PURIFICATION OF NARINGINASE BY AQUEOUS TWO PHASE SYSTEM

A PROJECT REPORT

Submitted by

AL AHMIRATHUS SYEDUSH SHIFA K (Register Number: 0810204001)

DEEPIKA P (Register Number: 0810204008)

KIRTHIKA J (Register Number: 0810204018)

in partial fulfillment for the award of the degree

of

BACHELOR OF TECHNOLOGY

IN

BIOTECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY

(An Autonomous Institution Affiliated to Anna University of Technology, Coimbatore)

ANNA UNIVERSITY OF TECHNOLOGY, COIMBATORE
COIMBATORE-641 047

APRIL 2012

ANNA UNIVERSITY OF TECHNOLOGY COIMBATORE, COIMBATORE

BONAFIDE CERTIFICATE

Certified that this project report **"PURIFICATION OF NARINGINASE USING AQUEOUS TWO PHASE SYSTEM"** is the bonafide work of "**AL AHMIRATHUS SYEDUSH SHIFA K (Register Number: 0810204001), DEEPIKA P (Register Number: 0810204008) and KIRTHIKA J (Register Number: 0810204018)"** who carried out the project work under my supervision.

SIGNATURE
SUPERVISOR
Mr.S.Sivamani
Assistant Professor (Senior Grade)
Department of Biotechnology
Kumaraguru College of Technology
Chinnavedampatti
P. O. Box No. 2034
Coimbatore – 641 049

SIGNATURE
HEAD OF THE DEPARTMENT
Dr.A.Manickam
Professor and Head
Department of Biotechnology
Kumaraguru College of Technology
Chinnavedampatti
P. O. Box No. 2034
Coimbatore – 641 049

_____
Internal Examiner

_____
External Examiner

ACKNOWLEDGEMENT

We express our sincere and heartfelt thanks to our guide **Mr. S. Sivamani,** Assistant Professor (Senior Grade), Department of Biotechnology, Kumaraguru College of Technology, Coimbatore for his continuous encouragement and valuable guidance.

We would like to thank our Principal **Dr. S. Ramachandran** for the resources made available in this institution to carry out the project work successfully.

We would like to thank **Dr. A. Manickam**, Professor and Head, Department of Biotechnology, Kumaraguru College of Technology, Coimbatore. His gracious and ungrudging guidance all through our project work is highly acknowledged with gratitude.

We wish to convey our heartfelt thanks to our review committee members **Mr. M. Shanmugaprakash,** Assistant Professor (Senior Grade) and **Dr. P. Ramalingam**, Associate Professor, Department of Biotechnology, Kumaraguru College of Technology, Coimbatore who were helping us for our project work with their constructive and innovative ideas at crucial times.

We also thank **Mr. P. Muthukumaran,** Assistant Professor, **Dr. K. Kumaresan**, Assistant Professor (Senior Grade) and **Dr. T. Sathish Kumar,** Assistant Professor (Senior Grade), Department of Biotechnology, Kumaraguru College of Technology, Coimbatore and all our **teaching faculty** for their support and guidance and **non-teaching staff** who has supported during our project work.

AL AHMIRATHUS SYEDUSH SHIFA K DEEPIKA P KIRTHIKA J

ABSTRACT

A Single step purification technique was developed to purify the naringinase enzyme. *Aspergillus brasiliensis* MTCC 1344 was used to produce extracellular naringinase using agro industrial residues such as rice bran and cassava waste in solid state fermentation. Due to various applications of this enzyme in food and pharmaceutical industry, it is essential to purify the enzyme in an easy and economic way. Aqueous Two Phase System (ATPS) was selected to purify the naringinase enzyme, since it is an easy to scale up and inexpensive method. ATPS was constructed using PEG 4000 and ammonium sulphate salt. The binodal curve was constructed and points from the tie line were used for the system construction. The effect of partitioning behaviour of naringinase in aqueous two phase system has been studied. The partition coefficient of Naringinase and total protein decreases on increasing the molecular weight of PEG and increases on increasing the temperature The effect of pH (3-8), temperature (40-70□C) and Polymer Molecular weight (1000, 4000, 6000, 8000, 20000) for the system were studied and optimized. Optimum pH and temperature of the system was found to be 5.5 and 65□C respectively. Under these optimal conditions, the enzyme was purified with purification factor of 53.37-fold and 91.37% yield. The molecular weights of the subunits of an enzyme (α-L-rhamnosidase and β-D-glucosidase) were estimated to be 87 kDa and 80 kDa respectively by SDS PAGE Electrophoresis.

Keywords: Naringinase, Naringin, *Aspergillus brasiliensis*, cassava waste, rice bran, ATPS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVATIONS

MTCC	Microbial Type Cell Culture
ATPS	Aqueous Two Phase System
PEG	Polyethylene Glycol
SDS- PAGE	Sodium Dodecyl Sulfate- Poly Acrylamide Gel Electophoresis
DEG	Diethylene Glycol
NaOH	Sodium Hydroxide
SSF	Solid State Fermentation
BSA	Bovine Serum Albumin
$(NH_4)_2SO_4$	Ammonium sulphate
Ke,	Partition Coefficient of enzyme
KP	Partition Coefficient of protein
µg	Micro gram
ml	Milli liter
rpm	Rotations per Minute
kDa	Kilo Dalton
◦C	Degree Celsius
mM	Milli molar
h	Hour
MW	Molecular weight

CHAPTER 1
INTRODUCTION

1.1 GENERAL

Naringin is abundant in immature citrus fruits but its concentration decreases as fruit ripens (Yusof *et al*., 1990; Puri and Banerjee, 2000). Since naringin is the main bitter component of citrus juices, thus, its hydrolysis with a concomitant decrease in bitterness is of industrial importance. The naringin level can be reduced by technologies such as adsorptive debittering, chemical methods, treatment with polystyrene divinyl benzene styrene (DVB) resins and cyclodextrin. Because of the various drawbacks, the capabilities of non enzymatic debittering technologies are limited (Puri and Banerjee, 2000). In comparison, the enzymatic debittering technology is regarded as the most promising method with the advantages of high specificity and efficiency, and a convenient operation for removing the bitterness in large-scale commercial production. Enzymatic hydrolysis is a possibility to overcome the bitterness and obtain compounds with improved biological activities. Hence suitable debitterness can be achieved by treating the juice with an enzyme, naringinase, which directly hydrolyses naringin.

1.1.1 Mechanism of naringinase action:

Naringinase is an enzyme complex consisting of α-rhamnosidase (EC 3.2.1.40) and flavonoid β-glucosidase (EC 3.2.1.21). In typical processing, naringinase converts naringin to naringenin in a two-step process. The substrate naringin (4'- 5, 7'- trihydroxy flavonone- 7- rhamnoglucoside) is hydrolyzed by the α-rhamnosidase component to produce prunin (4'- 5, 7'- trihydroxy flavonone- 7- glucoside), which is then converted by the flavonoid β-glucosidase to naringenin (4'- 5, 7'-trihydroxy flavonone) (Chandler and Nicol, 1975; Habelt and Pittner, 1980).

Naringin can be hydrolyzed by α-L-rhamnosidase to rhamnose and prunin (one-third of the bitterness of naringin), which can be further hydrolyzed by β-D-glucosidase into glucose and tasteless naringenin.

