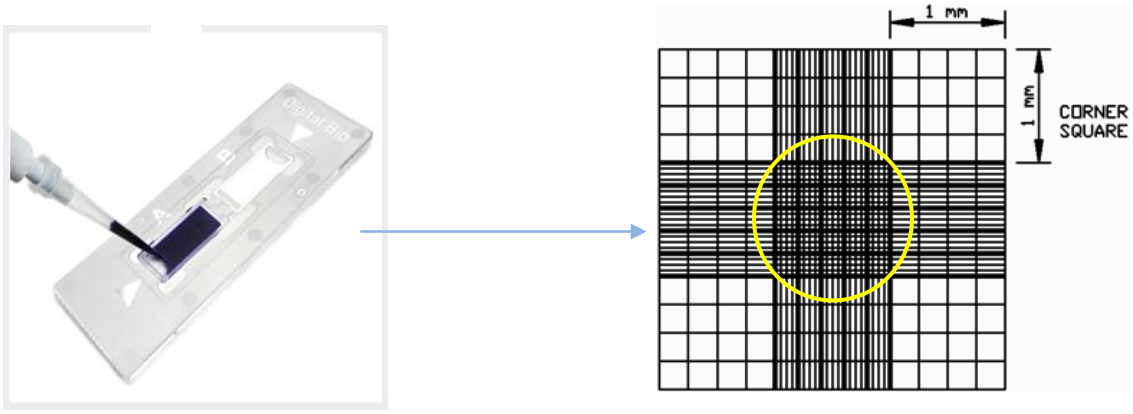


Figure 10.1: Haemocytometer disc & cell counting from Small Square

➲ Cell viability by Alamar blue assay

➢ Principle of Alamar blue assay

Alamar Blue is an indicator dye which incorporates an oxidation-reduction (REDOX) indicator that both fluoresces and changes colour in response to the chemical reduction, resulting from cell respiration (Hamid *et al.*, 2004, Mo *et al.*, 2008). Quantification of the reducing environment of the cells relates to the fact that when cells are metabolising they maintain a reducing environment within their cytosol and this reduced state can be measured spectrophotometrically through the conversion of fluorometric/colorimetic REDOX indicators. The reducing environment of the cells in the Alamar Blue assay is measured through the conversion of oxidised form to reduced form. This results in colorimetric (absorbance) and fluorescence changes. The oxidized

form of Alamar Blue is blue and non-fluorescent whereas reduced form is red and highly fluorescent.

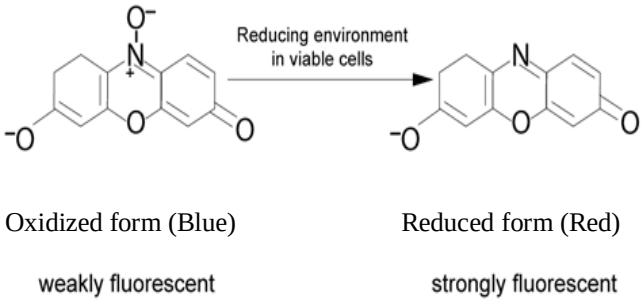


Figure 10.2 REDOX reactions from weak to strong fluorescence in cells by Alamar Blue

- **Materials and methods**

Adherent cells were released from their substrate by trypsinization and then resuspended cells at desired concentration. Ten micro litres of alamar blue reagent (Resazurin Sodium salt) was added to each well, including controls. The plate was then placed into the incubator for 1- 4 h. The cells were periodically viewed under an inverted microscope for presence of purple/red colour and measured at 560 nm in a microtiter plate reader. Fluorescence was also taken at the reference wavelength of 590 nm.

- **Determination of *in vitro* activation of mouse macrophages cells**

- **Activation of murine macrophages**

Murine macrophage cells (J774A.1) were cultured (100 µl) in 96 well plates at a concentration 50,000 cells/mL. The media used for cell culture was 10 % FBS, 2 mM L-Glutamine (1 % from the stock 200 mM) and 89 % RPMI. Cells were adhered by incubating the plates (96 well flat bottom; SARSTEDT) for 6 - 8 h at 37 °C and 5 % CO_2. Then 100 µL of IFN-γ was added to the wells at a final concentration 20 units/mL. After overnight incubation, supernatant were discarded and 100 µL of lipopolysaccharide (LPS) added at a final concentration 10 ng/mL. Plates were incubated for 9 h at 37 °C and 5 % CO_2. Plasma samples were heat-inactivated by warming the samples in a heating water bath at 54 °C for 1 h. After 9 h of LPS stimulation, the supernatant was removed from the

wells and heat-inactivated plasma samples (0.075 %) were added to the appropriate wells of the plate. The plates were again incubated overnight and the next day cell supernatants were collected and preserved at -80 ˚C for subsequent experiments.

