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# Enzymatic Conversion of RNA from Yeast Extract to Guanosine monophosphate (a flavoring agent)

*Master of Science Thesis in the Master Degree Programme, Biotechnology*

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Göteborg, Sweden, 2012

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Abstract

Ribonucleotides such as 5'-guanosine monophosphate (5'-GMP) and 5'-inosine monophosphate (5'-IMP) are important flavor enhancers. They improve the taste of food and have a synergistic effect with monosodium glutamate (MSG). 5'-GMP was synthesized enzymatically by hydrolysis of RNA to a mixture of Ribonucleotides by 5'-phosphodiesterase enzyme (5'-PDE). Yeast extract, which is produced from the extraction of yeast cells, consists primarily of amino acids, peptides, nucleotides and other soluble components of yeast cells. RNA content in yeast is typically in the order of 7-12% (w/w). Yeast cell of *Saccharomyces cerevisiae* as a by-product from bioethanol industry was used as a convenient starting material for production of yeast extract. This microorganism has a status as generally recognized as safe (GRAS), and are considered safe as food ingredient. Yeast extract was obtained from a simple method of autolysis process. 5'-phosphodiesterase enzyme (5'-PDE) were extracted and partially purified from germinated mung bean seeds. This partially purified enzyme was used for hydrolysis of RNA in the yeast extract to produce 5'-GMP-rich yeast extracts. The effect of autolysis time, enzyme concentration and hydrolysis (incubation) time on 5'-GMP content in the yeast extract was examined.

**Key Words**: RNA, hydrolysis, autolysis, yeast extract, enzyme, 5'-GMP, 5'-PDE.

Acknowledgements

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I would like to express my gratitude to Prof. Claes Niklasson, Department of Chemical Reaction Engineering, Chalmers University of Technology for providing me this opportunity to pursue a master thesis project through Linnaeus-Palme project. I would also like to thank Prof. Christer Larsson, Department of Chemical and Biological Engineering for being my examiner for this research work.

Further, this student exchange provided me with great opportunity to share knowledge between the students and teachers and also had a chance of experiencing each other's cultures. I would like to thank all of the lab technicians in Department of Biotechnology, Gadjah Mada University for helping me by providing with necessary strains, chemicals and glass wares during this research work. I owe a special debt to all who helped in the analytical laboratories to execute this research, without which my work would have not been consistent.

Last but not the least; I would like to thank my family and friends for their moral support and encouragement in carrying out this research work.

List of Abbreviations

| | |
|---|---|
| RNA - | Ribo Nucleic Acid |
| 5'-GMP - | 5'- Guanosine Monophosphate |
| 5'-IMP - | 5'- Inosine Monophosphate |
| 5'-PDE - | 5'- Phospho Diesterase |
| mM - | milli Molar |
| mL - | milli Litre |
| mg - | milli gram |
| M - | Molar |
| U/ml - | units per ml |
| g/L - | gram per litre |
| g/ml - | gram per milli litre |
| nm - | nano metre |
| MW - | Molecular Weight |
| µg/ml - | Micro gram per milli litre |
| µmol/ml - | Micro mole per milli litre |
| 4-npp - | 4- nitro phenol phosphate |

# Contents

# 1. Introduction

Monophosphate nucleotides have commercial applications in the food industry as flavor enhancers and are used as Flavor ingredients in packaged foods. Especially, 5'nucleotides such as 5'-GMP (Guanosine Monophosphate) and 5'-IMP (Inosine Monophosphate) are produced from enzymatic degradation of RNA during yeast autolysis (Aussenac, 2001). 5'-Nucleotides have been shown great importance in the biochemical industries due to its functional features and special chirality features which play a major role in the food flavor producing industries (Bercovici, 1995). Recently, the market growth rate for synthesis of nucleotides have increased from 5% to 7% and of more importance since they are used as active ingredients due to its wide applications in pharmaceutical, cosmetic and agricultural (food) products (Leuchtenberger, 2005).

## 1.1 Ribonucleotides

Ribonucleotides (RNA) play an important role in the food industry especially as food ingredients (as flavor enhancers). Ribonucleotides such as 5'-Guanosine Monophosphate and 5'-Inosine Monophosphate are important flavor enhancers. 5'-GMP can improve the taste of food and have a synergistic effect together with Monosodium Glutamate (MSG). Over the last 20 years, due to its flavor characteristics, 5'-Ribonucleotides have been shown great importance, especially that the three important amino acids such as L-glutamic acid (flavor enhancer – Monosodium Glutamate), L-aspartic acid and L-phenylalanine (starter material for peptide sweetener) in the food sector.

With a recent study from Brown, the market for the synthesis of amino acids grows at an annual rate of 7% due to its potential application in bio-pharmaceutical and food industries (Brown, 2005). Anticancer and Antiviral drugs such as acridine are synthesized with help of 5'-nucleotides (Khuttle, 2011). It is estimated that certain amino acids such as L-lysine, L-methionine, and L-tryptophan have a 56% market share and in case of food industries, Monosodium glutamate (MSG), is one of the most important flavor enhancers (Leuchtenberger, 2005).

Of the four nucleotides (5'-GMP, 5'-CMP, 5'-IMP, 5'-UMP) produced during incubation of yeast suspension containing RNA, 5'-GMP contains the flavoring properties which can be used widely in the food industries. When RNA is degraded by certain enzymes, 5'- Nucleotides (GMP, IMP, AMP, and UMP) can be produced. Following figure 1 is a schematic representation of the same.

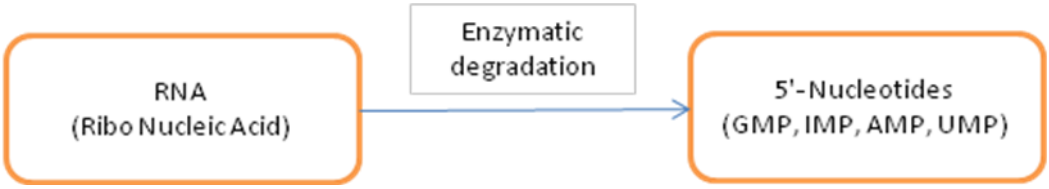


Figure 1: A simplified representation of enzymatic conversion of RNA to 5'- nucleotides

## 1.2 5'-Guanosine Monophosphate

Among the 4 available nucleotides (5'-GMP, 5'-CMP, 5'-IMP, 5'-UMP), 5'-GMP can associate or dissociate in both acidic and neutral aqueous solutions. 5'-GMP can improve the taste of food substance together with Mono Sodium glutamate. It is available in salt form and widely used in canned foods such as instant noodles, tinned vegetables and in various packaged food products. Together with MSG it can provide the taste of 'umami' (Ahluwalia, 1990).

GMP consists of a phosphate group, guanine base, pentose sugar ribose and hence referred to as ribonucleoside monophosphate. The structure of 5'-GMP is shown in the figure 2.

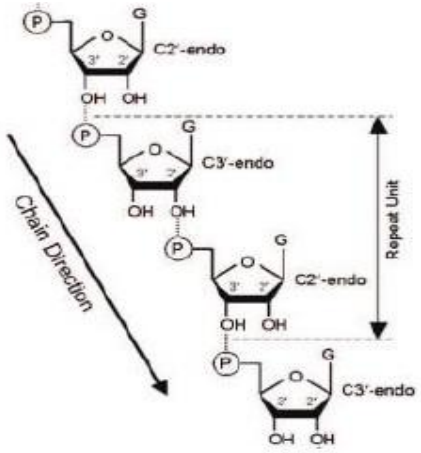


Figure 2: Structure of 5'-GMP (Wu, 2009)

## 1.3 Production of amino acids by Microbial Technology

Since 1980 there has been a rapid growth in cost-effective production and isolation of amino acids by microbial technology. There are four methods of production of amino acids and its derivatives namely extraction, fermentation, synthesis and enzymatic catalysis (Leuchtenberger, 2005). Due to its economical (cost of production) and environmental factors, fermentation and enzymatic production have numerous advantages. Figure 3 clearly shows the market growth rate for amino acids next to antibiotics during the years 2004 and 2009 (Maerz, 2005).

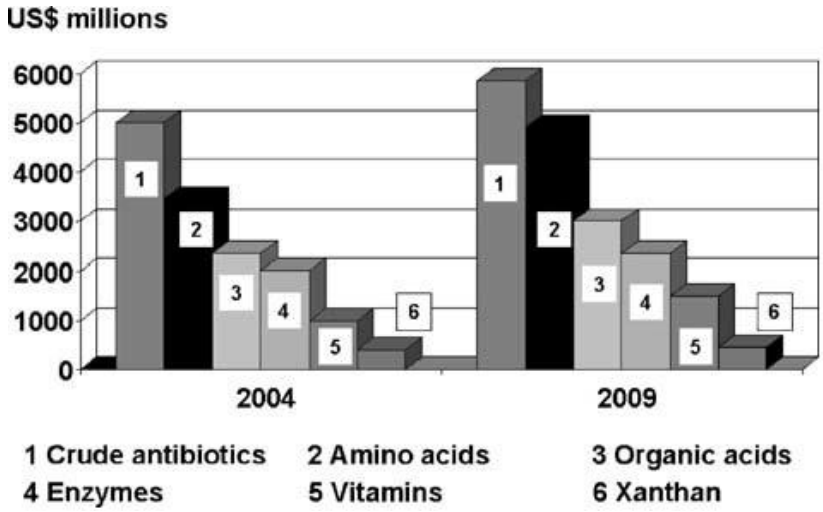


Figure 3: Showing the market growth rate of amino acids (Maerz, 2005).

Most of the amino acids produced are by enzymatic catalysis and fermentation technologies. Recent advancements in the field of Systems Biology also led to improve the production of amino acids by microorganisms (Marx, 2008).

## 1.4 Yeast cell and Extract

Yeast cells and yeast extract have been playing an important role in the food industries with applications in baking industries, as food ingredients, and flavor enhancers (AFLN, 1997). Around 6000 tons of dried yeast cell extract have been produced in Korea per year from Beer manufacturing industries (AFLN, 1997). Also, they are used as a flavoring agent in different food products such as in snack and canned foods (York, 1996). Yeast cells are composed of proteins, vitamins, minerals, RNA and lipid molecules.

When the yeast cells complete the growth period and enter the death phase, the process of autolysis occurs with endogenous enzymes present within the cell (Kollar, 1991).

In the old days, yeast extract was prepared by extracting RNA, by heating and hydrolyzing with 5'-PDE. However, this method was not economical due to the expensive enzymes employed during the extraction process. The enzymes were extracted either from *Penicillium citrinum* or *Streptomyces aureus*, microorganisms that do not have GRAS (Generally Recognized As Safe) status (Nagodawithana, 1994).

Yeast cell of *Saccharomyces cerevisiae* as a by-product of bioethanol industry is a convenient starting material for the production of yeast extract. This microorganism has GRAS status (Generally Recognized As Safe), therefore could be used a food ingredient together with monosodium glutamate [Querol, 2011].

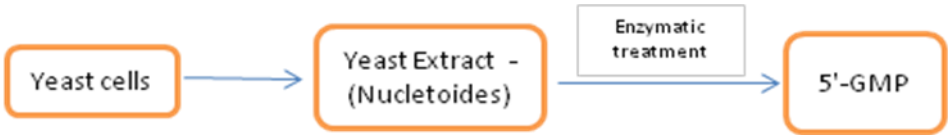


Figure 4: Simplified work flow of obtaining 5'-GMP rich yeast extract by Autolysing yeast cells

A detailed study report from WHO reveals that completely sequenced microorganisms (*S.cerevisiae*) among other microbial flora which are widely used in fermentation of food are published and reported to be free of known pathogenic traits (WHO, 2001). The report also added that utilization of microorganism in the production of agricultural and food products towards the enrichment in the nutritional value and safety of the food (Querol, 2011).

Yeast extract, which is produced from the extraction of yeast cells, consists primarily of amino acids, peptides, nucleotides and other soluble components of yeast cells. RNA content in yeast is typically in the order of 7-12% (w/w). It is obtained by breaking down the cell wall by enzymatic treatments. The two main important factors which influence the yeast cell autolysis are temperature and pH (Sombutyanuchit, 2001).

