Spring 2005

Optimization of sequential purification of beta-glucosidase from tricoderma reesei in aqueous two-phase system

Shalini Gautam
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The Van Houten library has removed some of the personal information and all signatures from the approval page and biographical sketches of theses and dissertations in order to protect the identity of NJIT graduates and faculty.
A novel sequential technique was developed for the purification of a valuable enzyme, beta-glucosidase, from microorganism *Tricoderma reesei*. The fungus *T. reesei* produces cellulose degrading enzymes, called cellulases: beta-glucosidase, endo-glucanase and exo-glucanase and low molecular weight proteins. For specific applications, the enzyme must be separated from other contaminants. The sequential technique, that included affinity precipitation with chitosan followed by separation with an aqueous two-phase system (ATPS), was implemented for the purification of beta-glucosidase from the culture filtrate of *T. reesei*.

The cultivation medium (nutrient) was optimized for the production of beta-glucosidase from *T. reesei* cell culture. Treatment of the crude extract of *T. reesei* with chitosan resulted in the precipitation of endo and exo-glucanases. During this separation step, beta-glucosidase activity was completely recovered in the supernatant. The enzyme was further purified from other proteins by partitioning in aqueous two-phase systems. Preliminary investigation with pure beta-glucosidase showed that the ATPS composed of PEG 4000, Potassium Phosphate salt and water is the best system for extracting the enzyme. The influences of system conditions, such as system pH and temperature, on the partition coefficients of beta-glucosidase and total proteins were evaluated in order to determine the most favorable condition for the purification of the enzyme from the
culture filtrate. For the range of pH (6.0-7.5) and temperature (25-55 °C) studied, a positive correlation was obtained between these two variables and the partition coefficients.

The development of reliable tools, that can predict equilibrium phase compositions and the partitioning behavior of the system components, is critical for protein purification in ATPS. Artificial Neural-Network models (ANN) offered a remarkable performance to predict equilibrium phase compositions and beta-glucosidase partition coefficients. In addition, the pilot plant study with the culture filtrate was carried out in a continuous two-stage counter-current aqueous two-phase extractor system. The pilot plant experiments demonstrated the feasibility of the continuous counter current extraction process of ATPS for large-scale purification of beta-glucosidase.
OPTIMIZATION OF SEQUENTIAL PURIFICATION OF
BETA-GLUCOSIDASE FROM TRICODERMA REESEI IN AQUEOUS
TWO-PHASE SYSTEM

by
Shalini Gautam

A Dissertation
Submitted to the Faculty of
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in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in Chemical Engineering

Otto H. York Department of Chemical Engineering

May 2005
OPTIMIZATION OF SEQUENTIAL PURIFICATION OF
BETA-GLUCOSIDASE FROM TRICODERMA REESEI IN AQUEOUS
TWO-PHASE SYSTEM

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"Purification of beta-glucosidase from Trichoderma reesei by sequential precipitation with reversible soluble-insoluble polymers and aqueous two-phase systems", AIChE conference, Nov. 8, 2004, Austin, TX.

S. Gautam and L. Simon,

S. Gautam and L. Simon,
"Artificial neural networks for the prediction of recombinant tissue-type plasminogen activator partition coefficients", AIChE conference, Nov. 6, 2002, Indianapolis, IN.

L. Simon and S. Gautam,
S. Gautam and L. Simon,
"Prediction of equilibrium phase compositions and \(\beta\)-glucosidase partition coefficient in aqueous two-phase systems", submitted to Chemical Engineering Communications.
To my beloved family for their love, support and encouragement
ACKNOWLEDGEMENT

Looking back at the time when I started my PhD at NJIT, I remember the moments of excitement and joy of being in a new country and a new environment. Although I should also admit that it made me a bit nervous, initially. But by God's grace, I had an opportunity to meet and associate with excellent people during this journey of four years, who not only have helped me in different endeavors but have also influenced me and have to a certain extent shaped my personality. I would begin by expressing my deepest gratitude to my advisor, Prof. Laurent Simon, who introduced me to this exciting area of research and provided me with most valuable guidance all through my doctoral studies. His perseverance, sincerity and hard working qualities will always serve as an inspiration to me.

Next, I would like to thank all my committee members, Prof. Marino Xanthos, Prof. Norman Loney, Prof. Dana E. Knox and Prof. Sanjay Malhotra, for providing me with valuable suggestions and fruitful discussions, leading to the present understanding of the topic. I would also like to thank the entire faculty in the Chemical Engineering department for teaching me the courses and helping me understand the fundamentals. I would also like to thank Mr. Thomas Boland for helping me with my research.

Although it is not possible to list all the wonderful people whom I have come across during these four years, some of the names that need special mention are Amit, Georgia, and Soniya. I will always cherish their friendship and will remember the great moments spent with my friends that made my stay at NJIT even more enjoyable.
Finally, I would like to thank my family for providing me with their unending love and support. It is hard to express my feelings in words for them. I wish that I can achieve and fulfill their dreams.
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<td>$T$</td>
<td>Absolute temperature (K)</td>
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<tr>
<td>$\Delta H_m$</td>
<td>Enthalpy change upon mixing (kJ)</td>
</tr>
<tr>
<td>$\Delta S_m$</td>
<td>Entropy change upon mixing (kJ)</td>
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<td>$\Delta G_m$</td>
<td>Gibb’s free energy (kJ)</td>
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<tr>
<td>$TLL$</td>
<td>Tie-line length</td>
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<tr>
<td>$K$</td>
<td>Partitioning coefficient</td>
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<td>$C_{top}$</td>
<td>Protein concentration in the top phase (g/ml)</td>
</tr>
<tr>
<td>$C_{bottom}$</td>
<td>Protein concentration in the bottom phase (g/ml)</td>
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<tr>
<td>$k$</td>
<td>Boltzmann’s constant</td>
</tr>
<tr>
<td>$n$</td>
<td>number of polymer or solvent molecules on the lattice</td>
</tr>
<tr>
<td>$p$</td>
<td>relative size of polymer with respect to solvent</td>
</tr>
<tr>
<td>$V$</td>
<td>specific molar volume of the mixed solution with respect to water</td>
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<td>$r$</td>
<td>volume parameter of component</td>
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<td>$f$</td>
<td>transfer function</td>
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<td>$b_j$</td>
<td>Bias of the unit $j$</td>
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<td>$a_i$</td>
<td>output of the unit $j$</td>
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<td>refractive index of the solution</td>
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<td>$W_s$</td>
<td>weight percent of potassium phosphate salt</td>
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<td>$W_p$</td>
<td>weight percent of PEG</td>
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<td>$d$</td>
<td>desired output</td>
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<td>$w_{ij}$</td>
<td>weight of the link connecting unit $i$ to unit $j$</td>
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<td>$V_T$</td>
<td>volumes of the top phase (ml)</td>
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<tr>
<td>$V_B$</td>
<td>volumes of the bottom phase (ml)</td>
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<td>$Y$</td>
<td>yield of beta-glucosidase</td>
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<th>Greek Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>selectivity coefficient</td>
</tr>
<tr>
<td>$\Delta \mu$</td>
<td>chemical potentials of the species</td>
</tr>
<tr>
<td>$\phi$</td>
<td>fraction of the lattice site occupied by the molecule</td>
</tr>
<tr>
<td>$\chi$</td>
<td>Flory interaction parameter</td>
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</table>
CHAPTER 1
INTRODUCTION

Biotechnological processes are environmentally friendly and utilize renewable resources to reduce the consumption of fossil resources, water and energy. Various enzymes are used in biological treatments. Enzymes are high molecular weight proteins secreted by living organisms. They are used to catalyze biochemical reactions (Hajny, 1969). Advances in genetic engineering have produced a large number of enzymes. Recently the cellulase enzyme, beta-glucosidase, has received attention due to its application in a variety of basic biological processes. The enzymatic catalysis of beta-glucosidase is implicated in biomass conversion, ethanol petrochemical production, development and regulation of chemical defense against pathogen attack, cellular signaling, growth and development in plants, and in other biotechnological applications (Cicek, 1999).

The commercial production of enzymes from microorganisms is rapidly increasing because of advances in separation techniques. Purification of the desired protein from the contaminated complex mixture accounts for 50-80% of the production cost (Pietruszka, 2000). However, in spite of these recent developments, most methods of protein separation are time consuming, costly and not applicable for large-scale operations (Scopes, 1994). New separation technologies that will decrease enzyme purification cost and improve the yield are indispensable prerequisites to expand the market for industrial enzymes.

Partitioning in aqueous two-phase system (ATPS) is an emerging technique, which has found applications in purification of various proteins and enzymes. The technique is inexpensive and meets the requirement of purity and selectivity. The
simplicity of the process and the low cost of the phase forming materials make it feasible for large-scale protein purification using appropriate scale-up techniques (Hustedt, 1988). However, the development of reliable tools, that can predict the partitioning behaviors of the system components and help in the selection of favorable conditions for large-scale protein purification, is critical.

Thermodynamic models have been developed to describe ATPS and to predict the partition coefficient of biomolecules present in the systems (Furuya, 1996; Sargantanis, 1997; Lin, 2003). Although these models have enhanced our understanding, their major limitations are their inability to predict system behavior and complex interactions among components of an aqueous biphasic system under various conditions. Artificial neural networks have the potential to overcome these disadvantages (Patnaik, 1999). Due to their nonparametric nature, these networks can account for intermolecular interactions and predict biomolecule partitioning in ATPS under different conditions (e.g. temperature and pH) (Baughman, 1994; Gao, 2002).
2.1 Beta-glucosidase

Beta-glucosidase is a major group among glycosylhydrolase enzymes with a molecular weight of 71 kDa and an isoelectric pH of 8.7 (Chen, 1992). The enzyme is found in seeds, plants, yeasts and is produced by many other microorganisms (Bhatia, 2002). The enzyme is also present in kidney, liver and intestines of mammals (Gascoigne, 1960). Beta-glucosidase catalyzes selective cleavage of glucosidic bonds by hydrolysis (Zouhar, 2001), functions at low pH values and is very stable (78% activity recovered after 145 h at pH 3.5 and 30 °C) (Gueguen, 1996).

Beta-glucosidase is a highly reactive enzyme used to catalyze the reaction of cellulose degradation to glucose by hydrolysis. This hydrolysis is utilized to produce various valuable products such as ethanol, petrochemicals, stone-washed textile and recycled paper etc. (Hajny, 1969). Existing hydrolysis techniques of cellulose degradation using chemicals are not very efficient due to high pressure and acid corrosion. Also, the production of side-products decreases the yield. Compared to a chemical catalyst, an enzyme is completely biodegradable and does not produce any pollutant after completion of the reaction. Beta-glucosidase is more efficient than an acid catalyst, as the energy requirement for the enzymes is less (Gascoigne, 1960). In addition, enzymatic reactions are more specific than chemical reactions. Enzymatic hydrolysis-based processes are conducted under mild conditions of temperature, pressure and pH, thus making recycling possible.
The cellulose degradation is carried out by the synergistic action of three cellulases (a group of enzymes to hydrolyze cellulose): (i) endo-β-glucanase, (ii) cellobiohydrolase (exo-β-glucanase) and (iii) β-glucosidase (Brumbauer, 1999). First, cellulose is converted to cellobiose by catalysis of endo, exo-glucanases. Second, β-glucosidase converts cellobiose into glucose. The rate-limiting step in cellulose degradation is catalyzed by beta-glucosidase (Juhasz, 2003). The rate of cellulose hydrolysis can be increased by supplementing commercial cellulase with pure beta-glucosidase. In some cases, when cellobiose is required as the final product (to be used as a sweetener for diabetics), removal of beta-glucosidase from the cellulase mixture is necessary to stop the conversion of cellobiose to glucose (Homma, 1993). Beta-glucosidase also plays an important role in the treatment of Gaucher’s disease resulting from a deficiency of the enzyme (Woodward, 1982). The following section outlines the applications of beta-glucosidase in various processes.

2.1.1 Applications of Beta-glucosidase

2.1.1.1 Cellulase Degradation. Celluloses are the most abundant substances (~5x10^10 tons/year) on earth. Agricultural and forest residues, energy crops, and about 40% of typical municipal garbage including newspaper and other paper products and other forms of cellulosic biomass provide potential renewable sources of chemicals and fuels (Ulger, 2001). Most organisms, except some animals and insects such as cattle, termites, and caterpillars, cannot digest cellulose, which limits the use of cellulose to satisfy the requirement of carbohydrates.
Cellulose is made up of glucose monomer units, linked together at beta-1, 4 locations as shown in Figure 2.1. Celluloses from different sources differ in their structures and bindings with other molecules.

![Figure 2.1 Beta-1, 4 linkage of glucose monomers in cellulose.](image)

Figure 2.2 shows the two kinds of hydrogen bonds of cellulose, one within the same molecule between the C₃OH group and oxygen and the other between the C₆OH group of the one molecule and the oxygen of the glucosidic bond of the other molecule.

![Figure 2.2 Hydrogen bonds in cellulose.](image)
The presence of hydrogen bonds gives a tightly packed crystalline structure to cellulose, this makes the molecule difficult to break down. During hydrolysis, water molecules are introduced to break these bonds. Two common methods are enzymatic and chemical hydrolysis. Hydrolysis of cellulose using acids and high temperature (chemical hydrolysis) is not environmentally friendly and can be expensive. Enzymatic hydrolysis is an economical process that uses low temperature and biodegradable enzymes. The degradation of cellulose to glucose by enzymatic hydrolysis requires three cellulolytic enzymes called cellulases. In the first step, endo-glucanase and exo-glucanase break the glucosidic linkage to convert cellulose to cellobiose; in the second step, beta-glucosidase converts cellobiose to glucose with the help of aspartic and glutamic amino acid residues, which break the beta-1,4 glucosidic linkage (Bauer, 1996; Tomaz, 1999):

\[
\text{endo, exo-glucanase} \quad \text{beta-glucosidase}
\]

\[
\text{Cellulose} \rightarrow \text{Cellobiose} \rightarrow \text{Glucose}
\]

2.1.1.2 **Ethanol Production.** Glucose can be fermented to produce ethanol to be used in gasoline. In the United States, each year, more than 1.5 billion gallons of ethanol are added to gasoline as an oxygenate, to improve vehicle performance and reduce air pollution (Levy, 2002). Ethanol is a very promising substitute for fossil fuels due to its low emission of carbon dioxide upon combustion and could eventually decrease our dependence on imported oil. There is an urgent need to develop economically feasible processes to use cellulose for ethanol production. For production of ethanol, biomass is disrupted to cellulose, then cellulases such as endo- and exo-glucanase and beta-glucosidase are used to convert cellulose to glucose and then glucose is fermented to ethanol by yeast. Ethanol can be processed further to produce other higher value petrochemicals such as acetone and butanol. A new method for production of ethanol
from paper mill waste fiber has also been invented using beta-glucosidase (Xin. 1993). Presently, a lack of low cost purified enzymes is the key barrier to the economical conversion of biomass to ethanol (Levy, 2002). The goal of biofuel research is to reduce the cost of these enzymes by employing cutting-edge and efficient biochemical technologies to produce bioethanol.