➲ **Griess test for NO measurement in activated macrophage cells**

The Griess test is a chemical analysis to detect the presence of organic nitrite compounds. Griess reagent relies on Griess diazotization reaction first described in 1858 by Peter Griess. Test for NO production is a simple and rapid test for classically activated macrophages (Mosser and Zhang, 2008). Nitrite is detected and analyzed by formation of red/pink colour upon treatment of a Nitrite (NO_2^-) containing samples with the Griess reagent. Due to the addition of sulphanilic acid, the nitrites formed a diazonium salt which then combines with α-naphthylamine to develop a pink-coloured water soluble azo dye.

➢ **Methods**

Griess reagent (50 µL) was added to 96 well plates. A similar volume of activated murine macrophage cell supernatants was added to the appropriate wells along with blank. On the other hand 50 µL of serially diluted $NaNO_2$ standards were also added to labelled wells for preparing a standard curve. Plates were then incubated at 37 ˚C for 20 minutes and absorbance measured at 540 nm. The concentration of NO of different samples was determined from the $NaNO_2$ standard curve.

➲ **Enzyme linked immunosorbent assay (ELISA)**

The purpose of an ELISA is to determine if a particular protein is present in a sample and if so, how much. ELISA's are usually performed on 96-well plates to allow for high throughput. It basically involves an unknown amount of antigen attached to the surface (of the 96-well plate). A specific antibody is applied over the surface to bind to the antigen. An enzyme is linked to this antibody, so one of the final steps involves converting this enzyme activity into a detectable signal (specific colour) so absorbance can be read (Figure 10.3). The magnitude of absorbance will show how much antibody was present. This

can be determined from the standard curve. Tables 10.1 and 10.2 describe the methods for the IL-10 and IL-12 ELISA's.

The first step involved coating the surface of the well with capture antibody. A mixture of FBS/PBS was then added to coat parts of the well that are not covered with capture antibody. Standards and samples were then added. The standards provided the data for the standard curve. The detection enzyme was next added and following a relatively short incubation period the Streptavidin Peroxidase enzyme was added so colour could be generated. The stop solution was phosphoric acid. Absorbance was then read. In between each step was an essential wash step. These wash steps varied in length depending on the step preceding it. The wash steps were carried to remove any elements from the previous step that may have affected the ELISA, for example any sample that was not attached to the capture antibody.