## 1.5 Glutamic Acid in Food Industries

Different scientific studies have been carried out on the microbial population towards the production of biochemicals. L-Glutamic acid together with 5'-GMP is one of the most important flavor enhancers in the food industries commonly used in Asian countries (Wang, 1993). Their characteristic properties and applications in the food industry had been a key factor to study its effect in food fermentation. They control the taste of fermented foods and help to reduce the toxic substances accumulated in the brain (Nandakumar, 2003).

## 1.6 5'-Phospho Diesterase Enzyme

5'-PDE is an enzyme that hydrolyzes RNA to a mixture of 5'- Ribonucleotides such as 5'-GMP, 5'-IMP, 5'-AMP and 5'-UMP. Due to its functional and structural properties and its distribution, they are often the targets towards pharmacological inhibition. It has its potential applications in the antivirus and anticancer treatments (Khutle, 2011). In the recent years, 5'-Ribonucleotides have been widely used in the pharmaceutical and food industries. Especially 5'-GMP is used as food additive in the most packaged food products (Khutle, 2011).

Various sources have been identified for extraction of this enzyme among which are barley rootlets and germinated mung bean seeds. Out of these two, mung bean seeds seemed to have higher enzymatic activity (Tyas, 2011). The type of enzyme to be used depends on various factors such as raw materials, their availability and method involved in extraction and purification of enzymes (Deoda, 2003).

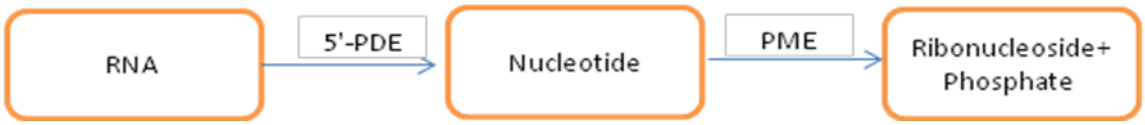


Figure 5: Enzymatic conversion of RNA from yeast extract to 5'- Nucleotides

In a recent research outcome, it has been reported that germination time influences the production of 5'-PDE Enzyme from germinated seeds. The three beans which were analyzed are black soybean, yellow soy bean and mung beans, out of which the mung bean has higher enzymatic activity compared to the other two (Utami, 2011).

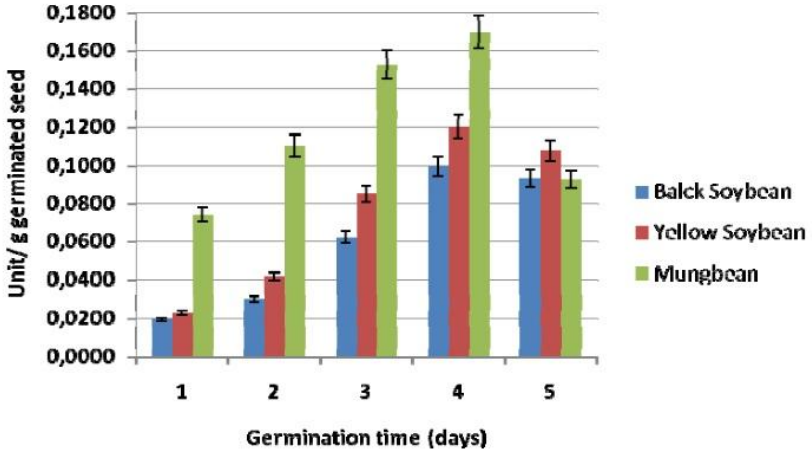


Figure 6: Activity of 5'-PDE from different germinated seeds (Utami, 2011).

## 1.7 Objectives

My objectives in this research included

1. To determine the growth curve of *Saccharomyces cerevisiae* and to study the rate of autolysis of yeast cells of *S.cerevisiae* in different growth phases.
2. To extract 5'-Phosphodiesterase enzymes from germinated mung bean seeds and purify them.
3. To autolyse yeast cells followed by hydrolysis of yeast's RNA by partially purified 5'-PDE enzyme.
4. To analyze the 5'-GMP content in the yeast extract by HPLC.

# 2. Theory

In this section, theoretical backgrounds for this research were described in detail.

## 2.1 Microbial Growth of cells

In batch cultures, the growth of microbial cells takes place in three main different phases namely Lag phase, Logarithmic (exponential) phase and stationary phase. The time required for the microbial cells to multiply depends on various growth factors such as the temperature, pH, and growth media (Madigan, 2009). The time taken by the microbial cells to double in size (or number) is known as generation time. The various phases of cell growth constitute the growth curve. Each phase of the microbial cells mentioned above is explained as below.

*Lag phase* is the initial phase and during this phase the cells undergo internal cytoskeletal and enzymatic changes in order to adapt to the new growth conditions. Also the synthesis of RNA and other molecules occurs during this period (Madigan, 2009).

*Exponential (Logarithmic) phase* is second phase where the biomass concentration increases exponentially with a constant growth rate.

When one or several nutrients are depleted, the cells reach a *stationary phase* where the growth rate is zero and there is not any production of biomass.

The stationary phase is followed by the *decline (death) phase*. During this phase, the cells start to decline. The typical growth curve of microbial population is represented in the following figure 7 with different phases (Madigan, 2009).

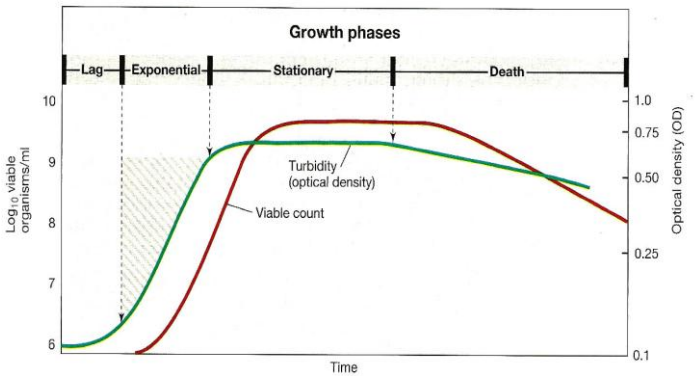

Figure 7: Different phases of microbial growth (Madigan, 2009)

Figure 8: A shaking incubator

## 2.2 Optical Density

The number of cells in a population is often determined by measuring the optical density (OD) of the cell culture. The total number of cells (total cell mass) in the microbial population can be indicated by its turbidity measurements. The spectrophotometer measures the scattered light at a certain wavelength, usually in between 540nm until 680nm for yeast populations (Wilson, 2010). The figure 9 shown below is a typical Genesys 20 spectrophotometer which is used for all optical density measurements during this research.

Figure 9: A spectrophotometer

## 2.3 Centrifugation

Centrifugation is a process of separating the biological macromolecules such as proteins, nucleic acids and their derivatives based upon their size, shape and density. This technique relies on the density difference between the biological macromolecules and the medium in which they are dispersed. During the process of centrifugation, the biological macromolecules or cells are sedimented by accelerating in a centrifugal field (Wilson, 2010).


Figure 10: A Laboratory Bench top centrifuge

## 2.4 Autolysis

Autolysis can simply be defined as 'self destruction'. The process of activation of cell's own degrading enzymes to solubilize the cellular components which is present within the cell is known as Autolysis (Tangluer, 2008). Proteases and Nucleases are the hydrolytic enzymes responsible for breakdown of biological macromolecules such as proteins, nucleic acids (Nagodawithana, 1994 & Sommer, 1998).

## 2.5 Yeast Autolysis

When the yeast cells are autolysed, cellular components are degraded and the breakdown products will be subsequently released in an extracellular environment (Charpentier, 2004). The research interest is now focused onto breakdown of protein molecules by autolysis reaction in yeast cells (Dziezak, 1987).

The process of autolysis of yeast cells can be divided into two steps.

1. The degradation of cellular constituents and
2. Degradation of cell wall

The mechanism of autolysis of yeast cell is described as follows.
Initially the cell endostructure is degraded and as a result the hydrolytic enzymes are released into intracellular space. During this stage, specific cytoplasmic inhibitors inhibit the released proteases, later which they are activated due to degradation of cytoplasmic inhibitors. Intracellular components hydrolyze together with hydrolysis products and at later stage, when their molecular masses are very low to cross pores in the cell wall, the hydrolytic products are released. The enzymatic degradation of intracellular macromolecules leads to an accumulation of hydrolysed products. The autolysed products are released in to the environment once the size of the cell wall pores becomes large enough. The autolysis process is usually induced by the changes in temperature, pH or osmotic pressure and /or addition of detergents (Alexander, 2006).

## 2.6 Enzymatic hydrolysis

Hydrolysis is a technique used to solubilize microorganism and this process is carried out either by acid or with proteolytic enzymes (Chae, 2001). The process of enzymatic hydrolysis can be carried out in different ways such as by using culture broth with microorganisms or by enzymes used for cell wall lysis or either by proteolytic enzymes (Lim, 1997). The selection of enzymes utilized in the enzymatic hydrolysis process depends upon various factors such as the source of raw materials and methods used in the extraction and purification process (Deoda, 2003). Furthermore it is very important to use appropriate conditions when using enzymatic treatments. Hydrolysis of RNA by enzymatic treatments is least complex, economical and gives higher yields compared to other methods of hydrolysis (Deoda, 2003).

## 2.7 Chromatography

The chromatography system consists of two phases: stationary phase and a mobile phase. Stationary phase is usually a solid, gel, liquid or their mixture which is immobilized whereas the mobile phase consists of liquid or gaseous substance passed through the immobilized phase to separate and identify the mixture of analytes (Wilson, 2010).

## 2.8 HPLC

High Performance Liquid Chromatography is a chromatographic technique used to identify or purify the individual components within a mixture. In this type of chromatography, it separates and identifies the molecules with relatively good sensitivity and only a minimal amount of sample is required for analysis. The principle behind this technique is that the analytes pass back and forth between the stationary and mobile phase and the distribution coefficient result in identification of the substances (Wilson, 2010). The high pressure inside the system is the unique characteristic responsible for high performance liquid chromatography.

Figure 11: Showing High Performance Liquid Chromatography instrument with PC

# 3. Materials and Methods

**Materials**

All the chemicals used for the experiments were obtained from Merck, Germany and were of analytical grade. The composition of the medium, buffers and other solutions used in the experiments can be found in Appendix section A.

## 3.1 Yeast strain and Cultivation

Yeast cell of *Saccharomyces cerevisiae* (Strain ID: 2534) was used in the experiment for preparation of yeast extract and further autolysis process. It was kindly provided as a strain in agar slant tubes from Food & Nutrition Collection centre for Gadjah Mada University, Centre for Food and Nutrition Studies. The strain was streaked on PGY Agar medium and incubated at room temperature for 14 hours. Later, this agar slant (Figure 12) was maintained by sub culturing and storage at 4 °C on PGY Agar Medium and utilized for rest of the experiments. Aseptic conditions were maintained throughout all the experiments to avoid any bacterial contaminations.

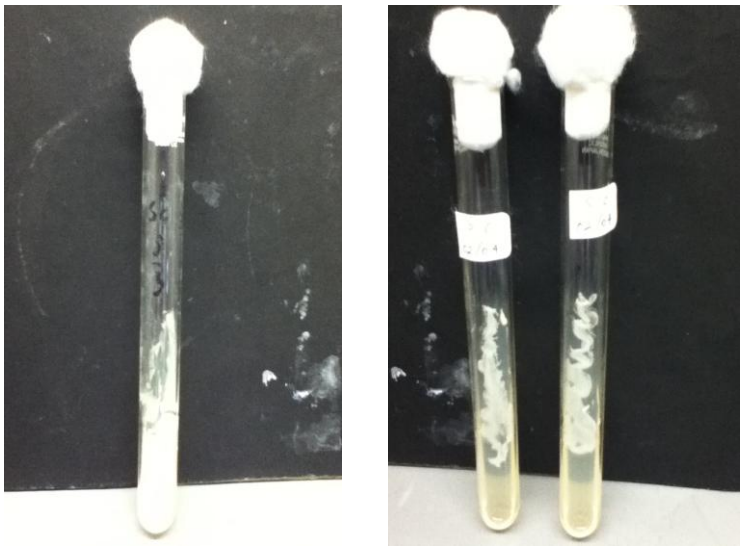

Figure 12: Figure on the left is yeast strain and on the right is the maintained sub cultured yeast cells.