2.1.1.3 Food Industry. Beta-glucosidase is highly beneficial for the beverage industry since it can be used as an immobilized enzyme for flavor enrichment of wine and fruit juice (Gallifuoco, 1998). Beta-glucosidase, present in plant tissues, hydrolyzes beta-glucosidic flavor precursors that enhance flavor, aroma and food quality. Enzyme hydrolysis is also used to obtain overall increase in solid yields, increase clarity, decrease the viscosity of the raw juice slurry, and increase fruit juice recovery.

Beta-glucosidase helps glycoside precursors called terpenes to produce aromatic potential in fruits (Gueguen, 1996). In addition to a free fraction of volatile terpenes, some nonodorous and nonvolatile bound terpenes, present in the fruits, are an important source of fragrant compounds. In general, bound glycoside forms of terpenes are more abundant than the free ones. Endogenous beta-glucosidase activates these free precursors to release aroma during fruit maturation. Endogenous beta-glucosidase is not sufficient to liberate the whole aromatic potential because of its low activity. Exogenous beta-glucosidase is then used to help the process. These enzymes are more efficient than acid hydrolysis in the liberation of bound terpenes as they do not affect the natural aromatic properties. The possibility of rapidly developing the aroma of terpenes with the help of beta-glucosidase in a continuous process may therefore be of great interest to fruit juice industry.
Beta-glucosidase is used to synthesize glycercyl glucoside (Glycoside) from glycerol and glucose (Roode, 2001). Glycosides are nonionic surfactants, which can be used in cosmetics and foodstuffs since they are biodegradable and food grade. Compared to enzymatic synthesis with beta-glucosidase, chemicals cannot be used to produce glycercyl glycosides in the food industry because of toxicity problems. The enzyme is also used to decrease food viscosity in the animal's gut, increase vitamin uptake and carbohydrate digestibility, break down gums in dough structure for baking purposes.

**2.1.1.4 Textile Industry.** The use of cellulase started in the textile industry in the 1980s to give cellulose based fabrics and garments a uniform finish. Beta-glucosidase and other enzymes are used for stone washing as alternatives to original stones for cotton fabrics (Belghit, 2001). Cotton and cotton blend fabrics contain small cellulosic fuzz and pills. These fuzz and pills are removed by applying cellulases during mechanical agitation. Cellulase treatment also gives softness, luster and superior color brightness to the garment. Use of cellulase for softness and stonewashing reduce the need of harmful chemicals. The enzyme is added in detergents also to maintain brightness after washings.

Beta-glucosidase is also used during the production of a new fiber, lyocell. Lyocell has greater strength than other fibers and the manufacturing process is environmental friendly. However, fibrillation is a common problem in lyocell fabrics, especially after repeated laundering. This problem can be alleviated by cellulase treatment.

**2.1.1.5 Paper Industry.** Pulp and paper processing is one of the largest users of biomass today. The per capita consumption of paper in the United States exceeds 300 kg annually, and paper consumption increases with the standard of living. One can expect
the paper industry to increase in other countries as emerging economies continue to grow. However, the established pulping processes are relatively inefficient and environmentally costly, so new processes are needed.

Enzymes have widespread uses in the paper industry. Beta-glucosidase is used in two important processes, de-watering and de-inking. The need is to make the use of beta-glucosidase economically feasible.

During de-watering step, cellulases can be used in combination with pressure, vacuum and heat to remove water (Jeffries, 1997). This also helps in lowering the energy consumption in mechanical pulp production by improving the beatability (the ease with which pulp can be beaten to achieve the desired properties), drainage and solubility of the chemical pulps.

De-inking is a major constraint in paper recycling. During recycling of the paper ink is removed from paper using chemicals. De-inking is a challenging problem as the toners are difficult to remove. Cellulase enzymes can be used for ink removal as an alternative to chemicals. Cellulase treatment enhances the brightness of the paper with lower residual ink and increases pulp drainage rates. The use of enzyme also decreases the application of bleaching chemicals and surfactants that pollute the environment. Enzymatic de-inking could become commercialized because the technology fits easily into current fiber recycling practices. The process is less costly and more efficient than chemical de-inking.

2.1.1.6 Defense against Pests. Plants evolve defense mechanisms against pests based on storing and releasing toxic chemicals (Cicek, 1999). The defense chemicals are typically beta-glucosidase, stored in different sub-cellular or tissue compartments. When
pests attack the plants, these enzymes hydrolyze the substrate to release bitter and toxic chemicals. These substances inhibit the entry, growth and spread of pests. The enzymes are potential targets for engineering enhanced crop protection, reducing or eliminating the need for costly and ecologically undesired pesticides.

2.1.1.7 Therapeutic Use. Beta-glucosidase has a therapeutic use in Gaucher's disease, caused by the deficiency of this enzyme in a variety of cells and tissues (Woodward, 1982). The deficiency in beta-glucosidase results in the accumulation of a toxic substance, called glucosyl ceramide, that enlarges the organs. Delivery of the enzyme to the target organ catalyzes the break-down of glucosyl ceramides and stops its accumulation.

2.1.1.8 Other Uses. Lignin is the second most abundant substance in the biosphere. Beta-glucosidase is used in plant tissues to hydrolyze the production of lignin precursor. Thus the enzyme can be used to improve wood strength and paper production.

Beta-glucosidase is also implicated in growth and development by releasing active hormones. The enzyme can be used for regulating and improving plant growth and development to enhance productivity (Zouhar, 2001).

Beta-glucosidase is involved in many plant mechanisms as a precursor, intermediate or synthesizer. One of these processes is glycosylation, used to either enhance solubility or to degrade substances.

2.1.2 Beta-glucosidase Production

A wide variety of microorganisms, plants and extracts from many animals contain beta-glucosidase. Microorganisms are the best source for commercial production of the enzyme as they posses several advantages (i.e., low cost and ease of production) over
other possible sources. Selected microorganisms can be fermented conveniently to obtain high yield of the enzyme. A few examples include funguses such as *Saccharomyces cerevisiae*, *Aspergilli niger*, *Tricoderma reesei*. *T. reesei* has been found to be a promising culture for the production of beta-glucosidase (Reczey, 1996). In addition to beta-glucosidase, *T. reesei* produces other cellulases such as endo-glucanase and exo-glucanase and low molecular weight proteins. Although optimum conditions are currently used to maximize the production of beta-glucosidase, the enzyme must be purified further for specific applications.

### 2.1.3 Need for Purification

The purification of beta-glucosidase is important for several reasons:

1. Beta-glucosidase is usually produced with other cellulases and proteins. These contaminants must be removed or minimized.

2. Extracycelial level of beta-glucosidase, present in commercial cellulase and obtained from culture filtrates, is low for practical applications. Therefore it is necessary to use a relatively high enzyme concentration in order to achieve a sufficiently high rate of substrate conversion (Rajoka, 2003).

3. During the degradation of cellulose to glucose, the action of beta-glucosidase may be inhibited by the presence of glucose. Also, a large quantity of enzyme may be lost through thermal inactivation. Therefore, extra beta-glucosidase is often required.
4. The rate-limiting step in cellulose degradation is catalyzed by beta-glucosidase. Therefore, optimal concentration of the enzyme is required to obtain high yield of glucose production. If the amount of beta-glucosidase is not sufficient, conversion of cellobiose to glucose will stop (Chen, 1992).

5. When commercial cellulase is used for enzymatic hydrolysis of cellulose, extra beta-glucosidase is required for the hydrolysis of the produced cellobiose.

6. Immobilized form of the enzyme is used to produce the aromatic potential of must, wine, saffron and other fruits.

7. Purified beta-glucosidase is capable of hydrolyzing coloring agents found in foods of vegetable origins.

8. For therapeutic application of the enzyme for Gaucher’s disease, purification is a crucial requirement.

2.2 Purification Methods

2.2.1 Chromatography and Electrophoresis Techniques

Berghem and Pettersson were the first to purify beta-glucosidase from the fungus *T. reesei* (Berghem, 1974). Later, several conventional techniques such as chromatography (CM-sepharose, CL-6B cation-exchange and DEAE Bio-gel anion-exchange chromatography), isoelectric focusing, electrophoresis (SDS-polyacryl-amide) and foam fractionation, applied in the field of protein chemistry, were used to purify beta-glucosidase (Chen, 1992; Josep, 1995; Lambert, 2003).

These techniques have limitations, due to the excessive number of unit operations and complicated procedures required to achieve the desired purification. Purification methodologies based on chromatography are difficult to implement on a large scale due to problems related to the enzyme elution, fouling and cost of the column, and the size of the process. Also, these techniques are very time consuming. As a result, the use of these separation methods is severely limited.

2.2.2 Affinity Precipitation

Hydrophobic groups and charged amino acid residues are present at the protein surface. These groups provide affinity towards a specific polymer for precipitation. A method to purify beta-glucosidase had been investigated using sequential precipitation with two reversibly soluble-insoluble compounds, chitosan and Eudragit S-100 (Agarwal, 1996). Chitosan, a form of chitin, is the main component of crab and shrimp shells. The polymer contains glucosamine units that have high positive charge densities in acidic solutions. Chitosan was used to bind and remove negatively charged cellulases (endo, exo-glucanases) by precipitation. Further, Eudragit S-100, a copolymer of methylacrylic acid
and methacrylate, was used as an affinity ligand for the precipitation of beta-glucosidase with the polymer. T. Homma et al. (1993) also used Chitosan to remove beta-glucosidase from cellulase for the production of cellobiose - used as an indigestible sweetener for diabetics. After precipitation of glucanases with chitosan, beta-glucosidase activity remained in the supernatant. This technique presented a suitable procedure for the removal of beta-glucosidase-free cellulases from the filtrate. However, the method is inefficient at separating the target enzyme from low molecular weight proteins. In addition, elution of Eudragit S-100 bound beta-glucosidase is complicated for large scale operations.

2.2.3 Aqueous Two-phase Systems (ATPS)

The availability of enzymes at a reasonable price is very critical for the enzyme industry and for the consumer. Compared to conventional downstream processing methods, such as centrifugation, electrophoresis and chromatography, ATPS is inexpensive and easy to scale-up for protein removal (Albertsson, 1986). Liquid-liquid extraction is a suitable unit operation technique for purification of labile substances such as proteins, although organic solvent systems are not suitable due to the insolubility and deterioration of protein activity.

Two hydrophilic but immiscible polymers (such as PEG and Dextran) form a biphasic liquid system, when mixed above some critical concentrations. Proteins added to the system partition to one of the phases preferentially. The systems made of polymers offer low osmotic pressure, low interfacial tension and high water content (70% -90%) in both phases. As a result, they provide a protective physical and chemical environment for biological materials (Walter, 1985).
Application of the process is not widespread in the industry because of the high cost of the phase forming polymers. The other challenge is to separate the desired protein from polymer solutions after extraction in one of the phases. Systems containing one polymer and a strong electrolyte are quite promising, as they offer advantages of low capital cost, energy, labor, high activity yields, and have proved useful for large-scale production using appropriate scale-up technique. Cost of the process can be further minimized using recycle of the polymer (Kula, 1982). In general, enzymes recovered through aqueous two-phase separation meet purity requirement of industries such as textile and paper industries. In the case of pharmaceuticals where further purification is often required, the ATPS extraction can be followed by chromatography (Hustedt, 1988).

Johansson et al. (1998) and Brumbauer et al. (1999 & 2000) used Polyethylene Glycol (PEG), Dextran and salt solutions to separate proteins and glucanases to the PEG-rich top phase, while beta-glucosidase was concentrated in the Dextran-rich bottom phase. Ulger et al. (2001) also used this ATPS to remove proteins from the culture filtrate of \textit{T. viride} to the top phase. However, the polymer Dextran is an expensive chemical and its solution is highly viscous. Therefore, the use of this polymer is difficult during scale-up. In the present study, Dextran was replaced by potassium phosphate salt to overcome these limitations.

Furthermore, the studies of ATPS did not account for the influences of the system pH and temperature on the purification process. The partitioning behavior of an enzyme or protein in ATPS is specific and depends on a host of factors, including the system pH and temperature. A particular challenge in protein purification by ATPS partitioning is to fine-tune the operation parameters so that the compound of interest is directed in one of
the phases with negligible contaminant molecules. Although several studies have been conducted on the influences of polymer molecular weights and concentration, system pH and temperature on various proteins (Forcinity, 1991; Silva, 2002; Shang, 2004), no published report has addressed the synergistic effects of the system pH and temperature on beta-glucosidase partitioning in PEG/potassium two-phase systems.
CHAPTER 3
BACKGROUND

3.1 Phase Separation

The microbiologist Beijerinck (1896) first discovered formation of ATPS with gelatin and agar. Albertsson studied different polymers and electrolytes to form ATPS for separation of plant organelles and viruses, including the effect of polymer molecular weight, concentrations on the binodal curve Albertsson (1971, 1986). Currently, application of ATPS is getting attention in chemical, pharmaceutical, and food industries. This technique is successfully applicable in purification of various proteins and enzymes at large scale (Husteld, 1998; Costa, 2000).