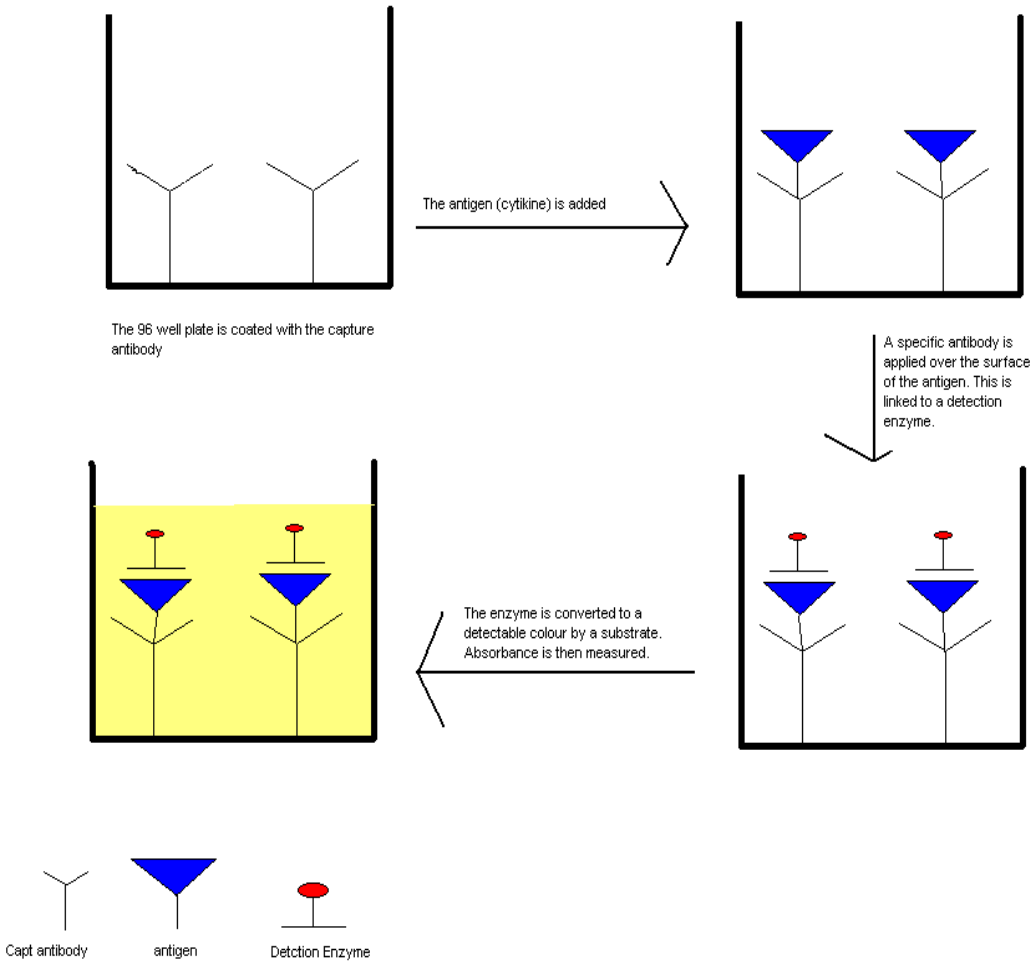


Figure 10.3 The type of ELISA employed here is known as a sandwich ELISA

Table 10.1 Method for IL-10 ELISA

Step No.	Name of Step	Diluent	Concentration of antibody	Volume/well	Duration of Incubation
Step 1	Capture	Binding buffer	Capture Antibody (Conc. =1/500)	50 µL	Overnight at 4 ^{0}C
Step 2	Wash	X4 in Tween/PBS			
Step3	Block	10 % FBS/PBS		100 µL	2 h at room temperature
Step 4	Wash	X4 in Tween/PBS			
Step5	Add standards and samples	10 % FBS/PBS	IL-10 standard (3 ng/mL)	50 µL	Overnight at 4 ^{0}C
Step 6	Wash	X4 in Tween/PBS			
Step 7	Detection	10 % FBS/PBS	Biotin rat anti-IL-10 (1/500)	50 µL	1 h at room temperature
Step 8	Wash	X6 in Tween/PBS			
Step 9	Add Enzyme	10 % FBS/PBS	Streptavidin Peroxidase (SA-HRP) 1/1000	50 µL	1 h at room temperature
Step 10	Wash	X8 in Tween/PBS			
Step 11	Add Substrate	TMB		100 µL	20 minutes
Step 12	Add Stop	1M Phosphoric acid		100 µL	5 minutes
Step 13	Read absorbance at 460 nm				

Binding Buffer = 0.71g $NaHCO_3$ + 0.16g Na_2CO_3 to 100 mL H_2O

Table 10.2 Method for IL-12 ELISA

Step No.	Name of Step	Diluent	Concentration of antibody	Volume/well	Duration of Incubation
Step 1	Capture	Na_2HPO_4	Capture Antibody (Concentration=1/100)	50 µL	Overnight at 4 ^{0}C
Step 2	Wash	X4 in Tween/PBS			
Step3	Block	10 % FBS/PBS		100 µL	2 h at room temperature
Step 4	Wash	X3 in Tween/PBS			
Step5	Add standards and samples	10 % FBS/PBS	IL-12 standard (3 ng/mL)	50 µL	Overnight at 4 ^{0}C
Step 6	Wash	X4 in Tween/PBS			
Step 7	Detection	10 % FBS/PBS	Biotin rat anti-IL-10 (1/1000)	50 µL	1 h at room temperature
Step 8	Wash	X6 in Tween/PBS			
Step 9	Add Enzyme	10 % FBS/PBS	Streptavidin Peroxidase (SA-HRP) 1/2000	50 µL	1 h at room temperature
Step 10	Wash	X8 in Tween/PBS			
Step 11	Add Substrate	TMB		100 µL	20 minutes
Step 12	Add Stop	1M Phosphoric acid		100 µL	5 minutes
Step 13	Read absorbance at 460 nm				

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