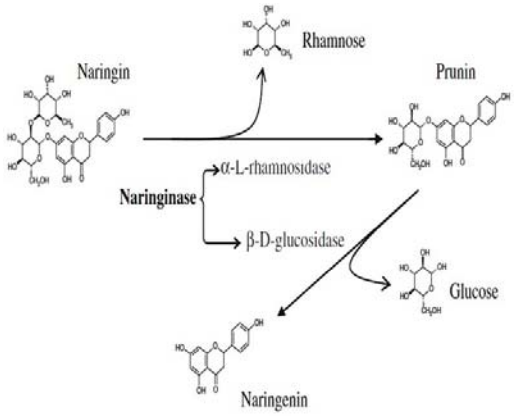


Figure 1.1 Stepwise degradation of naringin by the action of naringinase expressing α-L-rhamnosidase and β-glucosidase activities

The structures of hydrolyzed products of naringin (prunin, L-rhamnose, naringenin, and D-glucose) are shown in Figure 1.1 .Although naringinase are not common enzymes, there are several industrial applications like debittering of citrus juices (Thomas *et al*.,1958; Ono *et al*.,1978; Habelt and Pittner, 1983), deglycosylation of the novel glycopeptide antibiotic, chloropolysporin was achieved successfully by the rhamnosidase activity of Naringinase (Sankyo,1988). The enzyme is used to produce L-rhamnose which is a chiral intermediate in organic synthesis and it is used as a pharmaceutical and plant protective agent (Daniels *et al*., 1990).

1.1.2 Production of naringinase:

Production of naringinase from *Penicillium sp.* was originally suggested, but restricted for its industrial application in concern of high production cost, and particularly, the incomplete hydrolysis of naringin in citrus juice processing (Puri *et al*., 2001). In contrast, *Aspergillus niger* fermentation has shown more potential for industrial production of naringinase because the microorganism has been listed in the FDA's approved microbial category and proven safe for food and medicinal uses. Also, the process is easy to be scaled up for industrial application, and easy to increase the naringinase yield by optimizing the cultivation parameters (Puri *et al*., 2005). Therefore, it was considered as the most important source of naringinase for food use (debittering citrus juice) regarding the safety characteristics and the feasible culture process of *Aspergillus niger*.

The production of naringinase is inducer-dependent and continuous addition or stepwise addition of an inducer increases naringinase production. Whereas, replacement of the inducer with other carbon sources supports the growth of the organism but no enzyme is produced. Microbial naringinase may be produced both by submerged culture and solid-state fermentation. Compared to studies with submerged culture, solid-state fermentation has been barely investigated for production of naringinase. However, there is substantial scope for this mode of production, as revealed by demonstrated automation capabilities and operating experience with many other large-scale solid-substrate fermentation processes (Chisti, 1999). The moisture level plays a vital role in SSF, determining the success of the process. The importance of the moisture level in SSF media and its influence on microbial growth and product biosynthesis can be attributed to the impact of moisture on the physical properties of the solid substrate.

Enzyme production increased with the level of naringin, with the maximum titer corresponding to 10 mg naringin/mL nutrient solution. Increasing the naringin concentration did not have any significant effect on enzyme production. *Aspergillus niger* produced maximum naringinase levels (413 U/g) when Rice bran was moistened with nutrient solution at a ratio of 1:1, and the levels declined with further increases in the substrate-to-moisture ratio. Low moisture content is known to decrease the solubility of the nutrients and lead to low substrate swelling and higher water tension. Moisture levels higher than certain critical level cause low oxygen transfer, decreasing porosity, decreasing microbial growth, and lower metabolic activities in SSF. An increase in inoculum age generally improves growth and growth-related enzyme activities of the organism, up to a certain level. With further increase in inoculum age (216 h), there can be a reduction in enzyme activity as the organism enters into a decline phase. This indicates that the inoculum age has the desired effect on SSF.

1.1.3 Purification of enzymes by ATPS:

Usability of an enzyme in an industrial application is strongly depending on the characteristic properties of the enzyme. The conventional techniques involve multistep procedures which are time-consuming, expensive, and also result in lower yields. Hence, there is a need for efficient and economical bioseparation technique which can achieve high purity and increased recovery. Aqueous two-phase extraction (ATPE) is one such potential alternative technique (Raghavarao *et al*., 1998). It is a technique where extraction, concentration and partial purification can be integrated in a single step of liquid–liquid extraction. Aqueous two-phase systems (ATPS) are powerful and versatile that have been employed as an efficient tool in several biotechnology processes for the partitioning of biomolecules like proteins, enzymes, nucleic acids, animal, plant and microbial cells. The basis of separation is the selective distribution of a given biomolecule between the phases, depending on the characteristics of the phase system, properties of the biomolecule and the interaction between them. The partitioning of biomolecules in a standard biphasic aqueous system depends on pH, temperature, surface properties, size and concentrations of the biomolecules, types of employed polymers, molecular weight of the phase forming polymers, the concentration and type of the salt, and pH. The partitioning of biological materials is a result of Van der Waals and ionic interactions of the biomolecules with the surrounding phase. Hydrophobic interaction and hydrogen bonding too plays an important role in the partitioning. The two immiscible aqueous phases are generated by addition of substances such as polymers and salts to an aqueous solution. The top phase is rich in one polymer and the bottom phase is rich in other polymer or salt. The two phases both containing water at high proportion (about 80-99% by weight) and possess extremely low interfacial tensions. Two types of aqueous two-phase systems are commonly used:

i. Polymer-polymer two-phase system
ii. Polymer-salt two-phase system

Compared to the traditional separation and purification techniques including salting out and chromatographic methods, ATPS have several advantages such as low processing time and energy consumption, high capacity and yield, biocompatibility, easy to scaling-up and non-toxic, biocompatible environment for the biomolecules, lower interfacial tension, and scope for continuous operation. There are several reports for partitioning and recovery of different biomolecules and enzymes in ATPS using different systems like toxin, green fluorescent protein, insulin, lipase, alcohol dehydrogenase, galactosidase and invertase from different sources. Partitioning in ATPS is an emerging technique that has applications in purifications of various proteins and enzymes. The simplicity of the process and low cost of the phase forming materials make it feasible for large-scale protein purification. The PEG and salt concentrations in the ATPS were selected based on the critical concentrations obtained from the binodal curves at specified temperatures. Mono and dibasic salts were used to adjust the pH of ATPS.

1.1.4 Characterization of naringinase:

An enzyme isolated from *Penicillium* sp. is a glycoprotein which is electrophoretically homogenous determined by electrofocussing and SDS gel electrophoresis studies (Gabor and Pittner, 1984). The enzyme possesses both α-rhamnosidase (EC 3.2.1.40) and β-D-glucosidase (EC 3.2.1.24) activities. The pH optima for the two activities were 4.5 and 3.0, respectively (Gabor and Pittner, 1984). Using gel filtration, electrophoresis and immobilization (Schalkhammer and Pittner, 1986) determined the holoenzyme and subunit structure for this enzyme. Using data on the kinetic parameters K_m (Michaelis constant), V_{max} (the maximum rate), and K_i (the inhibition constant) for glucose and rhamnose (Schalkhammer and Pittner,1986) ascertained the existence of two catalytic sites. The chemical modification of both the active site regions supported the existence of an essential tyrosine site, as being necessary for both the activities. Modification of lysine group at one of the sites increased glucosidase activity by up to 5-fold.The commercially available naringinase of *Aspergillus niger* also has both α-rhamnosidase and β-D-glucosidase activities (Roitner *et al*., 1984). The ratio of these activities varies with the protein concentration and the pH. The rhamnosidase activity is nearly independent of pH in the range from 3 to 7, whereas glucosidase activity shows a distinct optimum that varies between pH 4 and 6, depending on the pretreatment used. The enzyme complex can be separated into various oligomers by gel filtration. The naringinase of *A.niger* appears to be a single enzyme with two active sites, one for the α-L-rhamnosidase activity and the other for β-D-glucosidase activity.