Aqueous two-phase system is composed of two different polymers (e.g. PEG/Dextran) or a polymer and a salt of high concentration (e.g. PEG/Phosphate salt). The two phases form when components are mixed above critical concentrations to produce an immiscible mixture. The phase separation occurs due to the difference in density and viscosity of the two phases (Walter, 1985). ATPS provides an excellent environment for the phase separation as both the phases contain large proportion of water. The interfacial tension of the two phases is very low, which provides high interfacial contact area for the mass transfer. Phase separation can be explained thermodynamically on the basis of entropy of mixing; whenever the interactions between polymer segments overcome the entropy of mixing, two phases separate.
3.1.1 Thermodynamic Theory

Phase separation phenomena can be explained using the laws of thermodynamics. For a closed system at constant temperature and pressure, equilibrium is reached when the total Gibb’s free energy is at a minimum. The change in the Gibb’s free energy is defined as:

\[ \Delta G_m = \Delta H_m - T\Delta S_m \]  

(3.1)

where \( T \) is the absolute temperature; \( \Delta H_m \) and \( \Delta S_m \) are the enthalpy and entropy changes, respectively. For mixing to occur, \( \Delta G_m \) must be negative. The entropy, \( \Delta S_m \), is a measure of the degree of randomness (or disorder) in the system as a result of the mixing process. The enthalpy of mixing, \( \Delta H_m \) is defined as the summation of individual net enthalpy changes associated with the formation of contacts between unlike components. These new contacts are generated at the expense of breaking the bonds between like components. Phase separation occurs, when the interaction energy (energy required to bring two molecules together) between unlike components becomes slightly positive (i.e., \( \Delta H_m \) is increased). The large size of the polymer reduces the entropy of mixing and results in the decrease of the term \( T\Delta S_m \). When this term is small, the enthalpy of mixing dominates the Gibb’s free energy, resulting in a positive change of \( \Delta G_m \). As a result, the system becomes unstable and separates into two phases in order to reach equilibrium (minimum \( \Delta G_m \)) by reducing the interactions between unlike molecules.

3.1.2 Phase Diagram

The different phases of a system at a given condition (polymer molecular weight, pH and temperature) can be represented by a phase diagram (see Figure 3.1 below). The phase compositions and required total composition of phase forming components can easily be obtained from the phase diagram. The coordinate axes are labeled with polymer and salt.
concentrations, usually, in the units of weight of the substance per 100 weight units of the mixture, percent by weight (w/w %). The ordinate axis is commonly used for the substance rich in the top phase. In the case of PEG/potassium phosphate salt system, the top phase is rich in PEG, while the bottom phase is more concentrated in salt. For a system with overall composition A, the top and bottom phase compositions are B and C, respectively.

Figure 3.1 Phase diagram of aqueous two-phase system.

3.1.3 Binodal Curve

The curve connecting the top and bottom concentrations, for different initial concentrations, is called the binodal curve (Figure 3.1). The binodal curve divides the graph into two regions. The region above the binodal curve is heterogeneous with two immiscible phases and below the curve is homogeneous with one phase. The position of
the binodal curve is influenced by changes in the system conditions. The binodal curve helps to select the system conditions and to produce the required two-phase composition.

3.1.4 Tie-line

The line that connects the top and bottom concentrations of the components and passes through the total composition is called tie-line. The tie-lines are usually parallel; the length of the lines decreases with a reduction in total composition and eventually becomes zero at the critical point. At the critical point, the composition and volume of both phases are equal. The tie-line length is defined as:

\[ TLL = [(\Delta \text{Component}_1)^2 + \Delta \text{Component}_2^2)^{1/2}] \]  

(3.2)

where \( \Delta \) is the difference in the component concentrations between top and bottom phases.

3.1.5 Effect of Polymer Molecular Weight on Phase Separation

At low molecular weight, only tightly bound water molecules are associated with the PEG chain. As the molecular weight increases, the chain begins to fold on itself (adopts a secondary structure), forming segment-segment interactions, as it shares loosely bound water between adjacent segments (Harris, 1992). This interaction reduces the contact of the PEG chain with the water molecules and the phase separation occurs at lower concentrations of the phase forming components (e.g. PEG and phosphate salt) (Zaslavsky, 1995). An increase in PEG molecular weight enhances the \( \Delta H_m \) term compared to \( \Delta S_m \), resulting in a positive change in \( \Delta G_m \) that separates the PEG molecules from water to make the system stable with minimum \( \Delta G_m \).
3.1.6 Effect of Temperature on Phase Separation

The separation of two phases depends on the temperature. The increase in temperature of the system decreases the solubility of PEG in water. PEG possesses a low consolute temperature (LCT), or cloud point, of approximately 100 °C in water. Raising the temperature above 100 °C, results in insolubility of PEG in water and formation of two phases. Addition of salt to the PEG solution changes the cloud point temperature. The increase in salt concentration lowers the LCT dramatically (Harris, 1992). Therefore, in PEG/Potassium Phosphate salt systems, the two phases are formed easily at low temperature. The effectiveness of various anions to depress the cloud point temperature follows the order: I⁻ < Br⁻ < Cl⁻ < F⁻ < OH⁻ < SO₄²⁻ < CO₃²⁻ < PO₄³⁻ (Zaslavsky, 1995).

3.2 Protein Partitioning in ATPS

Albertsson first noticed the partitioning of chloroplast in an aqueous two-phase system of PEG, phosphate salt and water. The chloroplast partitioned accidentally to the PEG phase. This discovery led to research focused on aqueous two-phase partitioning of biomolecules and organelles. Albertsson (1986) defined partitioning as an unequal distribution of a substance between two phases, contrary to the Brownian motion that result in the formation of a homogeneous mixture.

3.2.1 Partition Coefficient

The partition coefficient of a molecule in a given ATPS is determined by

\[ K = \frac{C_{\text{top}}}{C_{\text{bottom}}} \]  \hspace{1cm} (3.3)

where \( C_{\text{top}} \) and \( C_{\text{bottom}} \) are concentration in the top and bottom phase, respectively. The partitioning coefficient of the substance in a phase system depends on the properties of
the substance and the phase forming components. The physiochemical interactions between all the materials, determine the partitioning behavior of the molecule in the two phases. At low protein concentration, the partition coefficient is independent of the amount of protein used in ATPS (Mistry, 1993). The contributing factors for biomolecule partitioning in two-phase systems are discussed below.

3.2.2 Factors Affecting Protein Partitioning

Protein partitioning in ATPS depends on the properties of protein and the two aqueous media. Factors contributing to the partitioning behavior of biomolecules are protein size, charge and hydrophobicity, polymer molecular weight, polymer and salt concentrations, pH and temperature of the system. The distribution of hydrophobic and hydrophilic residues, combined with charged and other polar groups on the surface of the protein molecule, determines protein solubility in the aqueous phases. Also, the solvating properties of water in the phases can be manipulated by changing the ionic strength, polymers or salt concentrations and temperature (Gupta, 2002). Changes in the above conditions alter protein solubility in the media and, thus, the concentrations in the top and bottom phases.

3.2.2.1 Protein Hydrophobicity (Salting Out Effect). The salting out effect depends strongly on protein hydrophobicity. The protein surface has non polar hydrophobic groups (Robyt, 1987). In contact with an aqueous solvent, the water molecules arrange themselves around the hydrophobic surface. This ordering is thermodynamically unstable as compared to the unsolvated protein plus free water molecules (i.e., the entropy of the system is decreased). Upon addition of a salt, the number of water molecules available for the solvation of the hydrophobic residues
decreases as the salt ions become solvated. Consequently, the exposed hydrophobic areas interact with each other. Proteins, with a large number of hydrophobic residues and a large surface area are allowed to interact with one another and aggregate in order to reduce water contact. Other hydrophilic proteins, with polar groups on the surface, remain in solution even at higher salt concentration. This is mainly due to the strong bonding with water molecules. In ATPS, the protein concentration in the two phases is affected by its hydrophobicity.

3.2.2.2 Protein Surface Charge. The solubility of proteins in an aqueous solvent is primarily determined by the polar interactions with the solvent, ionic interactions with the salt present and the repulsive electrostatic forces between like charged molecules (Scopes, 1994). Proteins are sequences of amino acids that carry charged groups depending upon their acid or basic character. The net electric charge on the protein surface is the sum of all the electric charges present on the amino acids. At a pH value above the isoelectric point, proteins have a net negative charge; their charge is positive below the isoelectric point. Close to the isoelectric point, a decrease in the protein surface charge reduces the electrostatic repulsion between the protein molecules. Protein solubility is reduced, as a result. The solubility of a protein generally increases as the pH moves above or below the isoelectric point. At pH values apart from isoelectric point, there is a greater net charge on the protein surface resulting in repulsion between protein molecules, keeping them in solution.

3.2.2.3 Interaction of Proteins with PEG. The addition of an organic polymer such as PEG to an aqueous media containing proteins reduces the water activity. The solvating power of water for a charged, hydrophilic protein molecule is decreased as the
concentration of polymer increases (Scopes, 1994). This can be described in terms of bulk displacement of water and partial immobilization of water molecules through hydration of the PEG. Ordered water structure around the hydrophobic areas on the proteins surface is displaced by the PEG molecules. Thus the solubility of the protein molecules decreases in the aqueous medium.

The proteins with a large number of hydrophobic groups have more interaction with the PEG molecules than the proteins with more hydrophilic groups. This results in the displacement of these proteins from the aqueous salt phase. An increase in the hydrophobic difference between the two phases by increasing concentrations of PEG or salt or both, concentrates the protein in one of the phases.

3.2.3 Effect of Polymer Molecular Weight on Protein Partitioning

Increase in the molecular weight of polymer excludes the protein molecule from vicinity of the polymer and forces the protein to the phase deficient in polymer. This is called excluded volume effect. The influence of molecular weight on protein partitioning can be explained by using free energy (Equation 2.1). As PEG molecular weight increases, the number of polymer segments per molecule increases. As a result, the enthalpy term increases compared to the entropy term as the latter depends only on the concentration of the molecules, while the former depends on the number of the interacting units. The interactions between a polymer segment and protein are less energetically favorable than the interactions between two polymer segments (Albertsson, 1987; 1990). This effect is more prominent as the number of polymer segment per molecule increases. Therefore, a high-molecular-weight polymer tends to increase protein exclusion, which decreases the concentration of protein in the PEG rich phase.
3.2.4 Effect of the System pH on Protein Partitioning

Charged amino acid residues are present at the protein surface. These amino acid groups are positively, negatively or neutrally charged, depending on the system pH, and influence the protein surface properties. As a result, a change in the system pH leads to a modification of the biomolecule global charge (Berggren, 2002). The presence of electrostatic interactions, due to the surface charge of the biomolecules and the charge of the two-phases, contributes to protein partitioning in ATPS.

3.2.5 Effect of System Temperature on Protein Partitioning

The solubility of the protein is a function of the system temperature. In general, proteins solubility decreases with an increase in temperature (Zaslavsky, 1995). This important phenomenon is commonly observed when a clear protein-salt solution, initially maintained at a low temperature, precipitates by warming. This effect may be attributed to the change in the composition of the two phases and in the hydrophobicity of the protein molecules.

3.2.6 Partitioning Strategy

The properties of the proteins and the aqueous biphasic system determine protein partitioning. A firm knowledge of the factors and the roles they play in directing the partitioning of biomolecules (target enzymes as well as contaminant proteins) is crucial and can be exploited to extract the desired enzyme from a mixture. One strategy, designed to increase the concentration of the target enzyme in one phase and concentrate the contaminants in the opposite phases, is to fine-tune the system parameters. These conditions can then be tested in a pilot plant-scale experiment to measure the process feasibility for continuous industrial operation.
3.3 Models for Prediction

3.3.1 Thermodynamic Models

The development of reliable tools that can predict equilibrium phase compositions and partitioning behaviors of the different components of the system is critical for large-scale protein purification. In the last few decades, a large body of literature has been published on the thermodynamic modeling of phase equilibrium and protein partitioning in ATPS (Furuya, 1995; Peng, 1995; Zafarani-Moattar, 2001). Baughman et. al (1994) categorized these models into four main groups (1) adsorption model based on thermodynamics of adsorption of polymers; (2) Ogston-based models utilizing the osmotic virial equation; (3) Flory-Huggins based models applying the Flory-Huggins equations; (4) perturbation models based on stastical-mechanical calculations. In this study, a Flory-Huggins model was used for the prediction of ATPS phase composition.

3.3.2 Flory-Huggins Theory

The thermodynamics of polymer solutions is described by Flory and Huggins (Walter, 1985). According to the Flory-Huggins theory, the solution can be represented by a lattice site. Each site is occupied by a solvent or solute molecule. For a mixture of two species in a solvent, the Gibbs energy of mixing $\Delta G$ is given by (Peng, 1995):

$$\frac{\Delta G}{kT} = n_1 \ln \phi_1 + n_2 \ln \phi_2 + n_3 \ln \phi_3 + (n_1 + p_2n_2 + p_3n_3)(\chi_{12}\phi_1\phi_2 + \chi_{13}\phi_1\phi_3 + \chi_{23}\phi_2\phi_3)$$  (3.4)

where component 1 is the solvent (water) and components 2 (PEG-4000) and 3 (phosphate salt) are the two solute species. The symbol $k$ is the Boltzmann’s constant, $T$ is the absolute temperature, $n$ is the number of solute or solvent molecules on the lattice, $\phi_i$ is the volume fraction of species $i$ in the solution and $p_i$ is the relative size of solute $i$ with respect to the solvent. The parameter $\chi_{ij}$ is the Flory interaction parameter, defined as
the maximum interaction energy between molecules $i$ and $j$. The change in chemical potentials (from a pure state) of species 1 and 2, $\Delta\mu_1$ and $\Delta\mu_2$, are obtained by differentiation of Eq. (2) with respect to $n_i$ (Peng, 1995):

$$\frac{\Delta\mu_1}{kT} = 1 + \ln \phi_1 - \frac{1}{V_x} + \chi_{12}\phi_2(1-\phi_1) + \chi_{13}\phi_3(1-\phi_1) - \chi_{23}\phi_2\phi_3$$

(3.5)

$$\frac{\Delta\mu_2}{kT} = 1 + \ln \phi_2 - \frac{p_2}{V_x} + p_2 \left[ \chi_{12}\phi_1(1-\phi_2) + \chi_{23}\phi_3(1-\phi_2) - \chi_{23}\phi_2\phi_3 \right]$$

(3.6)

$$\frac{\Delta\mu_3}{kT} = 1 + \ln \phi_3 - \frac{p_3}{V_x} + p_3 \left[ \chi_{23}\phi_2(1-\phi_3) + \chi_{13}\phi_1(1-\phi_3) - \chi_{13}\phi_2\phi_3 \right]$$

(3.7)

where, $\phi_1 + \phi_2 + \phi_3 = 1$, $\phi_1 = x_1 p_1/V_x$, $\phi_2 = x_2 p_2/V_x$, $\phi_3 = x_3 p_3/V_x$.