Inoculum culture was grown aerobically in 125 ml of Growth media in a 500 ml Erlenmeyer flask at 30 °C for around 12 hours with orbital shaking (BT300 Shaking Water Bath Incubator, Yamato) of 70 rpm, as shown in figure 13, in a shaking water bath incubator and this was later used to inoculate the experimental culture. The composition of growth media used in the experiments were shown below.

Glucose - 50 g
Yeast Extract - 5 g
Di Ammonium Sulphate ($(NH_4)_2SO_4$) – 1 g
Potassium di hydrogen phosphate ($KH_2PO_4$) – 1 g
Di potassium hydrogen phosphate ($K_2HPO_4$) – 1 g
Magnesium Sulphate ($MgSO_4$) – 1 g

The above chemicals were mixed in 1 liter distilled water and sterilized before use.

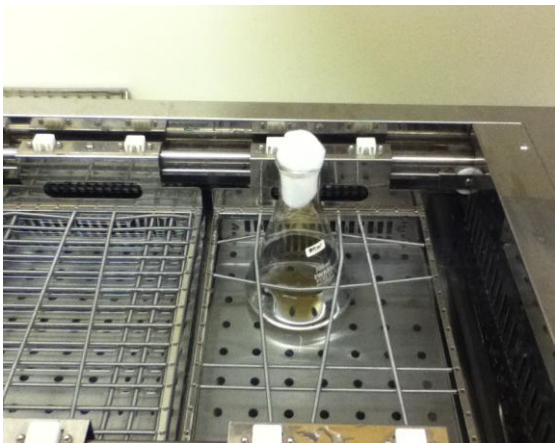

Figure 13: Starter Inoculum

## 3.2 Growth curve of *S.cerevisiae* and harvesting

The growth medium with following chemicals were prepared as shown in appendix A and sterilized (Glucose - 50 g, Yeast Extract - 5 g, Di Ammonium Sulphate ((NH4)2SO4 ) – 1 g, Potassium di hydrogen phosphate (KH2PO4 ) – 1 g, Di potassium hydrogen phosphate (K2HPO4 ) – 1 g, Magnesium Sulphate (MgSO4 ) – 1 g). The growth curve of *S.cerevisiae* was determined by culturing of yeast cells at 30 °C and 70 rpm for 30 hours by monitoring the optical density every 3 hours. Absorbance of the medium at 660 nm was 0.342 and this was used for setting the blank value in the spectrophotometer. The measurements were performed in duplicate and the average value was calculated.

Yeast cells of *Saccharomyces cerevisiae* were grown in Growth media, in 1 l Erlenmeyer flasks at 30 °C, 70 rpm until the cells reach the exponential phase and stationary phase. The process work flows were described in detail in Appendix Section C. The cells were then harvested by centrifugation (5804R Centrifuge, Eppendorf) at 4500 rpm for 20 minutes at 4 °C. The supernatant were discarded and the cells were washed with 0.85% NaCl solution. Later the Yeast cell pellet were suspended and maintained with 0.85% NaCl.

## 3.3 Autolysis of Yeast cells

To initiate autolysis, yeast cells suspended in 1 M NaCl solution were incubated at different temperatures such as 45 °C, 50 °C, 55 °C, and 60 °C in a water bath without shaking at different time points starting from 0 minutes until 2 hours with time interval of 30 minutes. Samples of autolysed yeast cells were withdrawn every 30 minutes and separated by centrifugation (4500 rpm for 20 min at 4 °C) into cell pellet and autolysate (supernatant) fractions. The amount of soluble protein content (supernatant) was analyzed by Bradford method using Bovine Serum Albumin as a standard (Appendix section B).

The process work flow to determine the effect of temperature of autolysis of yeast cells in different growth phase were described in Appendix section C.

## 3.4 Dry weight of yeast cells

Yeast cell suspensions (sample volume of 1 ml) in of 0.85% NaCl was poured onto a pre weighed porcelain cups and dried at 105 °C in a vacuum oven for overnight. The dry weights of the yeast cells at different growth phase were later weighed.

## 3.5 5'- PDE Enzyme Extraction

Mung beans were obtained from Peanut Research Institute, Malang through Centre for Food and Nutrition Studies, Gadjah Mada University.

### 3.5.1 Germination of mung beans

The mung beans were initially washed with tap water and soaked in water (47 °C) for 7 hours in a white cloth and subsequently water was removed. The mung bean was germinated at room temperature by putting them on sieve and being covered by wet cloth. During the second day the mung beans were soaked in water for 5 hours at 29 °C and later filtered using a paper filter (obtained from the local departmental store) to drain the water content. Water was sprinkled over the mung beans three times a day until the rootlets were observed.

Figure 14: Showing Germinated mung beans after the second day

### 3.5.2 Enzyme Extraction

5 g of Germinated mung beans were weighed and 40 ml of 50 mM acetate buffer (pH 5) maintained at 4 °C were added. The ratio of the germinated mung bean and the buffer is 1:8. Mung beans were later crushed in the mixer, filtered (using a tea filter obtained from a local departmental store) and the residue were discarded. The mung beans filtrate was centrifuged at 4500 rpm for 20 minutes at 4 °C. The residues were discarded and the supernatant (5'-PDE crude enzyme) was carefully collected by a pipette. It was transferred to a Falcon tube and enzyme activity was measured by Enzymatic Assay. The soluble protein content was determined by Bradford method using Bovine

Serum Albumin as a standard. The units/ml for 5'-PDE enzyme and µg/ml of protein content were determined, from which the specific activity of the enzyme (units/mg) was also calculated.

### 3.5.3 Partial Purification of 5'-PDE Enzyme

The whole experiment of partial purification of 5'-PDE enzyme was carried out in an ice bath maintained at 4 °C. 50 ml of the crude enzyme were taken in a beaker and ammonium sulphate was added to give a saturation of 60%. The precipitated crude enzyme was subjected to mild stirring by a magnetic stirrer for 15 minutes and allowed to stand for 30 minutes. The precipitated solution was centrifuged at 4500 rpm for 20 minutes at 4 °C. The supernatant was discarded and the precipitate formed was collected in a falcon tube and dissolved in 10 ml of 50 mM Acetate buffer of pH 5. This partially purified 5'-PDE enzyme was later used for enzymatic hydrolysis of yeast cell autolysis.

### 3.5.4 5'- PDE Enzyme Assay

To 0.1 ml of partially purified enzyme, 0.8 ml of 50 mM acetate buffer (pH 5) were added and incubated in a water bath maintained at 60 °C for 1 min. To the incubated sample, 0.1 ml of 10 mM 4-Nitrophenyl phosphate disodium salt hexahydrate were added as a substrate. This reaction mixture was incubated at 60 °C for 10 mins. The enzymatic reaction was stopped by adding 5% sodium carbonate solution. The reaction mixture was homogenized well and the total volume was made up to 3 ml by distilled water. A blank solution was also prepared without adding the enzyme solution to the sample. When the substrate was added, it produces a yellow colour due to presence of p-nitrophenolate ions and the absorbance at 400 nm was measured using a spectrophotometer. A standard graph of p-nitrophenol was also prepared in the range of 0-200 nmol. 5'-PDE enzyme is stable for one week (stored at 4 °C) which was confirmed through enzyme activity. One unit of the enzyme activity was defined as 1 µmol of the substrate converted per min at 60 °C.

## 3.6 Enzymatic Hydrolysis

The enzymatic hydrolysis were carried out by incubating the yeast cell extract together with 5'-Phospho Diesterase Enzyme (60% Precipitated) in a water bath without shaking at 50 °C at different time intervals ranging from 0 min to 30 mins. The samples treated with enzymes were taken at different time interval of 10 min and their enzymatic reaction was stopped by heating the samples in a boiling water bath for approximately 1 minute. 0 min autolysed sample was used as control without adding 5'-PDE enzyme. After which, the samples were centrifuged at 4500 rpm for 10 minutes at 4 °C. The supernatant was used as sample for analyzing the 5'-GMP concentration by HPLC. The amount of soluble protein content was also determined by Bradford method.

## 3.7 HPLC Analysis of 5'-GMP rich yeast extract

The nucleotide 5'-GMP concentration in the yeast extract was analyzed by High performance liquid chromatography technique using a 10 A VP Shimadzu HPLC as shown in Figure 15. A commercial GMP standard (0.5%) was obtained from PT. CJ Indonesia. The samples and the solutions were filtered through a membrane filter of pore size 0.45 µm (VWR, Germany) through an injector and collected in small eppendorf tubes prior to injection. The column (INSERTIL ODS-2) was made up of stainless steel and was silica-based and the water guard column was also placed before the main column. Elution was performed in presence of gradient system consisting a mixture of eluent A (50 mM $K_2HPO_4$, pH 5.45) and eluent B with 100% methanol. The volume of the injected sample was 20 µl and the flow rate was 1.5 ml/min. This flow rate was maintained throughout the whole run. Elution was performed at an ambient temperature of 25 °C. The compounds which were separated are quantified by comparing the peak areas with standard curve of GMP standard commercial. The calculations were shown in Appendix section. The separated nucleotides were quantified by comparing the peak areas with GMP standard.


Figure 15: Showing a High Performance Liquid Chromatography

# 4. Results and Discussion

## 4.1 Growth curve of S.cerevisiae

The standard yeast growth curve of *S.cerevisiae* can be observed in Figure 16. This standard curve has been used throughout the whole research work to examine the rate of autolysis in different growth phase of the yeast cells. After determining the absorbance of the growth curve at different time intervals with spectrophotometer, the dry weight of the yeast cells were solved from the equation y=0.529x and the Table 1 below shows the absorbance values and their corresponding dry weight of the yeast cells in different growth phase.

Table 1: Growth curve of yeast cells with absorbance values and corresponding dry weight of the cell at different time intervals

| Time (hrs) | Absorbance at 660 nm | DW - g/l |
|---|---|---|
| 0 | 0.074 | 0.140 |
| 3 | 0.119 | 0.225 |
| 6 | 0.442 | 0.836 |
| 8 | 0.812 | 1.535 |
| 9 | 0.986 | 1.864 |
| 10 | 1.132 | 2.140 |
| 12 | 1.644 | 3.108 |
| 14 | 1.934 | 3.656 |
| 16 | 2.059 | 3.892 |
| 18 | 2.136 | 4.038 |
| 20 | 2.158 | 4.079 |
| 22 | 2.107 | 3.983 |
| 24 | 2.121 | 4.009 |
| 27 | 2.105 | 3.979 |
| 30 | 2.127 | 4.021 |

The figure 16 shows the growth curve of yeast cells in growth medium. The primary axis on the left represents the absorbance values at 660 nm and the secondary axis in the right represents the dry weight of the yeast cells in g/l. From this curve it has been observed the logarithmic phase of the cells is up to 15 hours and the cells are in stationary phase until approximately 24 hours. These two growth phases are useful in the next experiment carried out to determine the rate of autolysis of yeast cells with temperature as a factor studied.