1.2 MOTIVATION:

Due to the availability of limited data, naringinase is the subject of much continuing research and it is commercially attractive. It is potentially useful in food, pharmaceutical, and flavoring applications; however, a limited supply of the enzyme restricts wider use. Commercially viable processes are required for large-scale production of the enzyme by fermentation. Only a few reports address fermentative production of naringinase at large scale and its subsequent use in debittering of fruit juice. There are no scientific reports available for purification of naringinase from *Aspergillus brasiliensis* MTCC 1344 using aqueous two phase extraction.

1.3 OBJECTIVES:

The objectives of this work are:

i. To identify suitable aqueous two-phase system for the partitioning of naringinase.
ii. To study the effect of process parameters such as polyethylene glycol (PEG) molecular weight, pH, temperature on the partition coefficient, activity recovery and purification factor, leading to an efficient downstream process for naringinase.
iii. To determine the molecular weight of the subunits using SDS PAGE.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 OPTIMIZATION OF TIME FOR THE NARINGINASE PRODUCTION:

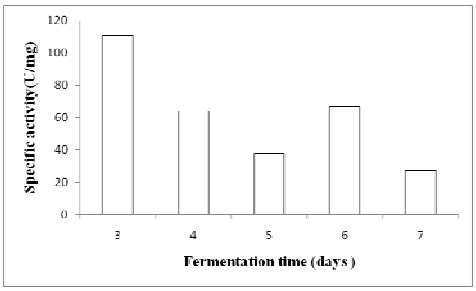


Figure 4.1 Effect of fermentation time on specific activity of naringinase production

From the Figure 4.1, it is found that enzyme production increased with incubation time, reaching a maximum at 3rd day and a further increase in the incubation period led to a decrease in the production of naringinase by *Aspergillus brasiliensis*. The decrease in activity after the incubation period may be due to denaturation of the enzyme, a decrease in naringin concentration, or an increase in glucose concentration, resulting from variations in pH and cellular metabolism during fermentation. (Shanmugaprakash *et al*., 2011)

4.2 pH OPTIMIZATION OF THE CRUDE ENZYME EXTRACT:

The optimum pH of naringinase (α- L - rhamnosidase and β`- D –glucosidase) was found by varying the pH values between 3.5 and 5.5, whereas the optimum temperature of each enzyme was determined at the optimum pH.

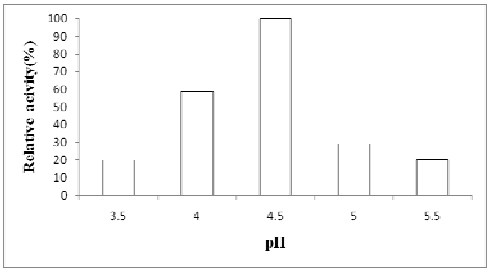


Figure 4.2 Effect of pH on relative activity of naringinase in crude enzyme extract

Figure 4.2 shows that, the optimum pH of *Aspergillus brasiliensis* MTCC 1344 producing naringinase activity was 4.5. This optimum pH of naringinase activity nearer to those of purified naringinase from *Aspergillus niger* 1344 (pH 4.0) (Puri and Kalra, 2005) and partially purified naringinase from *Penicillium decumbens* PTCC 5248 (pH 4.5) (Norouzian *et al*., 2000)

4.3 Temperature optimization of the crude enzyme extract:

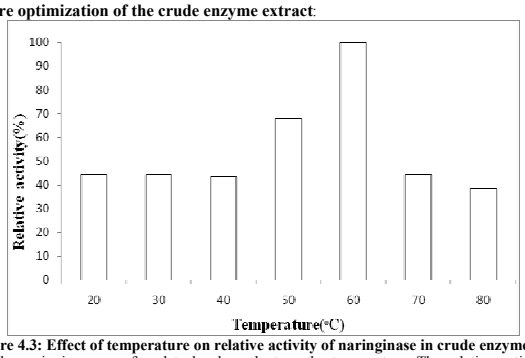


Figure 4.3: Effect of temperature on relative activity of naringinase in crude enzyme extract

The activity of the naringinase was found to be dependent on the temperature. The relative activity expressed in percentage of the maximum activity is presented in Figure 4.3 as a function of temperature. The optimum temperature was found to be 60□C. Also seen from the Figure 4.3, when the temperature was further increased the activity of the enzyme was reduced significantly. The enzyme activity declines at a temperature above 60□C (Puri and Kalra, 2005)

4.4 Effect of pH on partition coefficient of naringinase and protein in PEG-Ammonium sulphate system at 28□ C (U – Upper phase and L – Lower phase)

Table 4.4: Effect of pH on Partition coefficient of naringinase in ATPS

pH		3	3.5	4	4.5	5	5.5	6	6.5	7
Kp		0.583	0.48	2.57	0.188	0.38	**3.36**	0.18	0.365	0.94
Ke		0.33	0.68	1.175	0.47	0.31	**0.12**	0.17	0.179	0.28
Enzyme activity	U	95	34.68	95	60.83	87.5	65.83	29.175	31.68	72.5
	L	285	185	80.83	129.18	285	**530**	365.83	397.5	250.83
Specific Activity (U/mg)	U	226.19	99	263.88	304.15	336.54	70.03	132.61	68.87	113.28
	L	395.83	280.3	577.35	121.86	419.11	**1306.54**	434.42	315.48	368.87
Purification Factor	U	9.24	4.04	10.78	12.42	13.74	2.86	5.42	3.7	6.08
	L	16.17	11.45	23.58	4.97	17.12	**53.37**	17.75	16.95	19.82
Yield (%)	U	45.45	33.94	70.15	46.65	38.04	29.57	8.62	11.03	39.78
	L	54.55	66.06	29.84	53.35	61.95	**91.37**	70.43	88.97	60.22

pH is an important parameter which influences the partition coefficient of the ATPS. According to Alberttson, the partition coefficient of a charged bio material is influenced by short range (Vander Waals interaction) and long range (electrostatic interaction). Changing the pH can also alter the electrical charge of the molecules of the phase components. These factors can also alter the molecular interaction among the phase components and partition coefficient. When pH is increased or decreased, the enzyme tends to move to the top phase or bottom phase. It is mainly based on the isoelectric point (pI) of the enzyme. Effect of different pH values (3-7) on naringinase partition was studied. The maximum purification factor of 53.37 and yield of 91.37% were found at 5.5 at the lower phase. Due to high concentration of enzyme in the lower phase, partition coefficient of naringinase (K_e=0.12) was found to be low. Since other proteins are targeted to the upper phase, partition coefficient of the protein was obtained as higher value (K_p=3.36). Therefore the optimum system pH was found to be 5.5 (Mulimani and Naganagouda, 2008).

4.5 Precipitation of naringinase using Ammonium sulphate:

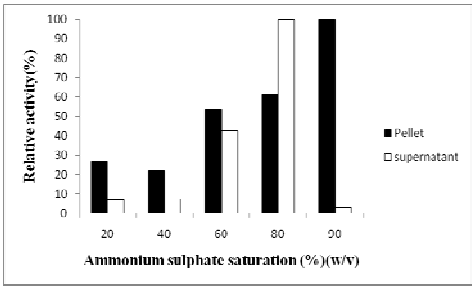


Figure4.4 Precipitation of naringinase using Ammonium Sulphate

The salting out effect depends on protein hydrophobicity. The protein surface also 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 unsolvated protein and free water molecules. Upon addition of salt, the number of water molecules available for the hydrophobic residues decreases as the salt ion become solvated. Consequently, the exposed hydrophobic areas interact with one another to reduce water contact.