$V_x = x_1 + x_2 p_2 + x_3 p_3$. $V_x$ is the specific molar volume of the mixed solution with respect to water. The parameters $p_2$ and $p_3$ are defined by $r_2/r_1$ and $r_3/r_1$, respectively; $r_i$ is the volume parameter of component $i$ and $x_i$ is the mole fraction of component $i$ (Peng, 1995).

### 3.3.3 Limitations of Thermodynamic Models

The equilibrium phase compositions and the partitioning behavior of a protein in ATPS are specific and depend on conditions such as polymers and salt concentrations, system pH, ion strength and temperature (Albertsson, 1986). This feature, however, makes the modeling of ATPS very difficult due, in part, to the complex nature of the theoretical models and the estimation of binary interaction parameters. Although significant efforts have been made to integrate system conditions into existing ATPS models (Furuya, 1995; Simon, 2004) there is a need to develop a model that combines contribution of the key factors in the partitioning of biomolecules.
3.3.4 Artificial Neural Network

In last few decades, the technique of artificial neural network has emerged as a potential problem solving methodology (Simon, 2001; Loney, 2003; Karim, 2003). The nonparametric ANN approach does not require any explicitly outlined set of rules. This eliminates the need to estimate interaction parameters of ATPS components, required by classical models. The interrelated network structure of ANN generally performs better than empirical models to solve complex and poorly defined problems in the presence of different variables (Patnaik, 1999). The highly nonlinear model of neural networks captures intricate relationships among ATPS variables without any assumptions (Djebbar, 2002).

3.3.5 Network Structure

The human brain is a complex network of layers of biological neurons (Dayhoff, 1990). A neuron is the computational unit of the network and typically has four parts. Dendrites accept inputs from the outside environment and send them to soma for processing. Axon turns processed inputs to outputs and synapses provide the electrochemical contacts between neurons. Information goes back and forth through neuron layers and thus the brain learns.

3.3.5.1 Network Processing Unit – Neuron. An artificial neuron network is a parallel-distributed information processing framework that imitates the work of a biological network. The processing units of the network, or neurons, are linked via unidirectional weighted connections. Each neuron takes an argument that is the sum of a weighted input and bias and produces an output using a transfer function. The transfer
function $f$, is typically a step function or a sigmoid function, which takes the argument $n$ and produces the output $a$.

The net input to a neuron is given by:

$$net_j = \sum_i w_{ij} x_i + b_j$$

where $x_i$ is the output from the previous layer, $w_{ij}$ is the weight of the link connecting unit $i$ to unit $j$, and $b_j$ is the bias of the unit $j$.

The output of the unit $j$ is given by:

$$a_j = f(net_j)$$

where $f$ is the transfer function of the neuron $j$.

![Figure 3.2](image)

**Figure 3.2** Neural network processing unit – neuron.

The central idea of neural networks is to adjust the weights so that the network exhibits some desired behavior (Diederich, 1990). The method to determine the weights and biases is called training. During the training process, a set of pattern examples is used, each example consisting of a known input and corresponding target output. The network is trained to minimize the network performance error (between network output and target outputs) by adjusting the weight or bias parameters. The error function represents a measure of the performance of the network. The examples are presented to
the network iteratively or in batch. The optimization continues until the network learns the underlying relationship between input and output variables. Each presentation of the whole example set is called an epoch. The training session is followed by a testing phase in which the performance of the ANN is determined by measuring the error for a new example.

3.3.5.2 Feed-forward Network. One of the most popular network structures, feed-forward neural network has been implemented in the literature (Bogdan, 1995; Simon, 2004). In a feed-forward neural network, the neurons are arranged in layers. The input layer consists of neurons corresponding to the process variables; the output layer is made of neurons representing the target output. Several hidden layers process the information from the input to output layer. Figure 2.5 represents an ANN with one hidden layer of neurons. Adjustments are made in the number of hidden layers, the number of hidden neurons and in their transfer functions to enhance the performance and robustness of the system. The output of each layer is fed into the next layer; therefore the structure is called a feed-forward network.

![Feed-forward artificial neural network](image)

Figure 3.3 Feed-forward artificial neural network.
3.3.5.3 Backpropagation Training Algorithm. Many different algorithms can be used to train the network. The Backpropagation algorithm is the most popular supervised learning algorithm for feed-forward neural networks. This algorithm calculates the changes in the weights of the final layer and computes the weights of the previous layer. Thus, it continues in a backward fashion to the input layer. The change in weight is calculated by taking partial derivative of the error function ($E$) in relation to each weight ($w$).

$$
\Delta w = - \epsilon \left( \frac{\delta E}{\delta w} \right)
$$

(3.10)

where $\epsilon$ defines the step size, called learning rate.

The procedure used to update the input weights for each neuron is specific to each algorithm. The resilient backpropagation algorithm eliminates any harmful effects due to the magnitude of the partial derivatives while measuring the change in weight (Igel, 2003). A derivative sign is used to determine the direction of the weight update. The update is increased when the derivative of the error function has the same sign for the two successive iterations and decreased when the derivative changes the sign. When the derivative is zero, the update value remains the same.
3.4 Aims and Objectives

The aims and objectives of this research are:

1. Study of the aqueous two-phase systems (PEG / potassium phosphate salt) to understand the system for biomolecule partitioning.

2. Study the partitioning behavior of beta-glucosidase in various ATPS for selection of the best ATPS.

3. Development of a sequential scheme for β-glucosidase purification from Tricoderma reesei cell culture.

4. Selection of the best system parameters for the separation of beta-glucosidase from the contaminants.


6. Pilot plant study of beta-glucosidase purification in ATPS for large scale process.
CHAPTER 4
MATERIALS AND METHODS

4.1 Materials

4.1.1 Proteins and Assay Materials
Pure beta-glucosidase and pure cellulase (including endo, exo-gluconase and beta-glucosidase) were purchased from Sigma Chemical Co. (Missouri, USA). The cell culture for preliminary experiments was a gift from the laboratory of Karim (Texas Tech University). The fungi Tricoderma reesei strain was purchased from American Type Culture Collection (Virginia, USA).

The substrate, p-nitrophenyl-β-D-glucopyranoside, was purchased from Sigma Chemical Co. (Missouri, USA) and Whatman No.1 filter paper (1x 6 cm) substrate from VWR international (West Chester, PA). Bicinchoninic acid (BCA) protein assay reagent kit was provided by Pierce Biotechnology (Rockford, IL).

4.1.2 Phase Forming Polymers
Polyethylene glycol of molecular weights 1450, 8000 and sodium chloride were obtained from J.T.Baker (Phillipsburg, NJ). PEG 4000; monobasic potassium phosphate were supplied by Mallinckrodt (Paris, Kentucky). PEG 6000 and dibasic potassium phosphate were purchased from Merck (Darmstadt, Germany). Dextran 500,000 was purchased from Fisher Scientific (Springfield, NJ).

4.1.3 Other Chemicals
Chitosan, potato dextrose agar and yeast extract were purchased from Sigma Chemical Co. (Missouri, USA). Eudragit was obtained from Rohm America LLC (Piscataway, NJ).
Lactose and glucose were obtained from VWR international (West Chester, PA) and acetate buffer from Electron Microscopy Sciences (Fort Washington, PA). All other chemicals were of analytical grade.

4.2 Analytical Methods

4.2.1 PEG Assay

PEG concentration was determined by refractometric method, using an Abbe refractometer. The interference of phosphate salt was taken into account using the following equation:

\[ n = 1.3278 + 0.0013W_s + 0.0013W_p \]  (4.1)

where \( n \) is the refractive index of the solution, \( W_s \) and \( W_p \) are the weight percent of the phosphate salt and PEG, respectively.

4.2.2 Phosphate Assay

Phosphate salt concentration was determined by measuring the conductivity of samples. A calibration curve was prepared using known salt concentrations.

4.2.3 Beta-glucosidase Assay

Beta-glucosidase activity was measured using p-nitrophenyl-\( \beta \)-D-glucopyranoside (PNPG) as a substrate. A 1.0 ml of Acetate buffer (pH 5.0, 0.1M) and 0.5 ml PNPG solution (20 mM) were mixed with the 0.5 ml sample solution and maintained at 37\(^0\) C for 15 min. A 2 ml sodium carbonate (0.2 M) solution was added to stop the reaction and the sample absorbance was measured at 400 nm. A blank solution was prepared by adding sodium carbonate to acetate buffer and PNPG solution first and the sample solution after 15 min. A standard calibration curve was prepared with pure beta-
glucosidase enzyme. One unit (IU) is defined as the amount of enzyme that produces 1 
μmol of PNPG per min under specified conditions.

4.2.4 Total Protein Assay
Total protein concentration was determined by following the bicinchoninic acid (BCA) 
assay at 562 nm (Smith, 1985). Bovine serum albumin served as a reference standard. A 
1 ml of working reagent was mixed with 50 μl sample and incubated for 30 min at 37 °C. The solution was cooled to room temperature and the absorbance (A) was measured at 562 nm using a UV- spectrophotometer (Genesys 10). A blank solution was prepared by adding 50 μl sample to 1 ml of reagent at the end of the cooling step. The absorbance of the blank sample (B) was then measured. The total protein absorbance was estimated by subtracting B from A.

4.2.5 Cellulase Assay
Total cellulase concentration was determined with filter paper assay method in FPU units (Wood et al.). A 1ml of citrate buffer (0.05M, pH 4.8) was added to 0.5 ml of enzyme suitably diluted in the same buffer. A strip of Whatman No.1 filter paper (1X6 cm) was added as a substrate and the reaction mixture was incubated at 50° C. After 60 min, 3 ml of Dinitrosalicylic acid reagent (DNS) reagent was used to stop the reaction. Reagent blank and control solutions were prepared in the same manner. Glucose standards were used to generate the calibration curve. The absorbance for all the solutions was read at 540nm. The filter paper unit was based on the release of exactly 2.0 mg of glucose equivalent from 0.5 ml of diluted enzyme in 60 min.
4.3 Procedures

4.3.1 Binodal Curve

PEG (Mol wt. 1450, 4000, 6000, and 8000) and potassium phosphate salt were dissolved in distilled water on a basis (w/w %) to make the stock solutions. The solutions were thoroughly mixed and preserved at 4 °C. Small volumes were withdrawn from stock solutions to obtain different ATPS at desired conditions. Graduated test tubes were used to measure the volumes of the phases after separation. Each sample was placed in a controlled temperature bath for 1 hr to ensure complete equilibration. To measure the phase compositions, the top phase was first removed using a glass pipette and subsequently the bottom phase without disturbing the interface. The densities were calculated by weighing known volumes on the analytical balance. Assays were performed on both the top and bottom phases to determine PEG and phosphate salt concentrations.

4.3.2 Total Protein Partitioning in ATPS

Stock solutions were prepared by mixing measured quantities of PEG 1450, PEG 4000, or PEG 8000 with potassium phosphate salt. Different ratios of K$_2$HPO$_4$ and KH$_2$PO$_4$ were used to obtain pH values of 4.5 and 5. A 0.5 ml of culture filtrate for total protein was added to 4.5 ml of ATPS. The pH values of the top and bottom phases were the same after separation. Therefore the ratios of K$_2$HPO$_4$/ KH$_2$PO$_4$ are assumed to be same in both the phases. This solution was stirred for 15 min and allowed to settle for 1 hr at a constant temperature (32° and 42°C). After separation, the concentration of protein in both phases was measured.
4.3.3 Beta-glucosidase Partitioning in ATPS

PEG 4000/Dextran, PEG 8000/Dextran, PEG 8000/potassium phosphate salt and PEG 8000/potassium phosphate salt aqueous two-phase systems were used to obtain different ATPS. A 0.5 ml of pure beta-glucosidase in acetate buffer (pH 5.0, 0.1 M) was added to 4.5 ml of ATPS solution. The system was mixed on a vortex mixer and incubated for 1 hr at 25 °C. The top and bottom phases were isolated and analyzed for beta-glucosidase activity.

PEG 4000 (8%) and phosphate salt (13%) ATPS was also used to study the kinetics of beta-glucosidase portioning. A 15 ml solution was investigated for the partitioning at pH 7.0 and 25 °C. Samples from top and bottom phases were taken at an interval of 5 min for 1 hr and analyzed for the enzyme activity.

4.3.4 Cellulase Precipitation

A 2 ml of enzyme (in acetate buffer, 0.01 M, pH 5.0) was added to 4 ml of Chitosan (5mg/ml in 0.1 M, pH 4.0 acetate buffer) and incubated for 2 hr at 50 °C. Tris-HCl buffer (0.5 M, pH 11.0) was added to adjust the pH to 7.0 for precipitating Chitosan. The solution was incubated for 15 min at room temperature (25 °C) and then centrifuged. The concentrations of total cellulase and beta-glucosidase were determined in the supernatant. The enzyme concentration was also measured in the solution at the same conditions, without using chitosan.