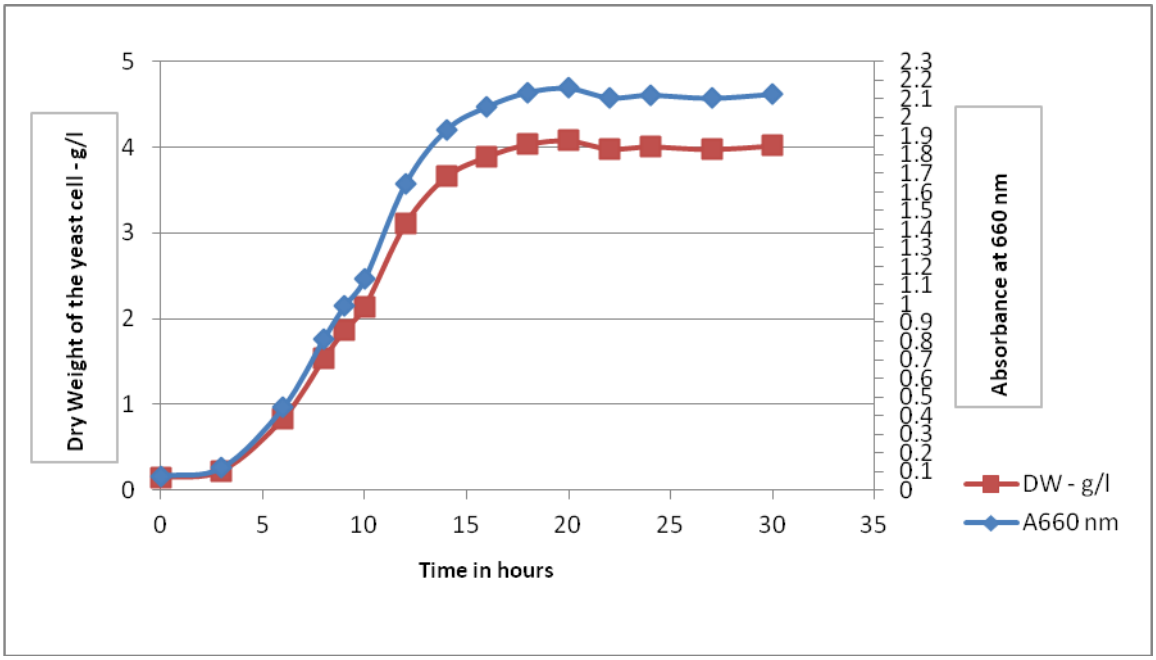


Figure 16: Showing the growth curve of S.cerevisiae

Growth curve of *Saccharomyces cerevisiae*

There was observed success in yeast cell culture. The yeast cell of Saccharomyces cerevisiae were successfully cultured in growth media and a growth curve was obtained as shown in Figure 16. The absorbance and dry weight of the yeast cell were calculated and the two growth phase which is of interest was identified. The cells continue to increase the cell number and their growth pattern can be observed in the figure 16 and we defined this phase as logarithmic phase (cell growth up to 15 hours). Also, once the glucose in the medium is utilized, the cells reached an apparent stationary phase. However, the cells are most probably not in stationary phase time but rather growing on ethanol but very slowly because of low oxygenation.

The condition for cell growth was chosen as 70 rpm and 30 °C under aerobic conditions and the samples were chosen after 15 hours. The growth phase of interest that is log and stationary phase were obtained successfully which were utilized in the rest of the experiments to identify the best conditions for yeast cell autolysis. Hence the cell growth during the logarithmic phase (15 hours) was chosen to study further due to the reason that molecules such as synthesis of RNA and other enzymatic changes occur in this phase.

## 4.2 Autolysis of Yeast cells

The effect of temperature on rate of autolysis of Yeast cell is important for enzymatic hydrolysis to produce 5'-GMP rich yeast extracts (Leutchenberger, 2005). Hence the yeast cells were autolysed with different parameters such as temperature and different time intervals to measure the protein content and corresponding dry weight of the yeast cells. The results of effect of temperature on the rate of autolysis of yeast cells during logarithmic phase (15 hours, obtained from growth curve) and stationary phase (24 hours obtained from growth curve) are shown in Table 2 (logarithmic phase) and Table 3 (Stationary phase).

Autolysis were carried out with temperature of 45 °C, 50 °C, 55 °C and 60 °C with time intervals 0 until 120 mins for different growth phases and their results are shown in Table 2 and 3. The absorbance values for the corresponding time points and the protein content (µg protein/ml) were calculated with help of Bradford constants. The standard graphs were shown in Appendix section.

Table 2: Showing the effect of temperature on rate of autolysis of yeast cells during logarithmic phase (15 hours)

| | protein content - µg protein/ml | | | |
|---|---|---|---|---|
| Time ( in mins) | 45°C | 50°C | 55°C | 60°C |
| 0 | 82.6 | 21.4 | 24.6 | 182.6 |
| 30 | 157.4 | 84.2 | 126.2 | 189.4 |
| 60 | 225 | 249.4 | 136.6 | 207 |
| 90 | 219.8 | 325.8 | 168.6 | 257 |
| 120 | 209 | 300.6 | 176.2 | 262.2 |

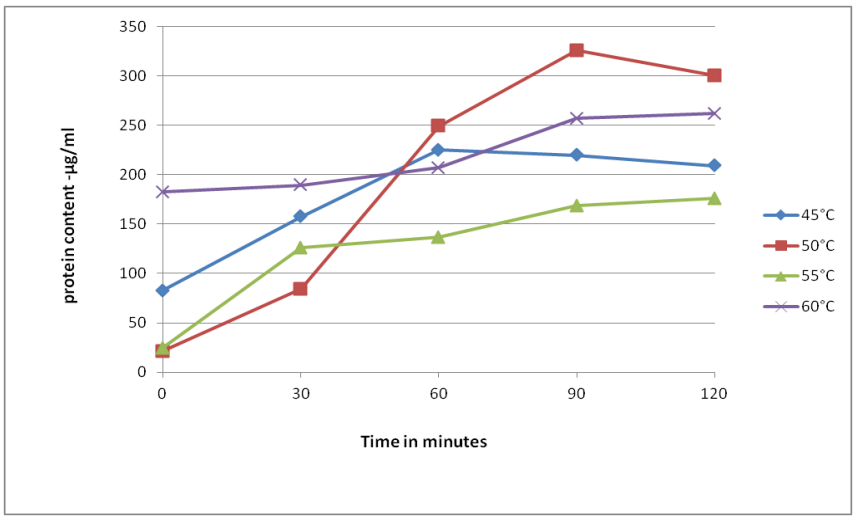


Figure 17: Showing the effect of temperature on rate of autolysis of yeast cells during logarithmic phase (15 hours). The primary vertical axis represents the amount of protein content and the horizontal axis represents the time in minutes

In the same way, the effect of temperature on the rate of autolysis of yeast cells during stationary phase was also determined and their results were shown in Table 3, showing the effect of temperature on the rate of autolysis of yeast cells during stationary phase and the amount of protein content in µg protein/ml.

Table 3: Effect of temperature on rate of autolysis of yeast cells during stationary phase (24 hours)

| | protein content - µg protein/ml | | | |
|---|---|---|---|---|
| Time (in Mins) | 45°C | 50°C | 55°C | 60°C |
| 0 | 48.2 | 43.4 | 59.8 | 302.2 |
| 30 | 65.4 | 89.8 | 117 | 213 |
| 60 | 86.6 | 118.2 | 130.2 | 230.2 |
| 90 | 133.8 | 137.8 | 177.4 | 225 |
| 120 | 161.8 | 150.2 | 150.2 | 261.4 |

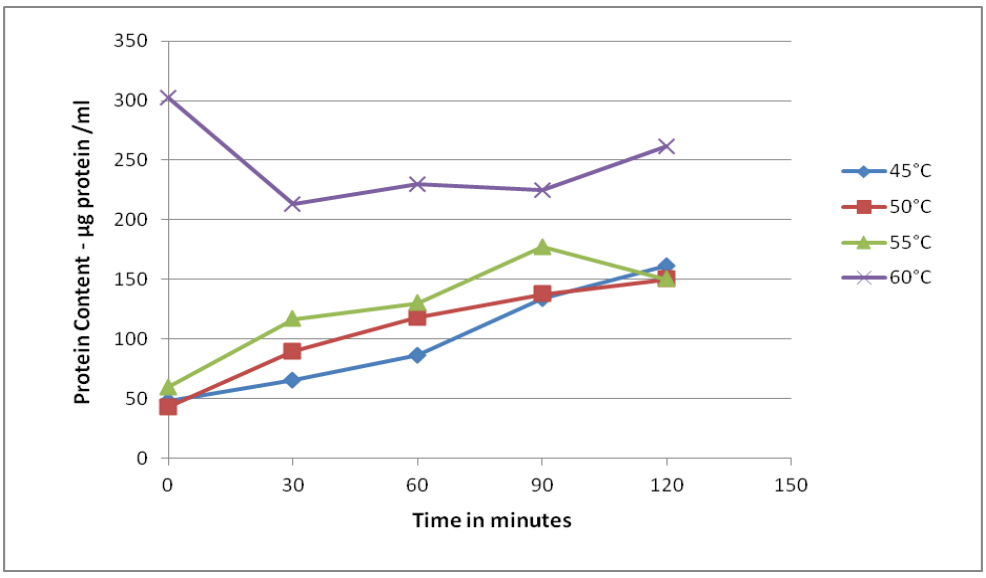


Figure 18: Showing the effect of temperature on rate of autolysis of yeast cells during stationary phase (24 hours). The primary vertical axis represents the amount of protein content and the horizontal axis represents the time in minutes.

It can be observed from the Table 2 that the maximum protein content (325.8 µg protein/ml) at 50 °C during the logarithmic phase-15 hours was higher while compared to the amount of protein content at various temperatures in stationary phase-24 hours. It is interesting to note that the amount of protein released during stationary phase at 60 °C is highest (302.2 µg protein/ml) among the other values. From the observation of graphs, effect of temperature on the rate of yeast cell autolysis at different growth phases it can be interpreted that the logarithmic phase is best suitable condition for the yeast cell autolysis at 50 °C. These parameters were noted for the enzymatic hydrolysis of yeast extract.

## 4.3 5'-PDE Enzyme Precipitation

Mung beans was germinated and 5'-PDE enzyme was extracted, partially purified and precipitated with ammonium sulphate to concentrations from 0%, 10%, 20%, 30% until 90%. The precipitation results together with 5'-PDE and 5'-PME enzyme activity, their specific activity and the ratio of PDE: PME were shown in Table 4. Later the crude enzyme was incubated for enzymatic assay together with substrate and the enzyme assay results are shown as below in Table 5. All the measurements for the experiment in enzymatic assay were performed in triplicate.

Mung beans soaked and germinated for three days showed the highest specific enzymatic activity of 0.45 U/mg. The partially purified 5'-PDE enzyme was precipitated with different concentrations of ammonium sulphate and measured the enzyme activity of 5'-PDE and its co-enzyme 5'-PME and their results are tabulated in table 4. It was identified that precipitation of partially purified enzyme around 60-70% of ammonium sulphate exhibited the maximum PDE activity of 0.572 U/ml with maximum enzyme recovery of 46.24%. The specific activity of 5'-PDE enzyme was 0.45 U/mg.

Similarly, the co-enzyme of 5'-PDE namely 5'-PME resulted in higher enzymatic activity of 35.94 U/ml at the same precipitation of around 60-70% with a recovery of 47.63% and with a specific enzymatic activity of 28.57 U/mg. An enzymatic assay experiment was carried out for 5'-PDE enzyme and their results are shown in table 5. Different protein concentrations were used in the enzyme assay experiments to determine at which concentration there is the maximum specific activity of 5'-PDE enzyme and their results were further utilized for enzymatic hydrolysis of yeast extracts.

The enzyme solution of 60% precipitate was taken in to consideration for 5'-PDE enzymatic assay. As we could see from Table 5, the 5'-PDE enzymatic activity of the crude enzyme exhibited the 0.107 μmol/ml where as the precipitated enzyme showed an enzymatic activity six times greater than the crude enzyme which is 0.628 μmol/ml. An experiment was carried out in parallel to determine the amount of protein content in the enzyme solution by Bradford method and their results are shown in table 6.

From the results, 5'-PDE enzyme with 60% precipitate exhibited a maximum protein content of 1216.6 µg/ml with a concentration of 1.217 µg/ml. Once the enzyme concentration and precipitation conditions were identified which is around 60% of ammonium sulphate concentration, the yeast cells were autolysed and then treated with 5'-PDE enzyme to produce the 5'-GMP yeast extracts.

Table 4: Showing the results of Ammonium Sulphate Precipitation of Partially purified 5'-PDE Enzyme

| Ammonium Sulphate Concentration % | volume (ml) | protein (mg/ml) | total protein (mg) | 5'-PDE Enzyme Activity | | | 5'-PME Enzyme Activity | | | Ratio (PDE:PME) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Activity (U/ml) | Specific activity (U/mg) | Recovery % | Activity (U/ml) | Specific activity (U/mg) | Recovery % | |
| 0 | 100 | 0.07 | 6.92 | 0.022 | 0.32 | 100 | 1.360 | 19.64 | 100 | 0.016 |
| 0-40% | 1.5 | 0.12 | 0.17 | 0.028 | 0.24 | 1.92 | 1.610 | 13.82 | 1.78 | 0.018 |
| 0-50% | 1.6 | 0.22 | 0.36 | 0.041 | 0.18 | 2.92 | 2.576 | 11.53 | 3.03 | 0.016 |
| 0-60% | 2.0 | 0.87 | 1.74 | 0.242 | 0.28 | 21.70 | 17.242 | 19.82 | 25.35 | 0.014 |
| 0-70% | 1.8 | 1.26 | 2.27 | 0.572 | 0.45 | 46.24 | 35.994 | 28.57 | 47.63 | 0.016 |
| 0-80% | 2.8 | 0.94 | 2.63 | 0.275 | 0.29 | 34.55 | 27.743 | 29.49 | 57.11 | 0.010 |
| 0-90% | 3.0 | 0.69 | 2.08 | 0.069 | 0.10 | 9.25 | 22.346 | 32.27 | 49.29 | 0.003 |

Note: For both the enzymes 5'-PDE and 5'-PME, Recovery (%) of the enzyme is the amount of enzyme extracted from after partial purification and calculated from the amount of total protein.