The proteins with large number of hydrophobic groups have more interaction with the PEG molecules than the protein with more hydrophilic groups. This results in the displacement of proteins from the aqueous salt phase and concentrates the protein in one of the phases. In order to ensure the naringinase is hydrophilic or hydrophobic in nature, ammonium sulphate precipitation study was carried out and precipitated maximum at 90% ammonium sulphate saturation. From the Figure 4.4, it is clear that enzyme surface is enriched with hydrophilic amino acid residues and this result was compared (Mulimani and Naganagouda, 2008).

4.6 Effect of PEG molar mass on naringinase partitioning:

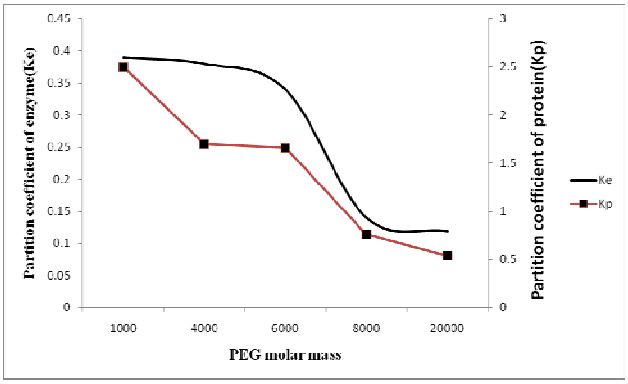


Figure 4.5 Effect of PEG molar mass on partition coefficient of naringinase and protein in PEG-Ammonium sulphate system at 28□C

Partitioning behaviour was determined for different PEG molecular mass (1000, 4000, 6000, 8000 and 20,000) at constant pH (5.5) and temperature (28□C). The effect of PEG molecular mass on partitioning of naringinase in PEG–Ammonium sulphate and water based ATPS is shown in Figure 4.5. It was observed that the partition coefficient of naringinase decrease with an increase in the PEG molecular mass. This was attributed to the fact that the hydrophobic nature of PEG increases with increase in molecular mass (Oliveira *et al*., 2008 and Tubio *et al*., 2006). From the naringinase precipitation study (from different ammonium sulphate salt cuts), the enzyme was precipitated maximum at 90% ammonium sulphate saturation. This indicates that the enzyme is relatively hydrophilic. It is evident that the enzyme surface is rich with hydrophilic amino acid residues which facilitate to move towards bottom phase and hence low K_e values were observed. Since naringinase is hydrophilic in nature, repulsive interactions between the polymer and the protein become stronger as the polymer molecular mass is increased, resulting in a distribution of the protein towards the bottom phase, because the relative hydrophobicity of the bottom phase is very low (Wu *et al*., 2000). A similar behaviour was also reported by Capezio *et al*., 2005, Boaglio *et al*., 2006 and Vaidya *et al*., 2006 for other ATPS systems. In addition, an increase in PEG molecular mass results in an increase in the polymer chain length which led to the decrease in the free volume. So that, the biomolecules will selectively partition to the bottom phase (Hatti-Kaul *et al*., 2000). Increase in the polymer weight results in the reduction of free volume of the top phase. This results in partition of biomolecules in the salt-rich bottom phase leading to decrease in partition coefficient.

4.7 Effect of Aqueous two phase system temperature on partition coefficient of naringinase and protein:

To study the effect of system temperature on naringinase purification, different temperatures of 40□C-70□C at constant pH 5.5 were performed and the partition coefficients of naringinase and total protein with respect to temperature were calculated. From the Figure 4.6, it is evident that there is gradual increase in partition coefficient of naringinase and total protein partition coefficients when the system temperature is raised from 40□C-70□C at constant pH 5.5.

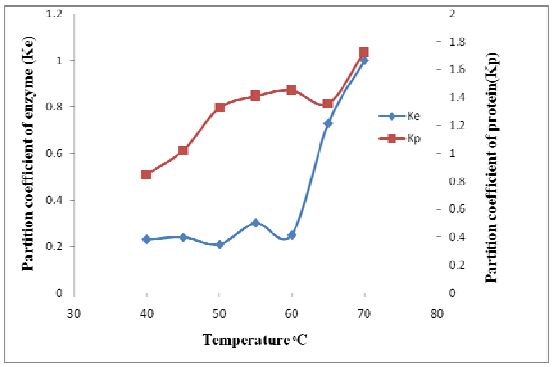


Figure 4.6: Partition coefficient of naringinase enzyme and proteins at various temperatures

With increased temperature, the binodal curve moved down and the larger two-phase region above the binodal curve resulted in increased differences in the phase compositions. The change in the 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 sulphate salt in the bottom phase. As a result, the number of water molecules available for solute solvation in the salt phase should have decreased, which also reduced the solubility of naringinase and other total proteins (salting out effect). This process increased the affinity of the biomolecule for the top phase, as is evident from the enhanced partition coefficients. From Figure 4.6, it is clear that the lowest enzyme partition coefficient (K_e) of 0.21 was observed. Mulimani and Naganagouda, 2008 have made similar observations for α-galactosidase extraction using ATPS containing PEG–phosphate salt.

4.8 SDS–PAGE analysis of naringinase:

The purity of the naringinase after ATPS process was analyzed by SDS–PAGE. As seen from the Figure 4.7, crude enzyme extract contained a variety of proteins with different molecular mass (Figure 4.7, lane 3). After partitioning with ATPS, nearly all the contaminating proteins were removed (Figure.4.7, lane 2). The purified naringinase gave two bands (α- L-rhamnosidase and β-glucosidase) with the apparent molecular mass was estimated to be 85 kDa and 80 kDa respectively (Puri and Kalra, 2005)

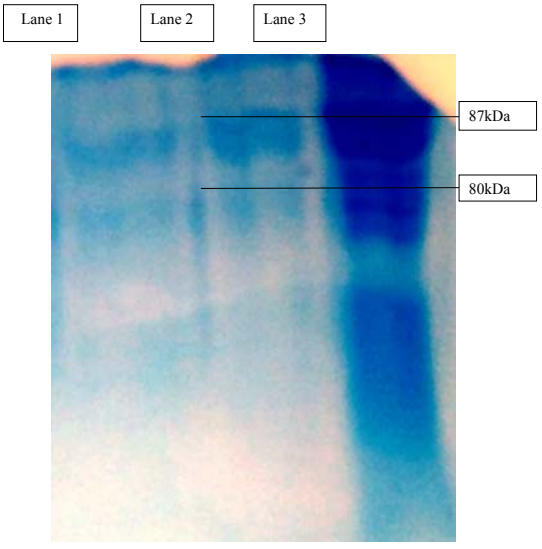


Figure 4.7 SDS –PAGE analysis of naringinase

Lane 1: Crude naringinase enzyme extract

Lane 2: Naringinase purified by ATPS

Lane 3: Bovine Serum Albumin (BSA)

CHAPTER 5
CONCLUSION

Aqueous two-phase extraction was demonstrated to be an efficient primary purification step for naringinase produced from *Aspergillus brasiliensis* MTCC 1344. The influences of various factors on the partitioning of the proteins have been studied. Partitioning of naringinase was carried out in PEG–Ammonium sulphate based aqueous two phase system. The partitioning of naringinase has shown decrease in partition coefficient value with increase in PEG molecular mass, due to increase in hydrophobic nature of PEG with higher molecular weight and with increase in PEG concentration, protein transfer to the salt rich (bottom) phase increases, due to decrease in free volume available for the protein in PEG rich phase. The partitioning of naringinase has shown increase in partition coefficient value with increase in temperature. When the temperature increases, the number of water molecules available for solute solvation in the salt phase should have decreased, which also reduced the solubility of naringinase and other total proteins (salting out effect). This process increased the affinity of the biomolecule for the top phase, as is evident from the enhanced partition coefficients. Purification of naringinase by ATPS resulted in a high yield of 91.37% and a 53.37-fold increase of enzyme purity with only single step operation. The results of ATPS developed at laboratory scale are very interesting for a first step extraction and purification of naringinase. The process is very cheap, simple and also powerful technique for recovery of naringinase from *Aspergillus brasiliensis*. The purified enzyme can be used in the food processing industry as the phase forming species PEG and salt are non-toxic and safe to use in food applications.