4.3.5 Preparation of Crude Extract

The fungi *Tricoderma reesei* strain was obtained from American Type Culture Collection (Virginia, USA). *T. reesei* was maintained on potato dextrose agar slant (39g/l) at 300 °C. After 14 days of incubation, the spores were transferred to flasks for inoculation.
The cultivation media consisted of 5g (NH₄)₂SO₄, 15g KH₂PO₄, 0.3g urea, 0.8g CaCl₂, 1.23g MgSO₄·7H₂O, 0.0027g FeSO₄·7H₂O, 0.0016g MnSO₄·H₂O 0.0014g ZnSO₄·7H₂O, 0.75g peptone, 10g/l glucose, 0.5g Tween 80, 0.3g yeast extract and 10g lactose in a liter of distilled water. Media was supplied with 10% (v/v) inoculum from T. reesei spores to start cultivation. The fermentation was carried out at 28°C on a rotary shaker at 150 rpm for 4-5 days. Samples were withdrawn and β-glucosidase activity was analyzed every 24 hr. At the end of the cultivation, the broth was centrifuged and the supernatant was stored at 4°C. Before starting the purification steps, the culture filtrate was tested for β-glucosidase activity, total cellulase and protein concentrations.

As shown in Figure 4.1, a two-step sequential purification schematic was developed for beta-glucosidase purification from T. reesei cell culture. First, the cellulase enzyme endo-glucanase and exo-glucanases were separated from the culture filtrate. Affinity precipitation with Chitosan, a reversibly soluble-insoluble compound was used to separate beta-glucosidase from other cellulases, selectively. Second, low molecular weight contaminant proteins were removed using aqueous two-phase system.

4.3.5.1 Affinity Precipitation. One milliliter of Chitosan (10mg/ml in 0.1 M, pH 4.0 acetate buffer) was mixed with 4ml of the culture filtrate and incubated for 2hr at 5°C. Then, Tris-HCl buffer (0.5 M, pH 11.0) was added to adjust the pH to 7.0 in order to insolubilize the chitosan. The resulting solution was incubated for 15 min at room temperature (25°C) and then centrifuged. Precipitated chitosan was then separated from the supernatant solution. The total cellulase and beta-glucosidase activities were determined in the supernatant. The enzyme activities in solution measured from this
method were compared with those obtained when chitosan was not added to the mixture.

Except for the chitosan, the same conditions were used in both studies.

**Figure 4.1** Sequential precipitation and aqueous two-phase separation technique for beta-glucosidase purification from *T. reesei.*
4.3.5.2 Culture Filtrate Partitioning in ATPS. Filtrate obtained after cell removal by centrifugation was used to prepare aqueous two-phase systems with PEG (8%) and phosphate salt (13%) in 10 ml graduated tubes. Calculated amounts of mono and dibasic Phosphate salts were supplemented first to 4 ml of the supernatant to obtain pH of 6.3. After mixing, PEG was added to the solution. The suspension was vortex mixed for 10 min and left for phase separation in a temperature bath for 1 hr at 25°C. The two phases were separated and the protein concentration and β-glucosidase activity were measured for the top and bottom phases. To measure enzyme purification in the top and bottom phases, the yield was calculated according to the equation

\[ Y_T = \frac{1}{1 + ((V_A / V_T)(1/K))}, \quad Y_B = \frac{1}{1 + ((V_T / V_B)(K))} \]  

where \( V_T \) and \( V_B \) are volumes of top and bottom phases. Since, the partitioning behavior of an enzyme or protein in ATPS is specific and depends on a host of factors, including the system pH and temperature. The effects of these parameters on the purification of beta-glucosidase were studied to determine the best conditions for directing the enzyme in one phase with negligible contaminant molecules. A procedure similar to the one described above was followed to obtain partitioning in ATPS at pH values of 6.0, 6.5, 7.0, and 7.5 and temperatures of 25, 35, 45 and 55°C. To select the optimal ATPS conditions for the enzyme purification, a selectivity parameter was also used as a criterion. The selectivity is defined as

\[ \alpha = \frac{K_{\text{protein}}}{K_{\text{glucosidase}}} \]  

where \( K_{\text{protein}} \) is the lumped partition coefficient of total proteins and \( K_{\text{glucosidase}} \) is the partition coefficient of beta-glucosidase.
4.4 Thermodynamic Modeling

Peng et al. (1995) applied the Flory-Huggins model for the two-phase system of water (1), PEG 4000 (2) and phosphate salt (3) with no added protein and used the parameters $p_1 = 1$, $r_1 = 0.92$, $r_2 = 145.7$, $r_3 = 1.59$. In this work, the equations were used to evaluate the interaction parameters in the aqueous biphasic systems. These parameters were estimated using the following objective function (Sargantanis, 1997):

$$Q_i = \sum_{k=1}^{N} \left( \sum_{i=1}^{3} \left( \frac{w_t \Delta \mu_i^{Top} - \Delta \mu_i^{Bottom}}{(\Delta \phi)^n} \right) \right)^2$$  \hspace{1cm} (4.4)

where $w_t$ is the weighing factor of equation $i$ (Equation (3.5), (3.6) or (3.7)), $n$ is an integer that prevents the algorithm to converge to the trivial solution (Sargantanis, 1997) and $N$ is the number of two-phase systems used to generate the bimodal. In this problem, $w_t$ was set to 1 and the denominator $(\Delta \phi)^n$ was not used. The superscripts correspond to the top and bottom phases. Equation (4.4) is based on the fact that when two phases are in equilibrium, the chemical potentials of a component in each phase must be equal. Several objective criteria could have been used. For a detailed discussion of how the selection of $Q_i$ affects the parameter estimates, see (Sargantanis, 1997).

After converting the phase equilibrium data to mole fractions, the only unknown parameters of Eq. (4.4) are $\chi_{12}$, $\chi_{13}$ and $\chi_{23}$. The function "NMinimize", from Mathematica® (Wolfram Research, Inc.), was used to calculate the global minimum of the function. A constrained optimization method ($0 < \chi_{12} < 1$, $0 < \chi_{12} < 20$, $0 < \chi_{12} < 20$), with initial values (Peng, 1995; Sargantanis, 1997): $\chi_{12} = 0.38$, $\chi_{13} = 5.10$, $\chi_{23} = 5.65$, was implemented to estimate the interaction parameters.
4.5 Artificial Neural Networks

The Neural Networks Toolbox of MATLAB® (The Mathworks Inc.) was used for the design and implementation of the networks. In general, network models could be designed in two ways for predicting two outputs. First, one network predicts both the outputs simultaneously. This topology is called an all-class-in-one-network (ACON). A second approach, called one-class-in-one-network (OCON) uses two networks to calculate two separate outputs. As discussed in the literature, with the same number of neurons, the ACON structure takes short time to converge compared to OCON (Kan, 1996). However, it has difficulty converging to an acceptable error level. Therefore, OCON structure was selected to predict the data.

The multiple layers feed forward networks (MLFN) with three layers (input, output and a hidden layer) were used as three layers perform sufficiently well when given enough neurons. The top phase compositions: PEG and water concentrations (outputs) were first predicted as a function of three variables: temperature, water and PEG concentrations in the bottom phase (inputs) (Figure 4.2). As mentioned above, two networks were developed. A third ANN model was built to predict the partition coefficient of beta-glucosidase (output) based on two input variables: system temperature and pH (inputs) (Figure 4.3).
Figure 4.2 Neural network architecture for prediction of PEG or water concentration in the top phase.

Figure 4.3 Neural network architecture for prediction of beta-glucosidase partition coefficient.
Table 4.1 shows the architecture of these three neural networks. Because of its simplicity, a Log-Sigmoid (logsig) transfer function was used in the hidden layer; a linear (purelin) function was used in the output layer. These two functions are:

Sigmoid Function: \[ f(x) = \frac{1}{1 + e^{-x}} \]  
(4.5)

Linear function: \[ f(x) = x \]  
(4.6)

Table 4.1 Architecture of the Neural Networks

<table>
<thead>
<tr>
<th></th>
<th>ANN 1</th>
<th>ANN 2</th>
<th>ANN 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Input layer</strong></td>
<td>PGE and salt conc. in bottor temperature</td>
<td>PGE and salt conc. In bottor pH, temperature</td>
<td></td>
</tr>
<tr>
<td><strong>Hidden layer</strong></td>
<td>5 neurons Logsig</td>
<td>4 neurons Logsig</td>
<td>5 neurons Logsig</td>
</tr>
<tr>
<td><strong>Output layer</strong></td>
<td>PEG conc. in top Purelin</td>
<td>Water conc. in top Purelin</td>
<td>Partition coefficient Purelin</td>
</tr>
<tr>
<td><strong>Epochs</strong></td>
<td>5000</td>
<td>5000</td>
<td>5000</td>
</tr>
</tbody>
</table>

Usually, an insufficient number of neurons in the hidden layer cannot cope with very complex problems, while excessive neurons lead to fluctuation and divergence. Therefore, an architecture consisting of one hidden layer with 4 or 5 neurons was used. The final topology was adopted after the network failed to learn with various numbers of neurons in the hidden layer.

The network performance was measured with the error function \( (E) \) defined as the mean square sum of the differences between the network \( (a) \) and desired outputs \( (d) \).

\[ E = \sum_j (d_j - a_j)^2 \]  
(4.7)
An efficient learning algorithm that converges to an optimum set of weights is critical to the success of the network. The gradient-descent technique-based back-propagation algorithm was adopted to minimize the error. This algorithm updates the weights and biases in the direction of the negative gradient of the performance function. To avoid local minima, due to the magnitude of the function gradient, resilient back propagation algorithm ("trainrp") was exploited using the sign of the gradient to determine the direction of the weight change with a controlled change in the magnitude. For all the networks, the inputs and outputs were normally distributed and the experimental data set was divided into training and testing sets randomly. The entire range was covered in the training to avoid extrapolation during the testing session. The number of epochs used for the training was not very large to circumvent over training of the network. The minimization process was terminated after a predetermined error limit was achieved. At the end of the training phase, an optimal set of connection weights was obtained.

4.6 Pilot-plant

A pilot scale plant with counter-current operation was studied in a two-stage mixer-settler for beta-glucosidase partitioning in ATPS. The mixer capacity was 320 ml with a variable speed mixer motor (40 to 2000 r/m) and settler capacity was 1400 ml. The flow scheme of two-stage continuous counter-current mixer-settler is shown in Figure 3.4. In each stage, the reactants are mixed for mass transfer and passed to the settler for phase separation. A total quantity of 4600 g solution with PEG 4000 (8%) and phosphate salt (13%) system was used for the phase separation at a pH value of 7.0 and a temperature of
25 °C. The mixers speeds and pump flow rates were adjusted by performing a few preliminary experiments to reach a steady interface level.

![Diagram of two-stage continuous counter-current mixer-settler](image)

**Figure 4.4** Two-stage continuous counter-current mixer-settler.

PEG and salt solutions were prepared separately by dissolving the compounds in 50% of the required volume of water. The PEG solution was introduced to mixer I and the salt, to mixer II (Figure 4.4). The counter-current scheme was produced by transferring PEG from stage I to stage II and phosphate salt in the reverse direction. The outlets of bottom phase of stage I and top phase of stage II were concentrated in salt and PEG, respectively. Pure beta-glucosidase was supplied to the mixer I with PEG solution. Samples were withdrawn every 30 min for 6 hrs from the top and bottom phases of both stages and the enzyme activity was measured.
Further, the pilot-plant was studied for the extraction of beta-glucosidase from *T. reesei* culture filtrate. In this experiment, PEG and phosphate salt were dissolved in the culture filtrate instead of water. The experiment followed the same procedure as described above. The samples were taken every 60 min and analyzed for beta-glucosidase and total protein concentrations.
5.1 Preliminary Results

The complex phenomenon of protein partitioning in ATPS is the result of many factors involving hydrogen bonding, charge interactions between molecules, van der Waals forces, hydrophobic interactions, and steric effects. These factors are related to properties of the system such as polymer molecular weight, system pH, and temperature, as well as properties of the protein such as size, surface charge, and hydrophobicity. This section of the chapter is based on preliminary experimental works that were performed in order to investigate the partitioning behavior of total protein and beta-glucosidase based on the system and protein properties.

In these experiments, the aqueous two-phase systems were formed by mixing poly(ethylene glycol) and potassium phosphate salt (Dextran was used in the place of potassium phosphate in a few systems). First, equilibrium composition data were collected after varying the polymer molecular weight and system temperature in order to understand the influence of these factors on the binodal curve. These experiments were conducted in the absence of total protein and beta-glucosidase. Second, the effects of the polymer molecular weight, system temperature and pH on total protein partitioning were studied. Third, pure beta-glucosidase was used in different polymer/water/polymer and polymer/water/salt solutions in order to identify the best aqueous two-phase components for extracting the enzyme. Fourth, the effects of pH, temperature, PEG and salt concentrations on beta-glucosidase partitioning were investigated using the system
selected in the previous step. The findings of this study were essential for devising a sound strategy for purifying the enzyme produced by _T. reesei_.

5.1.1 Aqueous Two-phase Systems

A typical aqueous two-phase system composed of PEG 1450/potassium phosphate salt ATPS was constructed at a constant temperature of 32 °C. The concentrations of phosphate salt and PEG were plotted as abscissa and ordinate, respectively, in units of weight percentage (Figure 5.1). The variations in the initial concentrations of PEG and salt generated a pair of top and bottom phase compositions (B and C) located on the binodal curve.

![Figure 5.1 Binodal curve of PEG 1450/Potassium Phosphate salt ATPS.](image)

The measurements of refractive index and conductivity (for PEG and salt concentrations, respectively, Section 4.2) showed the presence of all of the components
in both phases. However, PEG concentration was higher in the top phase while the salt concentrated in the bottom phase. No Separation could be obtained for concentrations below the binodal curve, as the system remained homogeneous in this region. Above the binodal curve, in the heterogeneous region, the system separated in two phases. As shown in Figure 5.1, the system total composition, A, resulted in two compositions B and C, for the top and the bottom phases, respectively. The system conditions affect the position of the binodal curve and, subsequently, partitioning of biomolecules. Development of the phase diagrams aided in determining suitable PEG and salt compositions, required for efficient partitioning.