## 5'-PDE Enzyme Assay

Table 5: Showing the 5'-Phosphodiesterase enzyme activity of crude and precipitated enzyme

| | Absorbance Values | | | Constant[c] | | Dilution Factor[d] | Concentration 4-npp[e] | Enzyme Activity µmol/ml |
|---|---|---|---|---|---|---|---|---|
| 5'-PDE Enzyme | Control[a] | Incubated[b] | Incubated-Control | a | b | | | |
| Crude Enzyme | 0.086 | 0.709 | 0.623 | -0.011 | 17.766 | 1.000 | 0.036 | 0.107 |
| Precipitated Enzyme (60%) | 0.049 | 0.410 | 0.361 | -0.011 | 17.766 | 10.000 | 0.209 | 0.628 |

Note: a- control without the addition of 5'-PDE enzyme, b- samples incubated together with enzyme, c- constants obtained from the linear equation of the standard curve of PDE enzyme, d – Dilution Factor-how much the samples were diluted, e- concentration of the substrate.

## 4.4 Protein content Analysis by Bradford Method

The protein content in the 5'-Phospho Diesterase enzyme was analyzed parallel to the crude enzyme assay and the results of the concentration of the protein content are shown in table 6.

**Table 6: Showing the concentration of protein content in the 5'-PDE Enzyme analyzed by the Bradford technique**

| Samples | Absorbance Values | Bradford Constant | | Dilution fold | Protein content µg/ml | Concentration µg/ml |
|---|---|---|---|---|---|---|
| | | a | b | | | |
| Crude Enzyme | 1.039 | -0.034 | 0.003 | 1.000 | 429.000 | 0.429 |
| Crude Enzyme (10X diluted) | 0.058 | -0.034 | 0.003 | 10.000 | 364.667 | 0.365 |
| Enzyme Precipitate 60% | 0.271 | -0.034 | 0.003 | 10.000 | 1216.667 | 1.217 |

Note: The Bradford constants (a,b) were obtained from the linear equation obtained from standard graph. Refer Appendix B2

## 4.5 Autolysis at 50 °C

From the effect of temperature and rate of autolysis at logarithmic phase (15 h) and stationary phase (24 h), the amount of protein content released is highest at 50 °C for log phase cells. Hence, in order to study in detail the amount of protein content at 50 °C with different time intervals such as from 0 until 30 minutes, autolysis was continued. The table 7 shows the results of autolysis at 50 °C and their corresponding protein content. The amount of protein content released during autolysis at 50 °C is higher when compared to longer autolysis time, due to the reason that the addition of 5'-PDE enzyme during the process of autolysis.

**Table 7: Shows rate of autolysis of yeast cells at 50°C during logarithmic phase with 0 until 30 minutes**

| Autolysis at 50 °C | |
|---|---|
| Time (in min) | **µg protein/ml** |
| 0 | 22 |
| 10 | 72 |
| 20 | 108.3 |
| 30 | 105.6 |

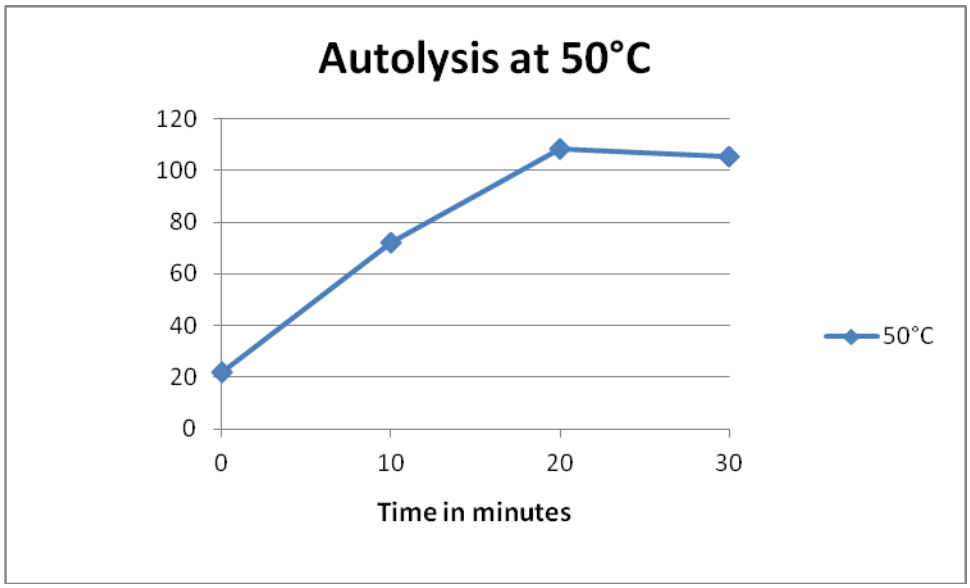


Figure 19: Shows the effect of temperature (50 °C) on the rate of autolysis from 0 to 30 minutes

From the results of effect of temperature on rate of autolysis of yeast cells during logarithmic phase, it was further analyzed with detailed time points (0 to 30 minutes) with optimum temperature (50 °C) identified through previous experiments to measure the amount of protein content. The results exhibit that autolysis at 50 °C and with 20 minutes, it produces high amount of protein content – 802.5 µg protein/ml. The effect of 5'-PDE enzyme during autolysis was also analyzed and the results show that higher amount of soluble protein content in treatment with 5'-PDE enzyme. As we could see from figure 20, autolysis with 5'-PDE enzyme exhibited higher protein content.

## 4.6 Effect of 5'-PDE enzyme

Once the parameters suitable for autolysis of yeast cell were identified, yeast extract was later examined with and without the presence of 5'-PDE enzyme during autolysis. The effects of enzyme at 50 °C were studied and the results are shown as below. The yeast cells were suspended in 50 mM Acetate buffer before autolysing at 50 °C. The results were shown in Table 8, 9 and in Figure 20.

Table 8: Showing autolysis at 50 °C with 5'-PDE Enzyme

| Time in mins | A 595 nm | Soluble Protein Content - µg protein/ml |
|---|---|---|
| 0 | 0.233 | 89 |
| 10 | 0.756 | 263 |
| 20 | 0.743 | 259 |

Table 9: Showing autolysis at 50 °C without 5'-PDE Enzyme

| Time in mins | A595 nm | Soluble Protein Content- µg protein/ml |
|---|---|---|
| 0 | 0.106 | 46.7 |
| 10 | 0.193 | 75.7 |
| 20 | 0.187 | 73.7 |

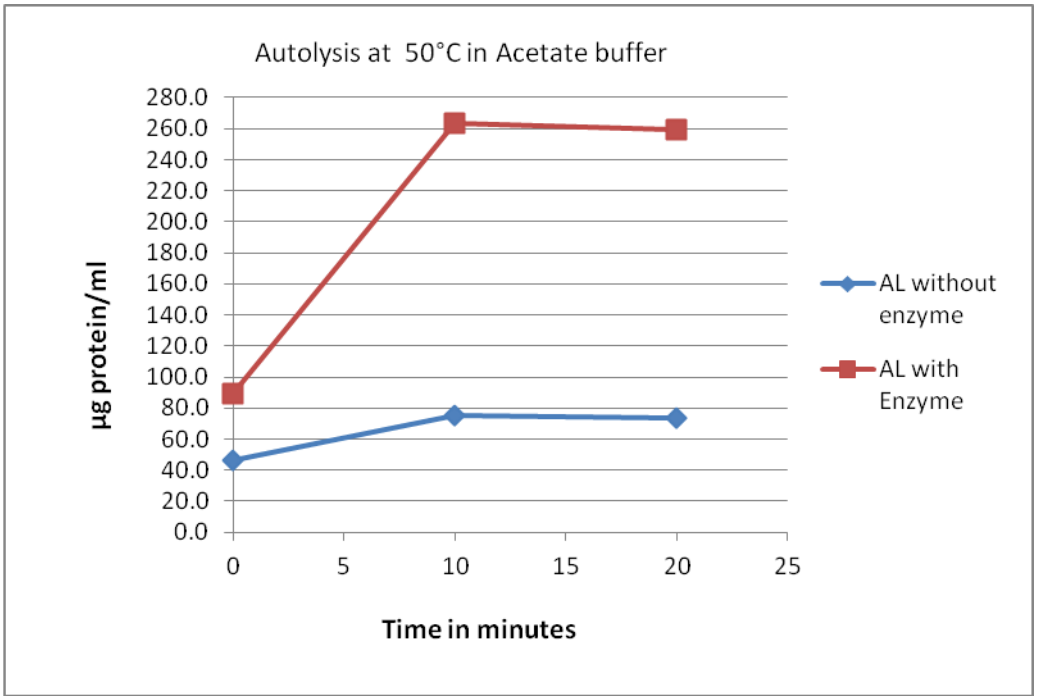


Figure 20: Effect of Autolysis in Acetate Buffer with and without 5'-PDE Enzyme

The amount of protein released during autolysis at 50 °C together with 5'-PDE enzyme is three times higher when compared to autolysis without addition of enzyme. From the results in the table 8 & 9, it is clear that autolysis together with enzymatic treatment (enzymatic hydrolysis) results in higher amount of protein content.

## 4.7 Enzymatic Hydrolysis

The yeast extract containing 5'-GMP was hydrolyzed by adding 5'-PDE enzyme (60% precipitate) together during autolysis of yeast cell extract at optimum parameters identified through above autolysis experiments. Before hydrolyzing the yeast extract, it was suspended in 50 mM Acetate buffer in pH 5 and then autolysed at 50 °C and the results of enzymatic hydrolysis are shown in Figure 21 and Table 10. The amount of soluble protein content was analyzed using Bradford solution as a standard and expressed in µg protein/ml.

Table 10: Showing the amount of soluble protein content after enzymatic hydrolysis of yeast cell extract

| 50°C<br>Time (in mins) | protein content - µg protein/ml |
|---|---|
| 0 | 18.5 |
| 10 | 27.0 |
| 20 | 30.8 |
| 30 | 42.0 |

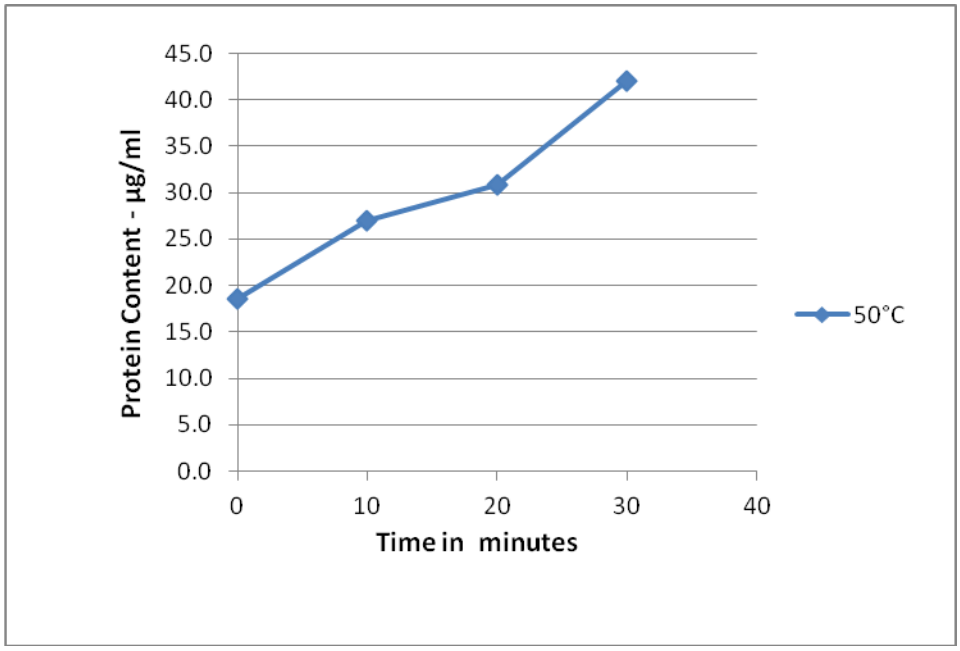


Figure 21: Showing the curve of enzymatic hydrolysis of yeast extract at 50 °C

## 4.8 5'-GMP Analysis by HPLC

In parallel to the enzymatic hydrolysis of 5'-GMP rich yeast extract, amount of nucleotide content (5'-GMP) were analyzed by High Performance Liquid Chromatography Technique and the corresponding chromatogram results were shown in the following figures. The amount of 5'-GMP was expressed in ppm. GMP commercial (250 ppm) were used as a standard. The concentrations of 5'-GMP in the hydrolyzed samples were calculated based on their peak area by comparing the peak area of the standard GMP. The results of the chromatogram are shown in Figure 22 until figure 26 and the areas of the samples with retention time were resulted in table 11.