REFERENCES

Albertsson, P.-A. (1986) 'Partition of Cell Particles and Macromolecules' John Wiley and sons, New York

Anna Colle´na, Merja Penttila, Henrik Sta lbranda, Folke Tjernelda, Andres Veidec. (2001) 'Extraction of endoglucanase I (Cel7B) fusion proteins from *Trichoderma reesei* culture filtrate in a poly (ethylene glycol)–phosphate aqueous two-phase system', Journal of Chromatography A, Vol. 943, pp.55–62

B. Bram and G. L. Solomons. (1965) 'Production of the Enzyme Naringinase by *Aspergillus niger*', Applied Microbiology, Vol. 13, No. 6, pp.842-845

Babak Mokhtarani, Ramin Karimzadeh, Mohammad Hassan Amini, SiaVash Darvish Manesha. (2008) 'Partitioning of Ciprofloxacin in aqueous two-phase system of poly (ethylene glycol) and sodium sulphate', Biochemical Engineering Journal, Vol. 38, No.2, pp.241–247
Biochemistry, Vol. 72, pp. 248–254

Boaglio A., Bassani G., Pico G., Nerli, B. (2006) ' Features of the milk whey protein partitioning in polyethylene glycol–sodium citrate aqueous two-phase systems with the goal of isolating human α-1 antitrypsin expressed in bovine milk', Journal of Chromatography B , Vol. 837, pp.18–23

Bradford MM. (1972) 'A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding', Analytical Biochemistry, Vol. 72, pp. 248–54

Caldini, C., Bonomi, F., Pifferi, P.G., Lanzarini, G., Galente, Y.M. (1994) 'Kinetic and immobilization studies on fungal glycosidase for aroma enhancement in wine', Enzyme Microbial Technology Vol. 16, pp.286–291

Capezio L, Romanini D, Pico GA, Nerli B. (2005) 'Partition of whey milk proteins in aqueous Two-phase systems of polyethylene glycol–phosphate as a starting point to isolate proteins expressed in transgenic milk', Journal of Chromatography B, Vol. 819, pp.25–31

Celal Ulger and Necdet saglam. (2001) 'Partitioning of industrial cellulose in Aqueous two-phase systems from *Trichoderma Viridae* QM9414' Vol. 36, No.11, pp.1075-1080

Chandler, B.V., Nicol, K.J. (1975) 'Some relationships of naringin: their importance in orange juice bitterness', CSIRO Food Res Quart, Vol. 35, pp.79–88

Chisti Y. Solid substrate fermentations, enzyme production, food enrichment. In: Flickinger MC, Drew SW, editors. Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and Bioseparation, Vol. 5. New York: Wiley, 1999. pp. 2446–2462

Daniels, L., Linhardt, R.J., Bryan, B.A., Mayerl, F., Pickenhagen, M. (1990) 'Methods for producing rhamnose', US Patent 4,933,281

Gabor, F., Pittner, F. (1984) 'Characterization of naringinase from *Penicillium* sp.' Hoppe Seyler-Zeitschrift für Physiologische Chemie , Vol. 365, pp.914–916

Göte Johansson, Kati Reczey. (1998) 'Concentration and purification of β-glucosidase from *Aspergillus niger* by using aqueous two-phase partitioning**',** Journal of Chromatography B: Biomedical Sciences and Applications, Vol. 711, pp.161–172

Griffith, F.P. (1969) 'Process for reactivating polyamides resins used in debittering citrus juice', US Patent 3,463,763

Habelt, K., Pittner, F. (1983) 'A rapid method for the determination of naringin, prunin, and naringin applied to the assay of Naringinase', Analytical Biochemistry, Vol. 134, pp.393–397

Hamid Shahbaz Mohammadi, Eskandar Omidinia and Heshmatollah Taherkhani. (2008) 'Rapid One-Step Separation and Purification of Recombinant Phenylalanine Dehydrogenase in Aqueous Two-Phase Systems', Biomedical Journal, Vol. 12, No.2, pp.115-122

He Guo-qing, Zhang Xiu-yan , Tang Xing-jun ,Chen Qi-he , Ruan Hui. (2005) 'Partitioning and purification of extracellularβ-1,3-1,4-glucanase in aqueous two-phase systems', Journal of Zhejiang University Science, Vol. 6, No.8, pp.825-831

Hui Ni, Feng Chen, Huinong Cai , Anfeng Xiao, Qi You, and Yunzhen Lu. (2012) 'Characterization and Preparation of *Aspergillus niger* Naringinase for Debittering Citrus Juice', Journal of Food Science, Vol. 71, No. 1, pp.C1-C7

Jorg Thommes, Markus Halfar, Holger Gieren, Simon CurVers, Ralf Takors, Rainer Brunschier, and Maria-Regina Kula. (2001) 'Human Chymotrypsinogen B Production from *Pichia pastoris* by Integrated Development of Fermentation and Downstream Processing. Part2. Protein Recovery', Biotechnology. Progress.journal, Vol. 17, No.3, pp.503-551

K .Naganagouda and V.H Mulimani,. (2008). 'Aqueous two-phase extraction (ATPE): An attractiVe and economically Viable technology for downstream processing of *Aspergillus oryzae* α-galactosidase', Process Biochemistry, Vol. 43, pp. 1293–1299

K. Ratanapongleka and J. Phetsom. (2011) 'Extraction in Two-Phase Systems and Some Properties of Laccase from *Lentinus polychrous'* World Academy of Science, Engineering and Technology Vol. 81,No.57,pp.812-815

Kanokpan Thammawat, Pongsuda Pongtanya , Vitchuporn Juntharasri and Patjaraporn Wongvithoonyaporn. (2008) 'Isolation, Preliminary Enzyme Characterization and Optimization of Culture Parameters for Production of Naringinase Isolated from *Aspergillus niger* BCC 25166', Kasetsart Journal: Natural Science, Vol. 42, pp. 61 – 72.

M.H Rashid and K.S Siddiqui. (1997) 'Purification and characterization of a β-glucosidase from *Aspergillus niger',* Food microbiology, Vol. 42, No. 6, pp.544-550

Marcela Zanella Ribeiro, Daniel Pereira Silva Michele Vitolo, Inês Conceição RobertoAdalberto Pessoa-Jr. (2007) 'Partial purification of glucose-6-phosphate dehydrogenase by aqueous two-phase poly(ethylene glycol)/phosphate systems', Brazilian Journal of Microbiology, Vol. 38, No.1, pp.78-83

Mirjana G. AntoV, Draginja M. Pericin, Milica G. Dasic. (2006) 'Aqueous two-phase partitioning of xylanase produced by solid-state cultiVation of *Polyporus squamosus',* Process Biochemistry, Vol. 41, No.1 , pp. 232–235.