5.1.1.1 Effect of PEG Molecular Weight. Binodal curves for different polymer molecular weights (PEG 1450, 4000, 6000, and 8000) were determined at a constant temperature 32 °C. As shown in the Figure 5.2, the main effect of increasing the molecular weight was to lower the binodal curve towards the origin. This resulted in an increase of the heterogeneous area of the phase diagram. With the increase in molecular weight of the polymer, the equilibrium polymer concentration increased while the equilibrium salt concentration decreased in the top phase. The reverse was true for the bottom phase. This observation indicates that as the PEG molecular weight increases, the self-interactions of PEG molecules become stronger than the interactions between PEG and other molecules. Therefore, the PEG molecules accumulated to one phase, removing other molecules from this phase, which also supports the discussion in Section 3.1.5.
This phenomenon can be explained more elaborately by noticing that the increase in the molecular weight of PEG is characterized by an increase number of monomers (\(-\text{CH}_2\text{-CH}_2\text{-O}\)-) in the PEG molecule. The number of \(-\text{OH}\) groups at the end of the molecule remains the same, although the number of \(-\text{O}\)- groups increases with the molecular weight. For a constant amount of PEG, an increased number of \(-\text{O}\)- groups enhances the intramolecular interactions in PEG segments, thereby, excluding the other molecules from its vicinity. Consequently, the PEG equilibrium concentration increased in the top phase while more phosphate ions migrated to the bottom phase. As the molecular weight of the polymer increased, this effect was amplified. Therefore, low PEG and salt concentrations are required for phase separation involving a high molecular weight PEG molecule.
5.1.1.2 The Effect of Temperature. Binodal curves of the PEG 4000/phosphate salt system at 22, 32, and 42 °C are shown in the Figure 5.3. The system temperature affected the composition of two phases of a given system and, hence, the position of the binodal curves. The observations are in agreement with the predictions made in Section 3.1.6, where it was mentioned that raising the temperature of a polymer solution results in a decrease in the polymer solubility. Therefore, for a PEG/salt system, increasing the temperature reduced the solubility of PEG in water as the molecules of latter were excluded from the top phase. One possible explanation for such a behavior could be that the ‘ether-oxygen’ of the monomer unit in PEG is strongly hydrated with two or three water molecules, which are H-bonded to it at relatively lower temperatures. At elevated temperatures, this stabilized local water structure, due to partial orientational polarization of water molecule in the vicinity of the polymer hydrophobic sites, gets disrupted because of the increased thermal motion (Kjellander, 1981). As a result, the solubility of PEG decreased and more PEG moved to the top phase, subsequently shifting the position of binodal curves as the temperature of the system is varied.

Several authors have reported the effects of the temperature on the tie-line length (Silva, 1997; Sivars, 2000). The tie line length (TLL) for a defined system is given by:

\[ TLL = \left[ \left( w^T_{\text{salt}} - w^B_{\text{salt}} \right)^2 + \left( w^T_{\text{PEG}} - w^B_{\text{PEG}} \right)^2 \right]^{\frac{1}{2}} \]  

(5.1)

where the weight percentages of the phase-forming components, k (k = Psalt, PEG), in the top (T) and bottom phase (B) are represented by \( w^T_k \) and \( w^B_k \), respectively.

It was observed that the two-phase region expanded and the binodal curves moved towards lower concentrations with the increase in temperature. Thus, the concentrations of PEG in the top and salt in the bottom phases increased (also the difference in the
concentrations of respective components increased in both the phases), resulting in increased tie-line lengths.

Figure 5.3 Effect of temperature on binodal curve.

5.1.2 Total Protein Partitioning in ATPS

An understanding of total protein partitioning behavior is required since beta-glucosidase has to be extracted from a total protein solution. To achieve this goal, several experiments were carried out to highlight the contributions of the system conditions on protein partitioning. These findings were later used to develop a model for predicting total protein partition coefficient.

Electrostatic interaction and protein hydrophobicity are the two main factors affecting partitioning of proteins in ATPS. The salt anions present in the bottom phase
attract the positively charged proteins due to electrostatic forces, while proteins with nonpolar groups and negative charges prefer the PEG-rich hydrophobic phase because of the repulsion caused by the salt anions (Shang, 2004; Sebastiao, 1996). In the experiments described in this section, total protein, because of the negative charge carried at a pH greater than 6.0 (above the isoelectric point) and also due to the presence of nonpolar hydrophobic groups on the surface favored the PEG-rich phase, resulting in a partition coefficient higher than 1.

5.1.2.1 Effect of PEG Molecular Weight on Total Protein Partition Coefficient. The partition coefficient of protein was measured in PEG 8% (w/w) and phosphate salt 13% (w/w) ATPS. Systems with different PEG molecular weights (MW) of 1450, 4000, and 8000 were employed. The effect of increasing molecular weight of the polymer on the protein partition coefficient is shown in Figure 5.4, which illustrates a decrease in protein partition coefficient with an increase in molecular weight. The effect was more pronounced for MW changes in the range of 1450 - 4000.

The polymer molecular weight changed the polymer-protein interactions resulting in a protein buildup in the salt phase. This effect was discussed in section 3.2.3 using Gibb's free energy equation. As the number of polymer segments per molecule increased with PEG molecular weight, the added interactions between PEG molecules excluded more protein molecules from the top phase and thus, lowered the partition coefficient. This is in agreement with the work reported by Albertsson et al. (1986), who showed that an increase in the polymer molecular weight causes the protein molecules to transfer to the phase deficient in the polymer.
5.1.2.2 Effect of Temperature on Protein Partition Coefficient. The effect of temperature on total protein partition coefficient (K) was investigated in ATPS (PEG/potassium phosphate salt). The results are presented in Figure 5.5, which shows the variation of the partition coefficient with molecular weight for different sets of experiments conducted at 32 °C, and 42 °C. It was observed that an increase in the temperature of ATPS was accompanied by an increase in the partition coefficient of total proteins in a PEG-8000/Psalt system (Walter, 1985). However, in the case of PEG 1450, there was a substantial increase in K at low temperature (32 °C), whereas for PEG 4000, the change in K, at lower temperature, was insignificant.
Figure 5.5 Effect of temperature on total protein partition coefficient.

Such a deviation from the expected trend is not clear. This could be attributed to a combined effect involving additional factors such as changes in the phase compositions and the "salting out" effect (discussed more elaborately in section 5.2.3.2).

5.1.2.3 Effect of pH on Protein Partition Coefficient. PEG/phosphate salt systems were also used to study the effect of protein partitioning in ATPS at different pH values. The pH values measured in both the phases were equal. This is due to the same ratio of K$_2$HPO$_4$/KH$_2$PO$_4$ in both the phases (Peng, 1995). Figure 5.6 shows the influence of pH (4.5 and 5.0) on the partition coefficient. The upper curve represents the variation of the partition coefficient with the molecular weight of the polymer at a pH of 5.0. Data collected for a pH of 4.5 are shown in the lower curve.
Figure 4.6 Effect of pH on total protein partition coefficient.

The protein partition coefficient of the protein increased significantly with the increase in pH. The increase in partition coefficient with system pH was relatively higher for PEG 1450 than PEG 4000 and PEG 8000. This effect is primarily attributed to the changes in the electrostatic charge on the protein surface and to the salt ions. This point is discussed in detail in Section 5.2.3.1.

5.1.3 Selection of ATPS for Pure Beta-glucosidase Partitioning

Partitioning studies were conducted to determine the best system for beta-glucosidase partitioning. Various ATPS such as two polymer systems, polymer and salt system, polymers with affinity ligands, polymers with electrolytes have been investigated to separate proteins from cell cultures. PEG/dextran and PEG/phosphate salt are frequently
used for proteins purification, since these phase forming components provide benign and non-disruptive environment for proteins.

In the present work, PEG/phosphate salt and PEG/dextran system (PEG MW 4000 and 8000) were used with NaCl (0.1 M), KSCN (0.1 M) and Eudragit S-100 (2 g/ml, pH 7.0). The presence of neutral salts NaCl and KSCN affects protein partitioning in ATPS (Mistry, 1993; Johansson, 1998). Eudragit S-100, a reversibly soluble-insoluble polymer, acts as an affinity ligand to selectively bind beta-glucosidase (Agarwal, 1996). This polymer carries a net negative charge and binds the positively charged enzyme predominantly by electrostatic interaction. Therefore, these components were added to ATPS to find the most suitable system for beta-glucosidase purification.

Table 5.1 shows the results obtained for beta-glucosidase partitioning in different systems. Beta-glucosidase molecule is hydrophilic with polar groups on the surface. The pH values of the systems were below the isoelectric point of beta-glucosidase (pI 8.7). Therefore, in most of the systems studied, the positively charged hydrophilic enzyme partitioned to the negatively charged bottom phase.

For the systems that included Eudragit, the partition coefficient was relatively high. This indicates that the enzyme moved to the top phase in the presence of this polymer. The systems with NaCl and KSCN also resulted in higher value of the partition coefficient compare to PEG/phosphate salt and PEG/Dextran systems. Although, PEG 4000/dextran system provided the lowest partition coefficient, the major drawback of dextran is its high molecular weight and high viscosity for industrial purpose. Also, dextran is a very expensive polymer. Therefore, PEG/dextran system was not used for further study.
Table 5.1 Beta-glucosidase Partition Coefficient in different ATPS

<table>
<thead>
<tr>
<th>Aqueous two-phase system</th>
<th>Beta-glucosidase partition coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG4000/Dextran</td>
<td>0.0609</td>
</tr>
<tr>
<td>PEG4000/Phosphate salt</td>
<td>0.1148</td>
</tr>
<tr>
<td>PEG4000/Phosphate salt/Eudragit S-100</td>
<td>0.5272</td>
</tr>
<tr>
<td>PEG8000/Dextran</td>
<td>0.0632</td>
</tr>
<tr>
<td>PEG8000/Phosphate salt</td>
<td>0.2376</td>
</tr>
<tr>
<td>PEG8000/Phosphate salt/Eudragit S-100</td>
<td>1.0057</td>
</tr>
<tr>
<td>PEG8000/Dextran/Eudragit</td>
<td>1.4655</td>
</tr>
<tr>
<td>PEG4000/Phosphate salt/Eudragit S-100/NaCl</td>
<td>1.3446</td>
</tr>
<tr>
<td>PEG4000/Phosphate salt/KSCN</td>
<td>0.3878</td>
</tr>
</tbody>
</table>

PEG 4000/phosphate salt system showed lower partition coefficient than other systems. In addition, phosphate salt is a commercially available product and its low cost makes it attractive for large-scale separations. Therefore, this system was selected in further investigations.

5.1.4 Pure Beta-glucosidase Partitioning in ATPS

Results from Section 5.1.3 show that a PEG 4000/phosphate salt aqueous two-phase system was suitable for isolating beta-glucosidase in the bottom phase. Therefore, this system was selected for further studies. In PEG 4000/phosphate salt systems, the salt anions present in the bottom phase attract the positively charged proteins due to the electrostatic forces. Beta-glucosidase possesses a positive charge at the pH values used in the experiments described in this section and partitioned to the anion-rich bottom phase.
The experiments were conducted at various pH and temperature in order to understand the influence of these factors on enzyme partitioning. These data would help to develop a model for the prediction of the enzyme partition coefficient. Other factors, such as the concentrations of PEG and phosphate salt, were investigated. Further, the kinetics of beta-glucosidase partitioning in ATPS was studied for the purpose of large-scale operations.

5.1.4.1 Effect of pH. PEG 4000 (8% w/w) and potassium phosphate (13% w/w) ATPS at pH of 6.5, 7.0, 7.5 and 8.0 were used to study the effect of the system pH on the partitioning of beta-glucosidase. The results are shown in Figure 5.7, which shows variations in beta-glucosidase partition coefficients as a result of pH and temperature changes.

An increase in pH values was accompanied by a sharp increase in the enzyme partition coefficient. This trend was observed for all the temperatures studied. In all the systems, the pH was lower than the isoelectric point of beta-glucosidase (pI 8.7), which ensures that the enzyme was positively charged (Fruton, 1958). Therefore, the enzyme should have partitioned favorably to the bottom phase (partition coefficient is less than 1). For a few systems, the partition coefficient exceeding 1.0 may be attributed to the effect of high temperature.
5.1.4.2 Effect of Temperature. PEG 4000/phosphate salt systems were used at 25, 40, 45 and 50 °C to investigate the effect of temperature on pure beta-glucosidase partitioning (Figure 5.8). The partition coefficient increased with the increase in temperature for pH values of 6.5 and 8.0. At temperatures below 45 °C, the partition coefficient was lower than 1.0 (relatively more enzyme favored the bottom phase than the top phase).

Above 45 °C at a pH of 8.0, the partition coefficient was higher than 1.0 (the enzyme concentration was higher in the top phase than the bottom). Due to the ‘salting out effect’ (Section 3.2.2.1), the increased salt concentration excluded beta-glucosidase from the bottom phase.
As a result, the partition coefficient increased with the temperature. This effect is discussed in detail in the section 5.4.3.2.

5.1.4.3 Effect of Polymer and Salt Concentrations. The concentrations of phase forming components also influence the partitioning behavior of the enzyme. These effects were studied using different concentrations of PEG 4000 at constant phosphate salt concentrations and vice versa at a system pH of 7.0 and a temperature of 25 °C.

The results demonstrate that the partition coefficient increased with increases in both, PEG (Figure 5.9) and salt concentrations (Figure 5.10). This finding can be explained by noticing the dependence of the equilibrium phase compositions on the initial
Figure 5.9 Effect of polymer concentration on beta-glucosidase partition coefficient.

Figure 5.10 Effect of Potassium Phosphate salt concentration on beta-glucosidase partition coefficient.
PEG and salt concentrations. Similar observations were made earlier by Zaslavsky (1995) who showed a system deviating from its critical composition as the polymer or salt concentrations were increased. Consequently, the difference between the compositions of the two phases (and the tie-line length from Equation 5.1) became larger.