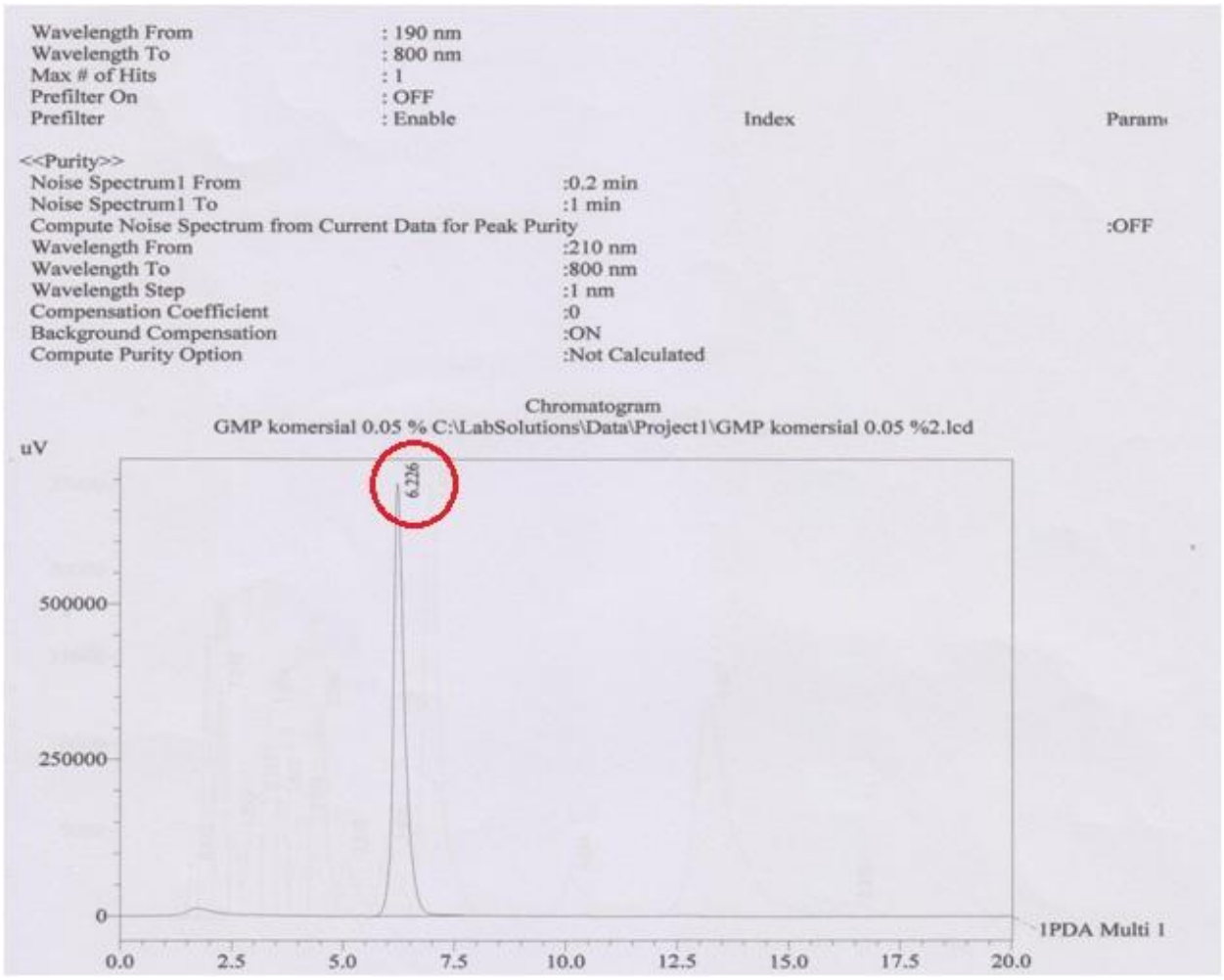


Figure 22: Chromatogram of GMP standard commercial, highlighted in red

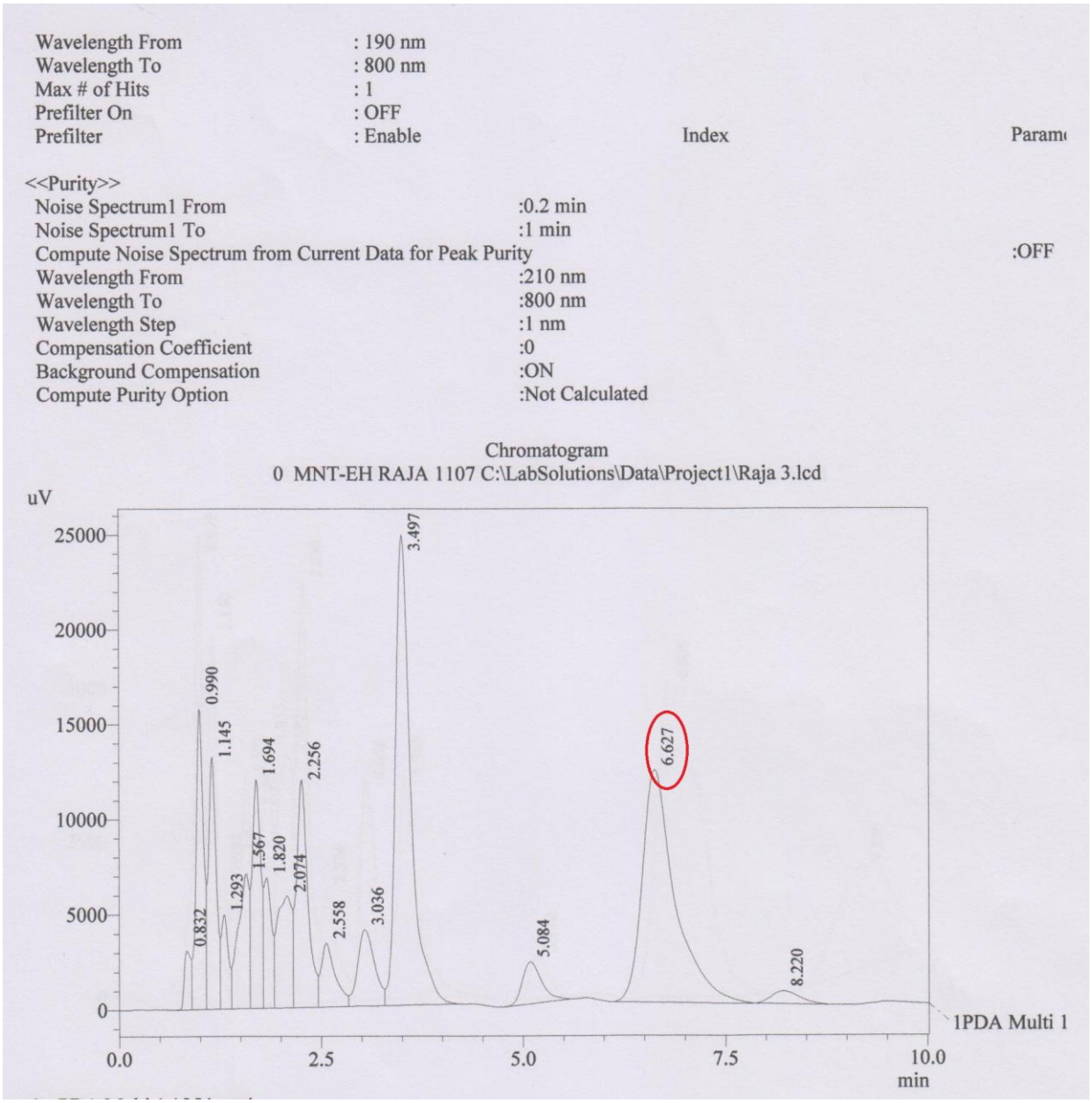


Figure 23: Chromatogram of 0 minute sample (control) without enzymatic treatment, the peak are of GMP is marked in red

| | | | |
|---|---|---|---|
| Wavelength From | : 190 nm | | |
| Wavelength To | : 800 nm | | |
| Max # of Hits | : 1 | | |
| Prefilter On | : OFF | | |
| Prefilter | : Enable | Index | Param |

<<Purity>>

| | | |
|---|---|---|
| Noise Spectrum1 From | :0.2 min | |
| Noise Spectrum1 To | :1 min | |
| Compute Noise Spectrum from Current Data for Peak Purity | | :OFF |
| Wavelength From | :210 nm | |
| Wavelength To | :800 nm | |
| Wavelength Step | :1 nm | |
| Compensation Coefficient | :0 | |
| Background Compensation | :ON | |
| Compute Purity Option | :Not Calculated | |

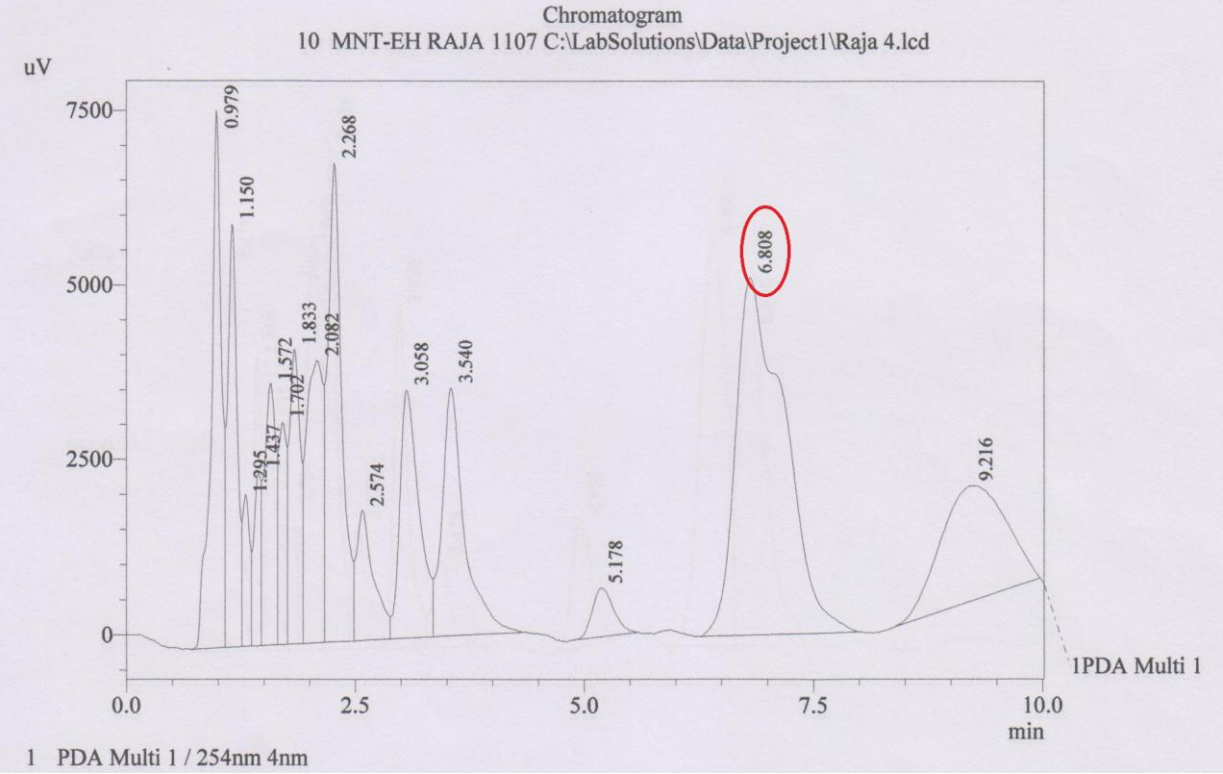


Figure 24: Chromatogram of 10 minute sample with enzymatic treatment

| | | | |
|---|---|---|---|
| Wavelength From | : 190 nm | | |
| Wavelength To | : 800 nm | | |
| Max # of Hits | : 1 | | |
| Prefilter On | : OFF | | |
| Prefilter | : Enable | Index | Param |