Mirjanag antov draginjam .periinand Stanan. Pejin. (2004) 'Pectinases partitioning in aqueous two-phase systems: and integration of the systems poly (ethylene glycol)/crude dextran and poly (ethylene glycol)/ammonium sulphate', Journal of the Serbian Chemical Society. Vol. 69, No.4, pp.299–307

Mukesh Kumar Sharma. (2011) 'Aqueous two phase extraction of Lipase from Rice Bran', Advances in Applied Science Research, Vol. 2, No.2, pp.265-271

Munish Puri and Sukirti Kalra. (2005) 'Purification and characterization of naringinase from a newly isolated strain of *Aspergillus niger* 1344 for the transformation of flavonoids', World Journal of Microbiology & Biotechnology, Vol. 21, No.5, pp.753–758

Munish puri and Uttam Chand Banerjee. (2000) 'Production, purification and characterization of the debittering enzyme Naringinase', Biotechnology Advances, Vol. 18, No 3, pp.207-217

Munish Puri, Anirban Banerjee, U.C Banerjee. (2005) 'Optimization of process parameters for the production of naringinase by *Aspergillus niger* MTCC 1344', Process Biochemistry, Vol. 40, No.1, pp. 195–201

Munish Puri, Aneet Kaur, R.S Singh, Wolfgang ,H.Schwarz, Amrit Kaur. (2010) 'One-step purification and immobilization of His-tagged rhamnosidase for naringin hydrolysis', Process Biochemistry, Vol. 45,pp.451-456

Muthiah Perumalsamy and Mohamed Iqbal Batcha. (2011) 'Synergistic extraction of bovine serum albumin using polyethylene glycol based aqueous system', Process Biochemistry, Vol. 46, No.2, pp.494-497

Mutuswamy Shanmugaprakash, Vaidyanathan Vinoth Kumar, Manickavassham Hemalatha, Vargese melbia, Pothiyappan karthik. (2011) 'Solid State Fermentation for the production of debittering enzyme naringinase using *Aspergillus niger* MTCC 1344', Engineering life science Vol. 11, No.3, pp.322-325

Norouzian D. A., Hosseinzadeh D.N., Inanlou and Moazami N. (2000). 'Production and partial

Purification of naringinase by *Penicillium decumbens* PTCC 5248'. World Journal of Microbiology And Biotechnology , Vol. 16, pp.471-473

OliVeira RM, Coimbra JSR, Minim LA, da Silva LHM, Fontes MPF. (2008) 'Liquid–liquid equilibria of biphasic systems composed of sodium citrate + Polyethylene (glycol) 1500 or 4000 at different temperatures', Journal of Chemical and Engineering Data, Vol. 53, pp.895–899

Ono, M., Tosa, T., Chibata, I. (1978) 'Preparation and properties of immobilized naringinase using tannin-aminohexyl cellulose', Agricultural and Biological Chemistry, Vol. 42, pp.1847–53
Oxidase (PPO) by partitioning in aqueous two-phase system', Biochemical Engineering Journal,

Paloma Manzanares, Leo H de Graaff, Jaap Visser. (1997) 'Purification and characterization of an α-l-rhamnosidase from *Aspergillus niger'*, FEMS Microbiology Letters, Vol. 157, No.2, pp.279-283

Pandey ,S.K and Banik,R.M. (2011)' Extractive fermentation for enhanced production of alkaline phosphatase from *Bacillus licheniforms* MTCC 1483 using Aqueous two-phase systems', Bioresource Technology, Vol. 102, No.5, pp.4226-4231.

R. Hatti-Kaul. (2000) 'Aqueous Two-Phase Systems. Methods and Protocols' Methods Biotechnology journal, Humana Press, New Jersey

RaghaVarao, K,S,M,S., Lakshmi, M.C., and Madhusudhan, M.C. (2012) 'Extraction and Purification of Lipoxygenase from Soybean Using Aqueous Two-Phase System', Food and Bioprocess Technology, Vol. 5, pp.193–199

Roitner, M., Schalkhammer, T., Pittner F. (1984) 'Characterization of naringinase from *Aspergillus niger*', Monatsh Cheme, Vol. 115, pp.1255–1267

Roitner, M., Schalkhammer, T., Pittner, F. (1984) 'Preparation of prunin with the help of immobilized naringinase pretreated with alkaline buffer', Applied Biochemistry and Biotechnology, Vol. 9, pp.483–488

Sankyo. (1988) 'Preparation of antibiotic chloropolysporin-C'. Japanese Patent 63,146,797

Schalkhammer, T., Pittner, F. (1986) 'Characterization of rhamno-glucosidase from *Penicillium* sp.', Biological chemistry Hoppe-Seyler, Vol. 367, pp. 364

Wilaiwan Chouyyok, Nuntiya Wongmongkol, Napa Siwarungson, Seeroong Prichanont. (2005) 'Extraction of alkaline protease using an aqueoutwo-phase system from cell free *Bacillus subtilis* TISTR 25 fermentation broth', Process Biochemistry, Vol. 40, No.11, pp.3514-3518

Wu YT., Pereira VA., Teixeira J. (2009) 'Recovery of endo-polygalacturonase using
Polyethylene glycol–salt aqueous two-phase extraction with polymer recycling', Bioseparation Vol. 9, pp.247–254

Yan xu, Michele Vitolo,Cristina northfleet de albuquerque, and Adalberto pessoa jr. (2003) ' Purification of Glucose-6-Phosphate dehydrogenase from Baker's Yeast in aqueous two-phase systems with free triazine dyes as affinity ligands', Applied Biochemistry and Biotechnology, Vol. 108, No.1-3, pp.853-865

Yong Chen, Yan-fa Meng, Jin-bao Tang, Tong-yi Sun, Feng-xiang Sun and Shu-juan Liang. (2010) 'Extraction of uricase from *Candida utilis* by applying polyethylene glycol (PEG)/ NH4)2SO4 aqueous two-phase system', African Journal of Biotechnology, Vol. 9, No.30, pp. 4788-4795

Yusof, S., Gazali, H.M., King, G.S. (1990) 'Naringin content in local citrus fruit', Food Chemistry', Vol. 37, pp.113–121

Shalini Gautam, Laurent Simon. (2006) 'Partitioning of β-glucosidase from *Trichoderma reesei* in poly (ethylene glycol) and potassium phosphate aqueous two-phase systems: Influence of pH and temperature', Biochemical Engineering Journal, Vol. 30, No.1, pp.104–108

Suppasith Klomklao, Soottawat Benjakul, Wonnop Visessanguan, Benjamin K. Simpson, Hideki Kishimura. (2005) 'Partitioning and recovery of proteinase from tuna spleen by aqueous two-phase systems', Process Biochemistry, Vol. 40, No.9, pp. 3061–3067

Taylan Karkas and secil Onal. (2012) 'Characteristics of invertase partitioning in poly (ethylene glycol)/magnesium sulfate aqueous two-phase system', Biochemical Engineering Journal, Vol. 60, No.23, pp.142-150

Thomas, D.W., Smythe, C.V., Labbee, M.D. (1958) 'Enzymatic hydrolysis of Naringin, the bitter principle of grapefruit', Food Research international Vol. 23, pp.591–598

Tubío G., Pellegrini L., Nerli BB., Picó GA. (2006) ' Liquid–liquid equilibria of aqueous two phase systems containing poly(ethylene glycols) of different molecular weight and sodium citrate'. Journal of Chemical and Engineering Data, Vol. 51, pp.209–212

V. Vinoth Kumar. (2010) 'Comparative studies on inducers in the production of naringinase from *Aspergillus niger* MTCC 1344', African Journal of Biotechnology, Vol. 9, No.45, pp. 7683-7686

Vaidya K B., Suthar KH., Kasture S., Nene S. (2006) 'Purification of potato polyphenol Oxidase (PPO) by partitioning in aqueous two-phase system', Biochemical Engineering Journal,
Vol. 28, pp.161–166

Vaidyanathan VinothKumar and Revathi Babu. (2010) 'Optimization of Fermentation Parameters for Enhanced Production of Naringinase by Soil Isolate *Aspergillus niger* VB07', Food Science and Biotechnology, Vol. 19, No.3, pp. 827-829