Changes in TLL affect the free volume available for a solute in a phase due to the changes in the number of water molecules for solvation. Since, the salt concentration increases in the bottom phase, less water molecules are available for solvation of beta-glucosidase. Hence, the solubility of the enzyme decreased in the bottom phase and it is directed to the more hydrophobic PEG environment. This “salting out” effect is more pronounced at higher PEG and salt concentrations.

5.1.4.4 Partitioning Kinetics. The time required for protein partitioning depends on the system conditions. This time is function of the density and viscosity of the phases and the time required for small droplets that are formed during mixing to coalesce into large drops. Using a PEG 4000/phosphate salt ATPS, the enzyme partitioning dynamics is shown in Figure 5.11. The partition coefficient decreased sharply (1.3 to 0.2) during the first 20 minutes and gradually for the next 10 minutes until equilibrium value was reached.
5.1.5 Cellulase Precipitation with Chitosan

Pure cellulase includes endo-glucanase, exo-glucanases and beta-glucosidase. The combination of these three enzymes is present in several sources of cellulase, including microorganisms. According to the purification technique outlined Section 4.3.5, chitosan was selected to separate beta-glucosidase from other cellulases selectively. Chitosan, a reversibly soluble-insoluble polymer, is a form of chitin that shows affinity to cellulases; endo-glucanase and exo-glucanase except beta-glucosidase. Pure beta-glucosidase, and cellulase enzymes were treated separately with chitosan to verify the binding quality of this polymer.

Table 5.2 shows the results obtained from precipitation of beta-glucosidase with chitosan. After inducing precipitation of chitosan by increasing the pH, the activity of the
pure enzyme remained unaffected in the supernatant. It confirmed that beta-glucosidase did not conjugate with chitosan.

**Table 5.2** Chitosan Precipitation with Pure Beta-glucosidase

<table>
<thead>
<tr>
<th>Time</th>
<th>Beta-glucosidase (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr</td>
<td>0.0032 ± 0.0003</td>
</tr>
<tr>
<td>Before 2 hr incubation</td>
<td>0.0032 ± 0.0007</td>
</tr>
<tr>
<td>After 2 hr incubation</td>
<td>0.0027 ± 0.0003</td>
</tr>
</tbody>
</table>

The next step focused on the affinity of the polymer for the cellulase. The results in Table 5.3 show that filter paper activity in the supernatant decreased after precipitation, although beta-glucosidase activity was unchanged. The reduction in FPU activity without any change in beta-glucosidase activity proved that chitosan bound endo and exo-glucanase, while the target enzyme remained in the solution. Thus, these preliminary experiments led to an important conclusion for purifying beta-glucosidase from other cellulase contaminants.

**Table 5.3** Chitosan Precipitation with Pure Cellulase

<table>
<thead>
<tr>
<th>Time</th>
<th>Cellulase (FPU/ml)</th>
<th>Beta-glucosidase (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr</td>
<td>0.020 ± 0.0007</td>
<td>0.0031 ± 0.0004</td>
</tr>
<tr>
<td>Before 2 hr incubation</td>
<td>0.020 ± 0.0007</td>
<td>0.0031 ± 0.0004</td>
</tr>
<tr>
<td>After 2 hr incubation</td>
<td>0.001 ± 0.0004</td>
<td>0.0032 ± 0.0004</td>
</tr>
</tbody>
</table>
5.2 Cell Culture

5.2.1 Cell Culture Growth

Cellulase enzyme beta-glucosidase can be produced by a number of microorganisms such as aerobic or anaerobic bacteria and fungi. The most widely used and best characterized fungus, *Tricoderma reesei*, was selected for the production of the target enzyme (Busto, 1996; Domingues, 2000). To improve the cultivation, carbon source and inoculum concentrations were adjusted for high activity of beta-glucosidase in cell culture.

![Graph showing enzyme activity over time](image)

**Figure 5.12** Beta-glucosidase activity in growing *T. reesei* culture.

In addition to beta-glucosidase, *T. reesei* produces other cellulase enzymes (endo-glucanase and exo-glucanase) and low molecular weight proteins. Figure 5.12 shows increasing beta-glucosidase activity in the cell culture with respect to time. The enzyme activity could not be detected at the beginning of the cultivation but increased sharply after 7 days. The maximum beta-glucosidase activity, 0.02 U/ml was found in the culture.
filtrate after 10 days of incubation. After the eleventh day, almost no increase was found and later the enzyme activity started decreasing.

5.2.2 Purification Scheme

Beta-glucosidase was purified from the culture filtrate of the fungus \textit{T. reesei}. The contaminants of glucanases and low molecular weight proteins were removed using sequential purification approach as shown in the section 4.3.5. Affinity partitioning with Chitosan, a reversibly soluble-insoluble compound was used to separate beta-glucosidase from other cellulases selectively. This step was followed by partitioning in aqueous two-phase system (ATPS), to purify beta-glucosidase from other low molecular weight proteins, as proteins showed a greater affinity to the top phase, while enzyme separated to the bottom phase.

5.2.2.1 Cellulase precipitation with Chitosan. Chitosan is a partially N-deacylated form of chitin and shows affinity to cellulases such as endo-glucanase and exo-glucanase except beta-glucosidase. Affinity precipitation technique with Chitosan was employed as a first step to separate beta-glucosidase from other cellulases selectively. The experiments were conducted on the culture filtrate and the activities of cellulase and beta-glucosidase were measured in the solutions with and without chitosan. Table 5.4. shows the data obtained from this first step of purification.

The culture filtrate consisted of beta-glucosidase and cellulase activities 0.0039 UI/ml and 0.094 FPU/ml respectively. Beta-glucosidase activity in the solution without treatment of chitosan was 0.0026 UI/ml. The experiment involving chitosan presented the same enzyme activity in the solution after chitosan precipitation. The filtrate precipitation
with chitosan recovered nearly 99.9% (in terms of activity units) of the enzyme in supernatant. This result confirmed that chitosan could not bind beta-glucosidase.

Table 5.4  Cellulase and Beta-glucosidase Activity in Supernatant of the First Step

<table>
<thead>
<tr>
<th></th>
<th>Volume (ml)</th>
<th>Cellulase activity (FPU/ml)</th>
<th>Beta-glucosidase activity (IU/ml)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>4.0</td>
<td>0.094 ± 0.001</td>
<td>0.0039 ± 0.0004</td>
<td>/ -</td>
</tr>
<tr>
<td>Filtrate in buffer without Chitosan</td>
<td>5.5</td>
<td>0.041 ± 0.008</td>
<td>0.0026 ± 0.0003</td>
<td>/ -</td>
</tr>
<tr>
<td>Filtrate with Chitosan</td>
<td>5.5</td>
<td>0.0095 ± 0.003 (77%)</td>
<td>0.0026 ± 0.0001 (99.9%)</td>
<td>/ -</td>
</tr>
</tbody>
</table>

The cellulase activity in the filtrate was 0.094 FPU/ml. The treatment of filtrate with chitosan reduced the cellulase activity in the solution from 0.041 FPU/ml to 0.0095 FPU/ml. Thus, almost 77% of the cellulase activity (FPU units) was precipitated with chitosan. Decreased activity of cellulase in the supernatant with chitosan precipitation implied that chitosan bound other cellulates. Remaining FPU activity in the supernatant after precipitation was due to beta-glucosidase.

5.2.2.2 Culture Filtrate Partitioning in ATPS. PEG 4000 and potassium phosphate salt were used as in the second step to purify the enzyme from other low molecular weight proteins. Earlier work with pure beta-glucosidase showed that PEG 4000 with pH 6-7 at room temperature (25 °C) provided better results for beta-glucosidase recovery in the bottom phase. Purification results with ATPS are summarized in the Table 5.5. The most beta-glucosidase activity was partitioned to the bottom phase with partition coefficient 0.29 (lower than 1.0) and protein content to the top phase with partition coefficient 1.27 (higher than 1.0). Culture filtrate with 0.016 IU ml⁻¹ activity partitioned
between 0.0026 IU ml\(^{-1}\) in the top phase and 0.0088 IU ml\(^{-1}\) in the bottom phase. Similarly, protein concentration of 1.057 mg/ml partitioned 1.2425 mg/ml and 0.9767 mg/ml into top and bottom phases, respectively. The recovery between top and bottom phases was 11%, 89% for beta-glucosidase and 80%, 20% for protein, respectively. Results with ATPS showed that the system was able to concentrate the enzyme and proteins in two different phases.

Table 5.5 Protein Content and Beta-glucosidase Activity in 8% PEG and 13% (w/w) Phosphate salt ATPS at pH 6.3 and Temperature 25 °C

<table>
<thead>
<tr>
<th></th>
<th>Volume (ml)</th>
<th>Protein content (mg/ml)/yield (%)</th>
<th>β-glucosidase activity (IU/ml)/yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>8.6</td>
<td>1.2563 ± 0.003 / -</td>
<td>0.0160 ± 0.0002 / -</td>
</tr>
<tr>
<td>Top phase</td>
<td>2.6</td>
<td>1.2425 ± 0.004 / 79.75</td>
<td>0.0026 ± 0.0003 / 11.43</td>
</tr>
<tr>
<td>Bottom phase</td>
<td>6.0</td>
<td>0.9767 ± 0.002 / 20.45</td>
<td>0.0088 ± 0.0002 / 88.57</td>
</tr>
</tbody>
</table>

The separation of beta-glucosidase and proteins in opposite phases could be explained, considering that in aqueous biphasic systems, composed of PEG and phosphate salt, the top phase is positively charged while the bottom phase is negatively charged (Silva, 2002). As a result, positively charged proteins are directed to the bottom phase, while negatively charged proteins concentrate to the top phase. Favorable partitioning of total protein and beta-glucosidase to the top and bottom phases, respectively, is better understood by examining their respective isoelectric points (IP). Since beta-glucosidase isoelectric point is approximately 8.7 (Chen, 1992), the enzyme is positively charged at the pH of 6.3 and, therefore, partitioned to the bottom phase.
(K\textsubscript{glucosidase} is less than 1). The negatively charged proteins (pI < 6.0) partitioned to the top phase.

5.2.3 Beta-glucosidase and Total Protein Partitioning in ATPS

PEG 4000/phosphate salt ATPS provides mild conditions for partitioning of beta-glucosidase and is suitable for scale-up. This system was implemented first at a pH of 6.3 and at room temperature to separate the enzyme from total protein for the initial study described in the previous section. The results showed that beta-glucosidase and contaminant proteins concentrated in the opposite phases with partition coefficient below and above 1, respectively. The difference in partition coefficient can be attributed to the difference in molecular weight of the total proteins and the enzyme, their respective hydrophobicities and surface charges, and electrostatic interactions between the charged biomolecules and phases. Fine-tuning of the system with pH and temperature to maximize the concentration of target enzyme in one phase and contaminants to the opposite phase, was a challenging task. To achieve this goal, the experiments were performed on the culture filtrate to determine the best conditions (with high total protein partition coefficient and low beta-glucosidase partition coefficient) for separating the enzyme from contaminants.

5.2.3.1 Effect of system pH on Beta-glucosidase and Protein Partitioning Coefficients. The culture filtrate was partitioned in ATPS at different pH values of 6.0, 6.5, 7.0 and 7.5 and at constant temperatures (25 and 55 °C), for the selection of suitable pH for beta-glucosidase purification. This pH range was selected, since a system pH lower than 6.0, (i.e. for very acidic environment), can denature the protein and higher pH
Figure 5.13 Effect of system pH on beta-glucosidase partition coefficient.

is undesirable for effective partitioning of the enzyme to the bottom phase (pl. refer section 5.1.4.1).

Figures 5.13 and 5.14 show the effect of pH on the partition coefficient of beta-glucosidase and protein in PEG 4000/phosphate salt ATPS. An increase in the partition coefficient was observed with the increase in pH (Figures 5.13 and 5.14).
The change in partition coefficient can be explained by considering the change in the net charge of the enzyme and protein surfaces, compared to their isoelectric points 8.7 and 4.7 respectively. As the pH of the system was increased, the beta-glucosidase surface charge became less positive (closer to isoelectric point 8.7). As a result, partitioning of the enzyme into the negatively charged bottom phase, decreased. Thus, the beta-glucosidase partition coefficient increases with the pH. A similar explanation applies for the observed increase in protein partition coefficient at relatively high pH. The increase in pH augments the negative charge of the protein surface above the isoelectric point. The negatively charged protein concentrates more in the top phase, hence the partition coefficient increases.
Another explanation supporting the observed effect is that with an increase in the pH of the solution, the concentration of \( \text{HPO}_4^{2-} \) also increases (due to increase in di basic phosphate salt concentration). The attraction between this anion (\( \text{HPO}_4^{2-} \)) and beta-glucosidase cation should increase with an increase in pH of the system. However, the concentration of \( \text{OH}^- \) also increases with pH. As a result, positively charged beta-glucosidase is partly neutralized, which causes a decrease in electrostatic interaction between the salt ion and beta-glucosidase. Hence the partitioning of the enzyme to the bottom phase also decreases. An increased concentration of \( \text{HPO}_4^{2-} \) anions at high pH repells the negatively charged proteins more to the upper phase. Therefore, the protein partition coefficient increases with pH.

### 5.2.3.2 Effect of Temperature on Beta-glucosidase and Total Protein Partitioning.

To select the favorable temperature for beta-glucosidase purification, different temperatures were used to generate different systems. The partition coefficients of beta-glucosidase and total protein with respect to temperature are plotted in figure 5.15 and 5.16, at different values of system pH. These figures show a monotonic increase in beta-glucosidase and total protein partition coefficients when the system temperature changes from 25 to 55 °C at constant pH. The effect of temperature on the binodal curve for PEG 4000 and phosphate salt was shown in the section 5.1.1.2.
Figure 5.15 Effect of the temperature on beta-glucosidase partition coefficient.

Figure 5.16 Effect of the temperature on total protein partition coefficient.
With the increase in temperature, the binodal curve moved down and the increase in two-phase region above the binodal curve resulted in increased difference in the phase compositions. The change in partition coefficients of the biomolecules with temperature may be attributed to this variation in the phase compositions. An increase in temperature was also accompanied by an increase in the concentration of phosphate salt in the bottom phase. As a result, in that phase, water molecules available for solute solvation should have decreased, which also reduced the solubility of beta-glucosidase and total protein (salting out effect). This process increased the affinity of the biomolecules for the top phase, as is evident by the enhanced partition coefficients (Figures 5.15 and 5.16).