<<Purity>>

| | | |
|---|---|---|
| Noise Spectrum1 From | :0.2 min | |
| Noise Spectrum1 To | :1 min | |
| Compute Noise Spectrum from Current Data for Peak Purity | | :OFF |
| Wavelength From | :210 nm | |
| Wavelength To | :800 nm | |
| Wavelength Step | :1 nm | |
| Compensation Coefficient | :0 | |
| Background Compensation | :ON | |
| Compute Purity Option | :Not Calculated | |

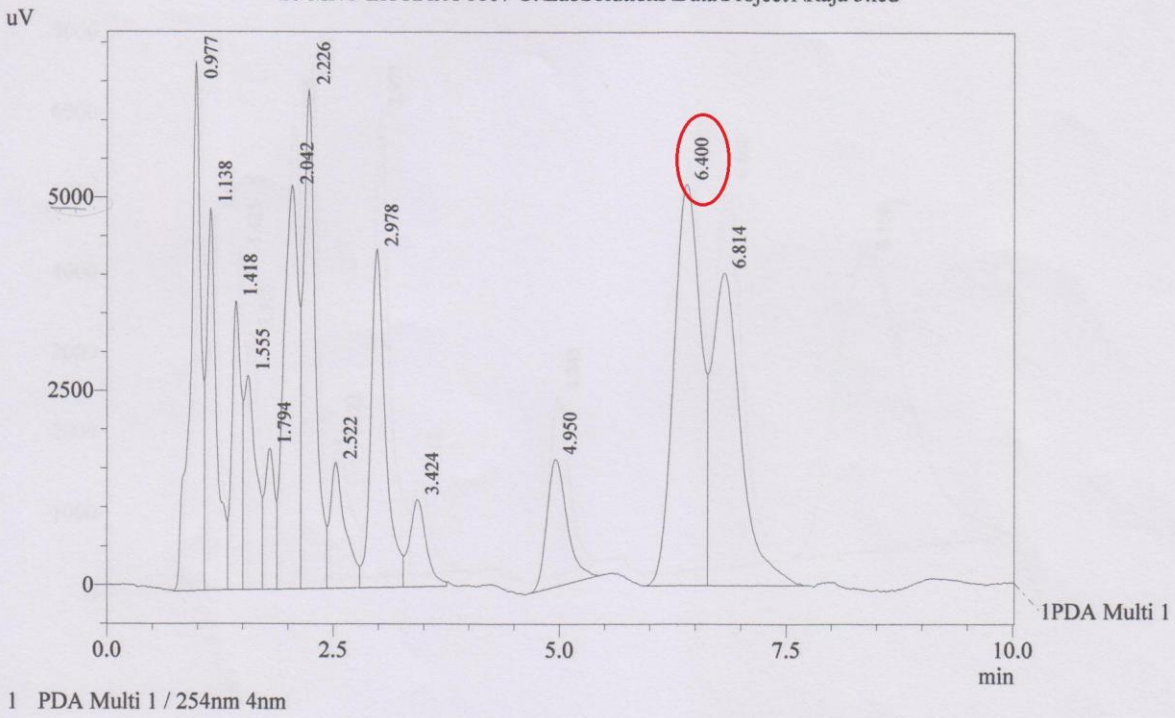


Figure 25: Chromatogram of 20 minute sample with 5'-PDE enzymatic treatment

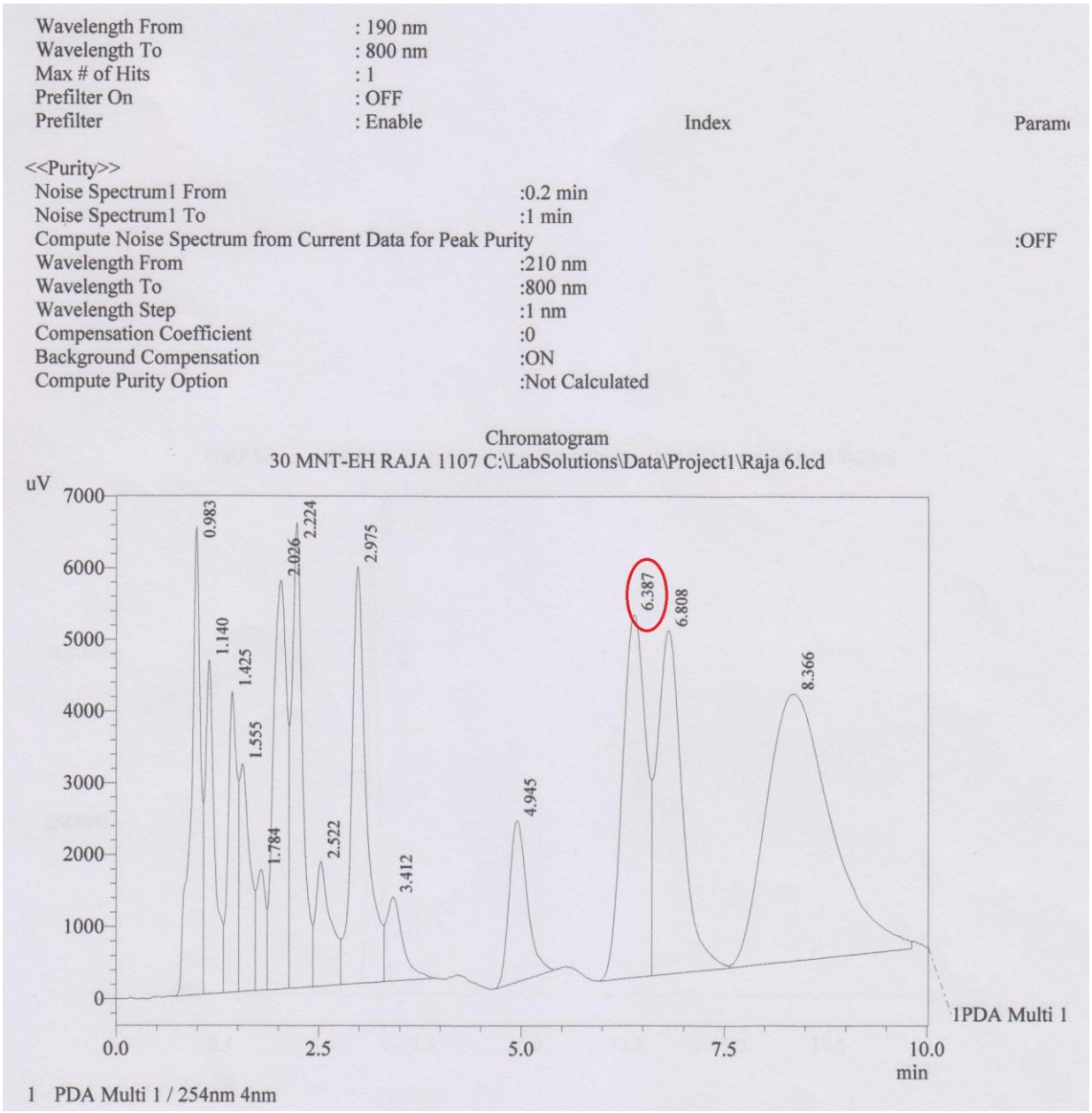


Figure 26: Chromatogram of 30 minute sample with 5'-PDE enzymatic treatment

From the chromatographic results, the peak area of the GMP standard commercial from the figure 22 were analyzed and were compared to the other peak areas of the 5'-PDE enzyme treated samples (figure 23-26). And the concentration of 5'-GMP in the yeast extract were calculated with below formula and the results are shown in below Table 11 and a graph illustrating were shown in Figure 27.

Table 11: showing the 5'-GMP concentration from the rich yeast extracts after enzymatic treatment.

| EH sample (time in mins) | Retention time | Peak Area | Concentration of 5'-GMP (ppm) |
|---|---|---|---|
| GMP Commercial | 6.226 | 12292272 | - |
| 0 | 6.6627 | 343750 | 13.982 |
| 10 | 6.808 | 188836 | 7.68 |
| 20 | 6.4 | 106776 | 4.343 |
| 30 | 6.387 | 102810 | 4.1818 |

Note: EH – Enzymatic Hydrolysis sample

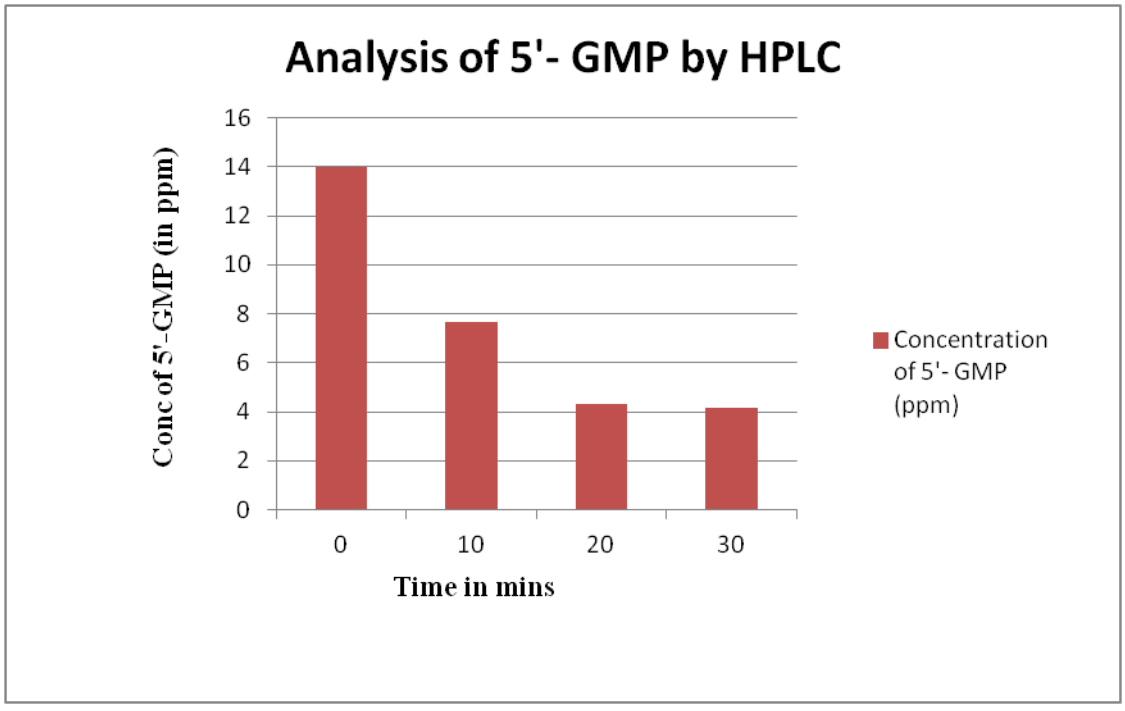


Figure 27: showing the 5'-GMP concentration in ppm with different time points

With optimum conditions identified from previous experiments i.e., autolysing at 50 °C during the 15 hours of growth of yeast cells, autolysis together with enzymatic hydrolysis were carried out and the soluble protein content were analyzed by Bradford technique and the results are tabulated in table 10 and figure 21. As the graph in the figure 21 displays the amount of protein content is higher during 30 minutes which is 703 µg protein/ml. However all the samples treated with enzyme were further analyzed by HPLC: It was noted that, autolysis at 50 °C together with 5'-PDE enzyme, the amount of protein content seems to increase while compared to previous experiments. We can interpret that enzymatic treatment results in higher levels of nucleotides. From the results of chromatogram figure 22 until figure 26, the concentration of 5'-GMP in the yeast extracts were calculated by comparing the peak area of the standard GMP commercial and the results are displayed in table 11. Here 0 minute sample was used a control (without adding enzyme). It is very interesting to note that the level of 5'-GMP in the yeast extract decreases. However, it is difficult to say that 5'-GMP is present in the yeast extract due to the reason that the sample has lot of impurities and the peak area is much larger which cannot be compared with the standard commercial.