Wenbo Zhi, Jiangnan Song, Jingxiu Bi , Fan Ouyang. (2004) 'Partial purification of α-amylase from culture supernatant of *Bacillus subtilis* in aqueous two-phase systems' Bioprocess and Biosystems Engineering, Vol. 27, No.1, pp.3-7

APPENDICES

Appendix 1

A1.1Growth Medium

Table A1.1: Composition of Czapek Yeast extract Agar –CYA

Composition	Quantity
Czapek concentrate	10ml
Di potassium hydrogen phosphate	1g
Yeast Extract	5g
Sucrose	30g
Agar	15g
Distilled Water	1L

Table A1.2: Composition of Czapek Concentrate

Composition	Quantity
Sodium Nitrate ($NaNO_3$)	30g
Potassium chloride (KCl)	5g
Magnesium Sulphate ($MgSO_4.7H_2O$)	5g
Ferrous Sulphate ($FeSO_4.7H_2O$)	0.1g
Distilled water	100ml

Appendix 2

A2.1Sodium Acetate Buffer Composition:

Stock Solution:

Solution A: 0.2M of acetic acid (11.5ml in 1000 ml)

Solution B: 0.2M of Sodium Acetate (16.4g in 1000ml)

"x" ml of solution A and "y" ml of solution B is diluted to 100ml with distilled water

x	y	pH
46.3	3.7	3.5
41	9	4
30.5	19.2	4.4
14.8	35.2	5
4.8	45.2	5.5

A2.2 Phosphate Buffer Composition:

Stock solution:

Solution A: 0.2M of monobasic sodium phosphate (27.8g in 1000ml)

Solution B: 0.2M of dibasic sodium phosphate (53.65g in 1000ml)

"x" ml of solution A and "y" ml of solution B is diluted to 200ml with distilled water

x	y	pH
87.7	12.3	6
68.5	31.5	6.5
39	61	7
16	84	7.5
5.3	94.7	8

Appendix 3

A3.1 Naringinase Assay

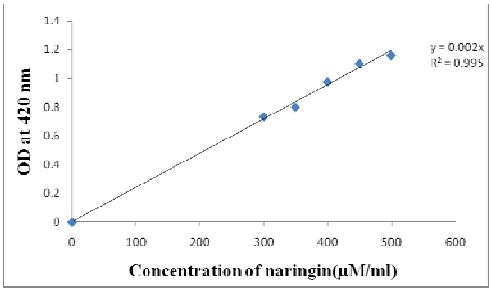


Figure A3.1: Standard Curve for Naringinase Assay

A3.2 Estimation of Protein by Bradford method:

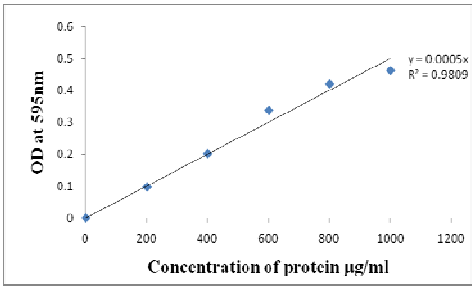


Figure A3.2: Standard Curve for Bradford Assay

Appendix 4

A4.1 Purification by using Aqueous Two Phase System (ATPS):

A4.1.1 Effect of PEG Molecular Mass on ATPS

Aqueous Two Phase System was constructed using Polyethylene Glycol (PEG) and Ammonium Sulphate and was maintained at 28ºC and different molecular mass of Polyethylene Glycol was used.

Table A4.1: Phase Composition for different PEG Molecular Weight

PEG Molecular Mass	Phase Composition	
	PEG (w/w%)	Ammonium Sulphate (w/w %)
1000	27	12.8
4000	31	7.3
6000	27.5	8.6
8000	28	7.8
20000	29	8.9

A4.1.2 Binodal curves for Different PEG Molecular Masses

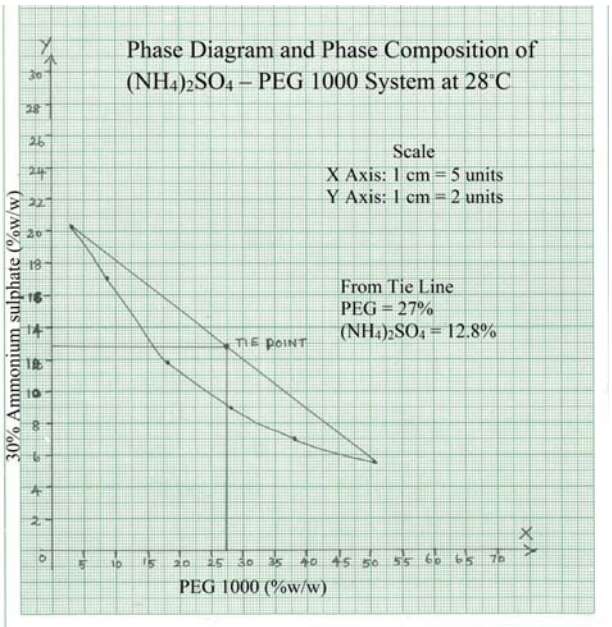
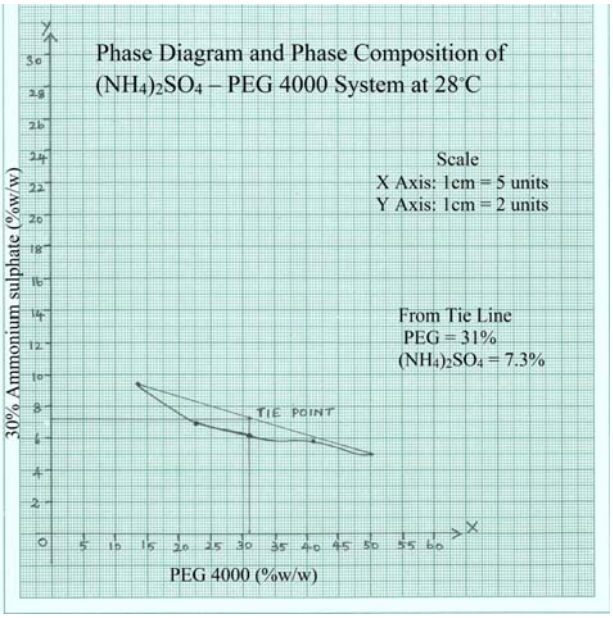


Figure A4.2 Phase Diagram and Phase Composition of $(NH_4)_2SO_4$ – PEG 1000 System at 28°C



FigureA4.3 Phase Diagram and Phase Composition of $(NH_4)_2SO_4$ – PEG 4000 System at 28°C

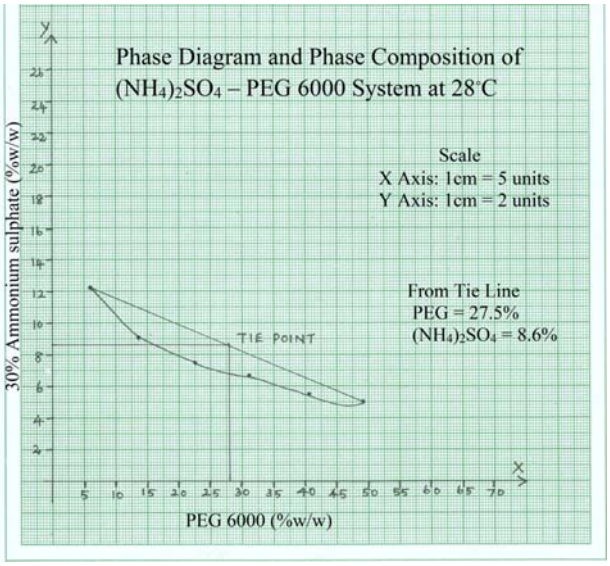


Figure A4.4 Phase Diagram and Phase Composition of $(NH_4)_2SO_4$ – PEG 6000 System at 28°C