5.2.3.3 Effect of System pH and Temperature on Yield. Figure 5.17 and 5.18 show the effect of pH and temperature on \( \beta \)-glucosidase yield in the salt-rich phase. In all experiments, the phase volume ratio \( (V_T/V_B) \), where \( V_T \) is the volume of the top phase and \( V_B \) is the volume of the bottom phase, was less than 0.5. This helped to increase the yield of the enzyme in the bottom phase with lower partition coefficients. A decrease in the yield of beta-glucosidase activity was observed for increased system pH and temperature. This decrease was more pronounced when the system pH was increased from pH of 6 to pH of 7.0, at constant temperature.

The variation in the yield was also more severe at higher temperature (35 °C) than at the lower temperature (25 °C). Therefore, it can be seen that the yield increases with lowering the pH together with the system temperature. At pH values less than 7.0 and temperature below 35 °C, the yield of beta-glucosidase in the bottom phase was greater than 92%.
Figure 5.17 Effect of system pH on yield of beta-glucosidase.

Figure 5.18 Effect of system temperature on yield of beta-glucosidase.
5.2.3.4 Selection of the Optimum System pH and Temperature of ATPS. The optimum separation condition can be determined from these experiments. Since an increase in pH and temperature is accompanied by a change in the partition coefficients of beta-glucosidase ($K_{\text{glucosidase}}$), and total protein ($K_{\text{protein}}$), in the same direction ($K_{\text{glucosidase}}$ from 0.23 to 0.50 and $K_{\text{protein}}$ from 1.0 to 1.29), it was necessary to compute the ratio $K_{\text{protein}}/K_{\text{glucosidase}}$ in order to find the best conditions to isolate the enzyme. A high $K_{\text{protein}}/K_{\text{glucosidase}}$ value promotes the concentration of contaminant proteins and beta-glucosidase in the top and bottom phases, respectively. This ratio was maximal at a pH of 6.5 and a temperature of 25 °C. Also, the high beta-glucosidase yield in the bottom phase (> 92%) at pH values less than 7.0 and temperatures below 35 °C supports the selection of these parameters.

5.3 Models for Phase Composition and Beta-glucosidase Partitioning

5.3.1 Flory-Huggins Model

Flory-Huggins interaction parameters between water (1), PEG 4000 (2) and potassium phosphate salt (3), were obtained using equilibrium data at seven different temperatures. Figure 5.19 shows $\chi_{ij}$ values at temperatures varying from 25 to 55 °C.

The parameters generated for all the systems were of the same order of magnitude to those estimated by Peng et al. (1995). Although the relationship between $\chi_{ij}$ and the temperature is not clear from the graph, the relatively high value of $\chi_{23}$ indicates a greater interaction energy between PEG 4000 and phosphate salt molecules. This supports the formation of two phases as extra energy is required to bring these molecules together.
The PEG and water concentrations of the top phase were calculated by minimizing the objective function represented by Equation 4.4. Calculated values are compared with experimental data in Figures 5.20 and 5.21. The wide scattering of the points about the regression line indicates a poor model performance (COR $\ll 1.0$). The significant difference between measured and model-predicted values could be attributed to underlying assumptions formulated in the Flory-Huggins model.
Figure 5.20 Flory-Huggins model predictions for PEG concentration in the top phase.

Figure 5.21 Flory-Huggins model predictions for water concentration in the top phase.
5.3.2 Limitations of the Thermodynamic Model

As discussed by Zaslavsky (1995), the shortcomings of the model may be attributed to the fundamental assumptions of the model such as ideal entropy of mixing and representation of an aqueous solution by lattice model. Also, the deviation from ideal solution accounted in the enthalpy term, is not sufficient. Besides, the model required constraints for solving the “reverse problem” (i.e., estimation of model parameters from experimental data). The constraints were needed to avoid the trivial solution and the negative values of the concentrations. In addition to that, a close guess to the equilibrium composition was required for the successful optimization.

Furthermore, the interaction parameters depend on the system conditions. For different temperatures, the model was not able to include all the binodal data in one regression. Interaction parameters were generated individually for different temperatures.

5.3.3 Artificial Neural Networks

Artificial neural networks were used to predict the equilibrium phase compositions of PEG and water in the top phase and the partition coefficient of \( \beta \)-glucosidase at various temperature and pH values. The data were randomized and divided into training and testing sets. During learning, the training set was fed to the networks and the weights were changed to minimize the error between the predicted and target values. Using the optimum weights from the training phase and the testing set (Figures 5.22 and 5.23), the performance of the ANN model was assessed. Both training and testing sample points were scattered closely about the regression line, which indicates the accuracy of the ANN model.
Figure 5.22 ANN prediction of PEG concentration in the top phase; o, test data; ж, training data.

Figure 5.23 ANN prediction of water concentration in the top phase; o, test data; ⬤, training data.
The correlation coefficients obtained for different models are shown in Table 5.6. A high correlation between experimental and predicted values was achieved. Compared to the thermodynamic model, the ANN model performed efficiently and circumvented the complex computation of interaction parameters. In addition, the computation time was reduced since it only took one ANN model to represent equilibrium data taken at various temperatures.

**Table 5.6 Correlation Coefficients of Flory-Huggins and ANN Models**

<table>
<thead>
<tr>
<th>Method</th>
<th>Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flory-Huggins</strong></td>
<td></td>
</tr>
<tr>
<td>PEG concentration in TOP</td>
<td>0.207</td>
</tr>
<tr>
<td>Water concentration in TOP</td>
<td>0.250</td>
</tr>
<tr>
<td><strong>ANN</strong></td>
<td></td>
</tr>
<tr>
<td>PEG concentration in TOP</td>
<td>0.938</td>
</tr>
<tr>
<td>Water concentration in TOP</td>
<td>0.943</td>
</tr>
</tbody>
</table>

For the prediction of beta-glucosidase partition coefficient, a thermodynamic model was not used because the addition of the biomolecule to the aqueous biphasic system increased the complexity of the model Equations and augmented the number of parameters to be calculated. Figure 5.24 shows the prediction of β-glucosidase partition coefficients using the ANN approach. There was a good agreement between calculated and measured values. The accuracy of the prediction is evident by the value of the correlation coefficient: 0.978.
5.4 Continuous ATPS Extraction of Beta-glucosidase in a Pilot-plant

The pilot plant studies with a continuous operation were carried to aid technology transfer to full-scale commercial applications. Pure beta-glucosidase and the culture filtrate were partitioned in PEG 4000 and phosphate salt ATPS in a continuous two stage counter-current aqueous two-phase extractor system (see section 4.6). After mixing in the mixers I and II, phase separation was accomplished in the settler columns I and II by settling under gravity. The flow rates were adjusted to provide sufficient time for settling.
Figure 5.25 Pilot-plant experiment for pure beta-glucosidase partitioning.

Figure 5.25 shows pure beta-glucosidase partition coefficient verses time curves in two settler columns. The run was conducted for about 6 hours. A drastic drop in partition coefficient was observed within 1 hr of the operation in the both columns. The equilibrium was achieved in approximately 2 hours. The short process time to achieve complete partitioning of pure enzyme showed compatibility of the process for commercial application. Pure beta-glucosidase partition coefficient (0.12) obtained for the pilot plant experiment was the same as for small test-tube scale experiment in identical system conditions.

The second process run in the pilot plant was purification of beta-glucosidase from culture filtrate of *T. reesei* fungus at pH 7.0 and 25 °C. Figure 5.26 and 5.27 show the data obtained for partitioning of beta-glucosidase and total protein, respectively. Beta-glucosidase partition coefficient became stable in 2 hours, while protein partitioning
Figure 5.26 Pilot-plant experiment for beta-glucosidase partitioning from *T. reesei* at pH 7.0.

Figure 5.27 Pilot-plant experiment for total protein partitioning from *T. reesei* at pH 7.0.
stabilized in about 2.5 hours. The results show that partition coefficients obtained for the pilot plant were different to those obtained for small scale experiment. Beta-glucosidase partition coefficient in the pilot plant (0.43) was higher than in the small scale experiment (0.26). Similarly, protein partition coefficient was higher in the pilot plant (1.23) than in the small scale experiment (1.03). This difference was expected due to the difference in the quantity of the processed volume of culture filtrate, equipment design and experimental procedure. However, these pilot plant experiments demonstrated the technical feasibility of the continuous counter current extraction process of ATPS for purification of beta-glucosidase at large scale.

![Partition Coefficient vs Time](image)

**Figure 5.28** Pilot-plant experiment for beta-glucosidase partitioning from *T. reesei* at pH 6.5.
Further, optimized conditions obtained in the section 5.2.3 were used to improve the purification of beta-glucosidase from the culture filtrate. Beta-glucosidase and protein partition coefficients obtained at pH 6.5 and 25 °C are shown in figures 4.28 and 4.29, respectively. Compared to the previous system (with pH 7.0) these results showed improved concentration of the enzyme in the bottom phase with a low value of partition coefficient 0.35. Although, the lower values of protein partition coefficient in this system were unfavorable, higher value of selectivity, by calculating the ratio of protein and beta-glucosidase partition coefficient, at pH 6.5 supports the system conditions.
A new sequential scheme of affinity precipitation and partitioning in aqueous two-phase system was developed to efficiently purify beta-glucosidase from the culture filtrate of *T. reesei*. Precipitation of β-glucosidase free cellulase with Chitosan successfully separated specific contaminants endo-β-glucanase and exo-β-glucanase from the crude preparation. The purification of beta-glucosidase from other protein contaminants in aqueous two-phase systems formed by PEG and potassium phosphate salt were also investigated.

The position of the binodal curve was affected by PEG molecular weight and temperature. The increased intramolecular interaction of PEG molecules at high molecular weight and disruption of local water structure at elevated temperature increased the PEG concentration in the top phase. The two-phase region expanded and the binodal curves moved towards lower concentrations with the increase in PEG molecular weight and temperature.

The preliminary work with total protein showed that due to the electrostatic interactions and protein hydrophobicity protein partitioning was influenced by system conditions. The increase in molecular weight produced a decrease, while increase in pH produced an increase in partition coefficient. However, the effect of temperature on partitioning was not very clear.

PEG 4000/Potassium Phosphate salt system provided the most suitable system for pure beta-glucosidase separation among the different ATPS considered. An increase in a host of system factors such as system pH, temperature, PEG and salt concentrations showed increase in beta-glucosidase partition coefficient. Treatment of pure beta-
glucosidase and cellulase with Chitosan showed affinity of Chitosan to endo-glucanase and exo-glucanase except beta-glucosidase.

Beta-glucosidase purification from crude extract of *T. reesei* reinforced the earlier findings obtained with preliminary work. The cultivation medium (nutrient) was optimized for the production of beta-glucosidase from *T. reesei* cell culture. The experiment involving chitosan resulted in the precipitation of endo and exo-glucanases. During this separation step, beta-glucosidase activity was completely recovered in the supernatant.

PEG 4000 and Potassium Phosphate salt were used as the second step to purify the enzyme from other low molecular weight proteins. The results showed that beta-glucosidase and contaminant proteins concentrated in the opposite phases with partition coefficient below and above 1, respectively. The concentration of target enzyme in one phase and contaminants to the opposite phase was maximized by fine-tuning of the system with pH and temperature. Increase in the partition coefficients were observed with the increase in pH for both enzyme and protein. The changes in partition coefficients were due to the change in the net charge of the enzyme and protein surfaces. Similarly, a monotonic increase in beta-glucosidase and total protein partition coefficients was obtained with the increase in system temperature. The change in partition coefficients of the biomolecules with temperature was attributed to the variation in the phase compositions due to the shifting of the binodal.

A decrease in the yield of beta-glucosidase acitivity was observed for increased system pH and temperature. At pH values less than 7.0 and temperature below 35 °C, the yield of beta-glucosidase in the bottom phase was greater than 92%. The optimum
separation conditions, a pH of 6.5 and a temperature of 25 °C were determined by computing selectivity.

Artificial Neural-Network models (ANN) predict the equilibrium phase compositions and beta-glucosidase partition coefficients very accurately. Compared to the Flory-Huggins theory, the neural-network approach required no parameter estimation and was able to incorporate the effects of the system temperature on equilibrium phase compositions. Although the thermodynamic framework was especially useful for quantifying polymer-solute, polymer-salt and salt-solute interactions, the major disadvantage of the Flory-Huggins model was the increasing number of parameters that could not be evaluated using experimental data and its failure to account for factors contributing to the partitioning of the biomolecule. The data-driven method was better suited to predict the partition behavior of the enzyme as a function of the system pH and temperature. The results showed that neural-network offered a remarkable performance with correlation coefficients close to unity.

The results obtained from pilot plant extractions were also found to be in good agreement with the small scale findings. The plant performance showed the practicability of continuous extraction of beta-glucosidase from culture filtrate.

Based on the results of the experiments and pilot plant study, there could be some future work which can be performed in order to transfer the technology to the production scale in the industry and to overcome some of the inherent limitations observed during the laboratory tests. The recommendations are as follows:

1. Ultrafiltration can be investigated for concentration of beta-glucosidase and removal of the phosphate salt from the product solution.
2. For therapeutic use of beta-glucosidase, the purification can be enhanced by coupling chromatography step with ATPS.

3. Magnetically enhanced phase separation can be explored to improve enzyme partitioning.

4. Recycling of PEG after beta-glucosidase extraction should be developed to make the process economically feasible for industrial purpose.

5. Pilot plant study including steady state and transient behavior of the system will facilitate to establish a fully integrated purification process. In addition, designing of control system for the plant will improve the efficiency of the extractor.
REFERENCES


47. Levy I., Shani Z., and Shoseyov O., Modification of polysaccharides and plant cell wall by endo-1,4-$\beta$-glucanase and cellulose-binding domains, *Biomolecular Engineering*, 19, 17-30 (2002).