The bar graph from the figure 27 clearly indicated there is a decrease in the level of 5-GMP in the yeast extract after enzymatic treatment. The presence of coenzymes such as 5'-PME affects the outcome as it could be seen from figures 22-27, where it is difficult to confirm the presence of 5'-GMP in the yeast extract. However, one could conclude that the during the enzyme extraction, precipitation and purification process, the byproduct of phosphor Diesterase enzyme, that is phosphor mono esterase might have influenced the enzymatic hydrolysis reaction. Overall, it seems that autolysis of yeast cell suspension with optimum conditions identified through the experiments, followed by the 5'-PDE enzymatic treatments results in the significant level of 5'-GMP content.

# 5. Conclusion

The general conclusions that can be drawn are,

- The growth phase of yeast cell culture during logarithmic phase (15 hours) yielded high levels of protein content.
- Autolysis at 50 °C during the logarithmic phase is best condition to autolyse yeast cells.
- The enzyme solution of 60-70% saturation of ammonium sulphate precipitation resulted in highest enzymatic activity.
- Autolysis together with 5'-PDE enzymatic treatments results in high level of yeast extracts containing 5'-GMP.

Question to be asked is countless. Few might be

- Is there an influence of PME enzyme during the enzymatic hydrolysis of yeast extract?
- What could be done to inhibit the co enzyme activity?
- How the presence of impurities in the yeast extract can be eliminated?

# 6. Future Work

Since the level of 5'-GMP in the yeast extract results from the enzymatic hydrolysis of yeast, one could try to inhibit the 5'-PME enzyme activity. This can be carried out by addition of an inhibitor during enzymatic hydrolysis which inhibits the co enzyme activity. Also, optimization process with the parameters concluded above can be carried out to produce higher level of 5'-GMP in the yeast extracts. It is also beneficial to optimize the growth parameters for the large scale production of 5'-GMP flavor enhancers.

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# 8. Appendix

### A. Growth Media, Buffers and Substrate Solutions

All the growth media prepared were sterilized in an autoclave at 121 °C for 90 minutes to avoid any bacterial or fungal contamination. All the mediums, buffers and solutions were prepared with double distilled water.

A.1 PGY Agar medium

For preparing 1 litre of PGY Agar medium, following chemicals were added to a beaker while stirring

Peptone – 7.5 g
Yeast Extract – 4.5 g
Glucose – 20 g
Agar – 15 g

The medium was transferred to small test tubes and then autoclaved. The Agar slant was prepared by heating the medium at 95 °C, and then inclined at 20 degrees for up to 20 minutes.

A.2 Growth medium – Yeast Cell culture

The growth media for yeast cell growth was prepared with following chemicals below and they are dissolved in one litre of distilled water.

Glucose - 50 g
Yeast Extract - 5 g
Di Ammonium Sulphate ($(NH_4)_2SO_4$) – 1 g
Potassium di hydrogen phosphate ($KH_2PO_4$) – 1 g
Di potassium hydrogen phosphate ($K_2HPO_4$) – 1 g
Magnesium Sulphate ($MgSO_4$) – 1 g

The pH was adjusted to 4 by adding glacial lactic acid (5 ml for 1 litre)

A.3 1 N Sodium Chloride solution (0.85% NaCl)

To prepare a volume of 100 ml of 1 N NaCl solution, 0.85 g was dissolved in 100 ml of distilled water.

A.4 0.1 M Acetate Buffer (pH 5)

Volume - 100 ml

A – 14.8 ml of 0.2 M solution of Acetic Acid
B – 35.2 ml of 0.2 M solution of Sodium Acetate

A and B were mixed in a beaker and 50 ml of distilled water was added to make a volume of 100 ml to give a concentration of 0.1 M

A.5 Ammonium Sulphate Precipitation

5.67 g of powdered ammonium sulphate was added to give a saturation of 20% in 50 ml of the enzyme filtrate. Likewise the amount was calculated to give various saturations as listed in Table 4.

A.6 10 mM 4-nitrophenyl phosphate di sodium salt hexahydrate

This solution was prepared by dissolving 0.09034 g in 25 ml of Acetate buffer to give a concentration of 10 mM.

A.7 5% Sodium Carbonate

To prepare 5% of Sodium carbonate solution, 5 g was dissolved in 100 ml of distilled water

**B. Standard Graphs**

B.1 Bradford Solution & Standard Graph

To prepare 1000 ml of the Bradford Reagent, dissolve 100 mg of Coomassie blue in 50 ml of 95% Ethanol and then add 100 ml of 50% Phosphoric Acid in a 1 litre measuring beaker. The volume was made to 1000 ml by adding distilled water. The coefficients of the equation were used as a Bradford constant to calculate the protein content in the solutions.
Standard curve for Bovine Serum Albumin for soluble protein content analysis

Table: Average of Absorbance values at 595 nm with various concentrations of BSA

| Standard curve of BSA for soluble protein content | | | |
|---|---|---|---|
| Blank = 1.020 | Absrorbance Values at 595 nm | | |
| Concentration of BSA mg/ml | 1 | 2 | Average |
| 0 | 0 | 0 | 0 |
| 20 | 0.017 | 0.012 | 0.0145 |
| 40 | 0.045 | 0.04 | 0.0425 |
| 60 | 0.136 | 0.136 | 0.136 |
| 80 | 0.158 | 0.142 | 0.15 |
| 100 | 0.209 | 0.209 | 0.209 |
| 120 | 0.23 | 0.223 | 0.2265 |
| 140 | 0.371 | 0.367 | 0.369 |
| 160 | 0.38 | 0.37 | 0.375 |
| 180 | 0.426 | 0.394 | 0.41 |
| 200 | 0.505 | 0.482 | 0.4935 |
| 220 | 0.472 | 0.48 | 0.476 |

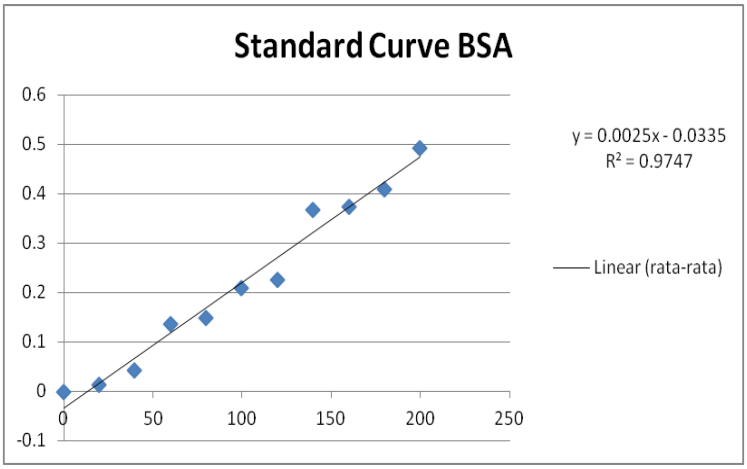


Figure: showing the standard curve of BSA for measuring the protein content

## B.2 Standard graph of p-nitrophenol

Table: showing the absorbance values at 400 nm for the concentration of p-nitrophenol

| Standard Curve for PDE | |
|---|---|
| Concentration p-nitrophenol (µmol/ml) | Absorbance - 400nm |
| 0 | 0 |
| 0.0025 | 0.028 |
| 0.005 | 0.076 |
| 0.01 | 0.157 |
| 0.02 | 0.349 |
| 0.04 | 0.7 |

A graph was plotted based on the absorbance values above and shown in below figure.

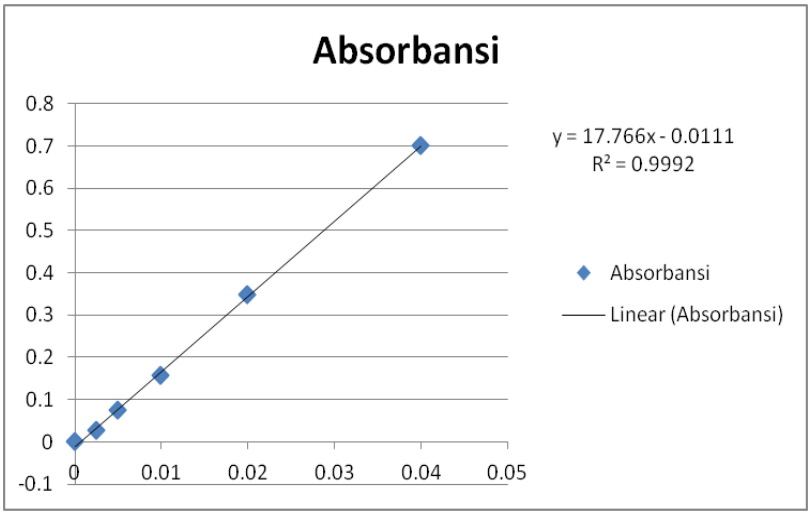


Figure: showing the standard curve for PDE enzyme. The constant values from the equation shown in the figure were used as constant to calculate the concentration.

**C. Process Work flow and procedure**

C.1 Yeast cell culture (Growth curve of S.cerevisiae)

- A loop of the yeast strain in Agar slant was inoculated in to 125 ml of growth media(500 ml Erlen meyer flask), incubated in a shaking water bath at 30 °C, 70 rpm for 30 hours.
- The absorbance values for the cell culture were noted every 3 hours until there observed the decline in the cell growth.
- The dry weight of the yeast cell at each time point can be calculated from the standard curve of absorbance and biomass dry weight.

Once the different growth phases of the cell culture were identified, the next step is to identify the rate of autolysis in logarithmic phase and stationary phase with different growth parameters.

C.2 Effect of temperature on rate of yeast cell autolysis

- Yeast strain was inoculated into 900 ml growth medium (3*300 ml in three different 1 L culture flasks)
- Incubated in shaking water bath for 15 hours (logarithmic phase) until the Absorbance at 660 nm is approximately 2 Å (30 °C, 70 rpm)
- The yeast cells were centrifuged in a table ultracentrifuge at 4500 rpm, 4 °C for 20 minutes, later the supernatant were discarded.
- The cell pellet were washed once with 0.85% 1 N Sodium Chloride solution and suspended in the same.
- The yeast cell suspension was then autolysed in a water bath at different temperatures ( 45 °C, 50 °C, 55 °C, 60 °C) and samples were withdrawn at different time intervals (0 until 120 minutes – every 30 minutes).
- 0.5 ml of the sample were taken in a small eppendorf tube and centrifuged at 4500 rpm for 20 minutes at 4 °C.

- The cell pellets were dried at 105 °C and the 0.1 ml of supernatant was analyzed for the soluble protein content by Bradford technique. (The sample was prepared by adding 0.1 ml of the autolysed sample together with 1 ml of Bradford Reagent and then measured the absorbance). A blank was also prepared without adding the sample to the Bradford solution.

Note: The same procedure and work flow was carried out to determine the effect of temperature on rate of yeast cell autolysis during the stationary phase except that the cells were grown until 24 hours. In the same way to determine the effect of enzyme on rate of autolysis, the procedure was followed for the experiments together with 5'-Phospho Diesterase enzyme.

C.3 Enzymatic hydrolysis

- Yeast strain was inoculated into 45 ml of growth media for 8 hours at 30 °C with 70 rpm and later transferred to a 700 ml (2*350 ml) of the medium for large scale culture.
- The cell growth was carried out for 15 hours (log phase) or 24 hours (stationary phase) depending upon the growth phase of interest.
- The yeast cells were later centrifuged at 4500 rpm for 20 minutes at 4 °C. The supernatant was discarded and the cell pellet was washed with 0.85% 1N Nacl solution and the cells were later suspended in 50 mM Acetate buffer pH 5.
- Autolysis is carried out in a water bath maintained at optimum temperature and then 5'-PDE enzyme was added to the yeast cell suspension and hydrolysis were carried out at 50 °C, and the samples were withdrawn every 10 minutes upto 30 minutes.
- The enzyme can be inactivated by heating the sample at 90 °C for 2-3 minutes.
- This sample was later taken in to HPLC analysis to determine the concentration of 5'-GMP in the yeast extracts.