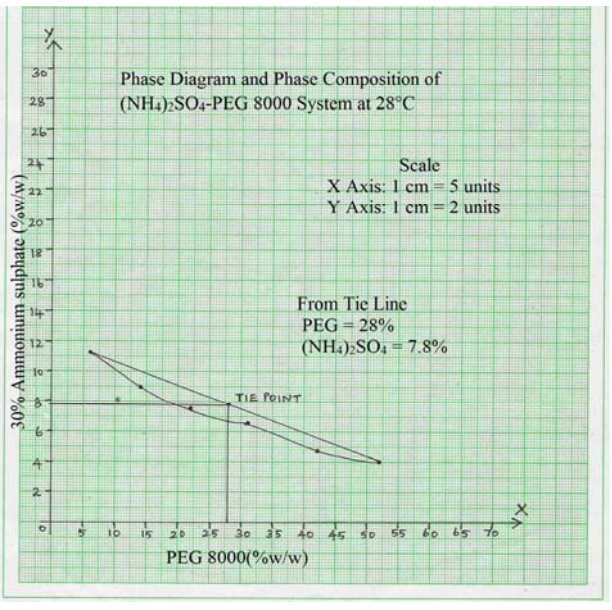


Figure A4.5 Phase Diagram and Phase Composition of $(NH_4)_2SO_4$ – PEG 8000 System at 28°C

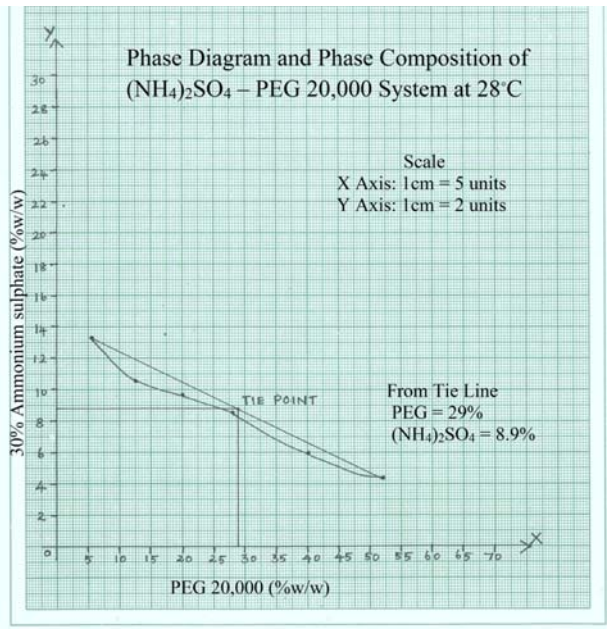


Figure A4.6 Phase Diagram and Phase Composition of $(NH_4)_2SO_4$ – PEG 20,000 System at 28°C

A4.1.3Effect of Temperature on ATPS:

ATPS was constructed using PEG 4000 and 30% Ammonium Sulphate and were maintained at different temperatures.

Table A4.2: Phase Compositions for different Temperature

Temperature(ºC)	Phase Composition

	PEG (w/w%)	Ammonium Sulphate (w/w %)
28	33.5	7.5
40	30	8.1
45	30.5	5.9
50	31.5	6.9
55	26.1	6.8
60	31	6.5
65	30.5	5.4
70	31	6.4

A4.1.4 Binodal Curves for PEG-$(NH_4)_2SO_4$ system at different Temperatures

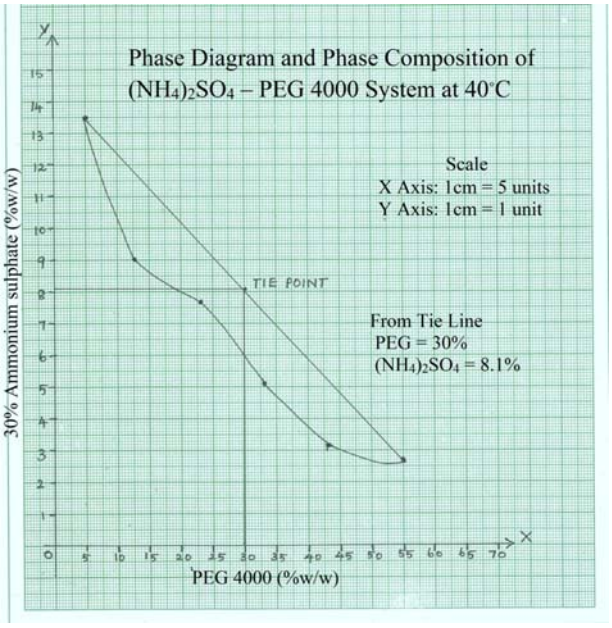


Figure A4.7 Phase Diagram and Phase Composition of $(NH_4)_2SO_4$ – PEG 4000 System at 40°C

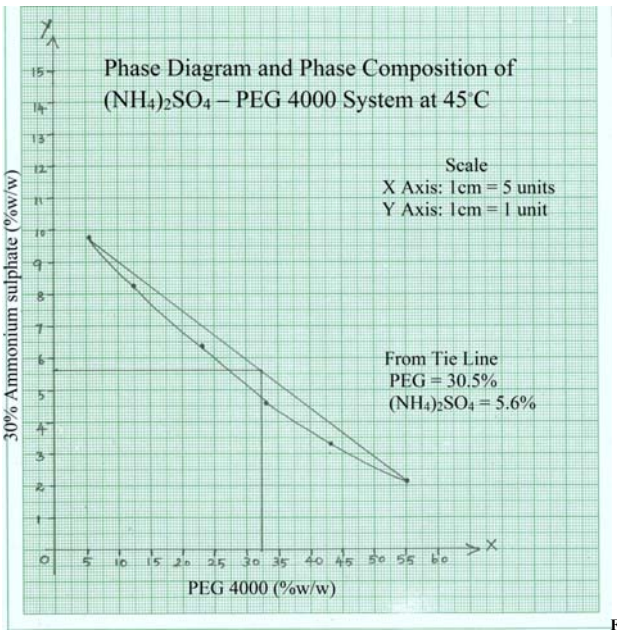


Figure A4.8 Phase Diagram and Phase Composition of $(NH_4)_2SO_4$ – PEG 4000 System at 45 °C

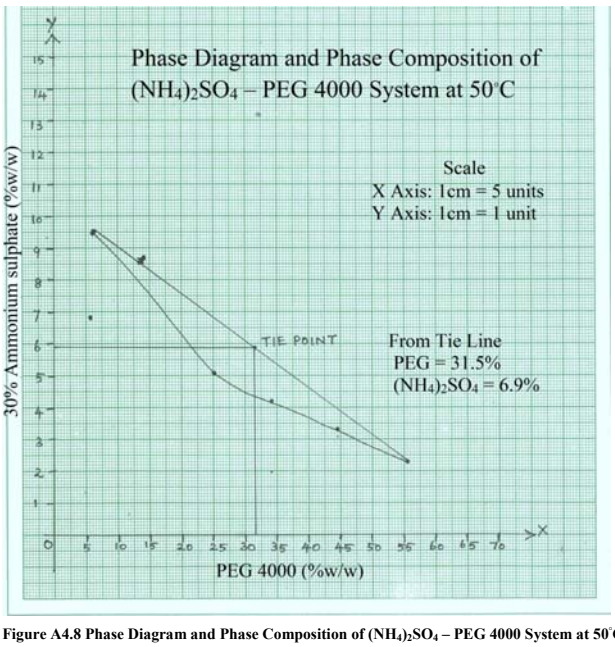
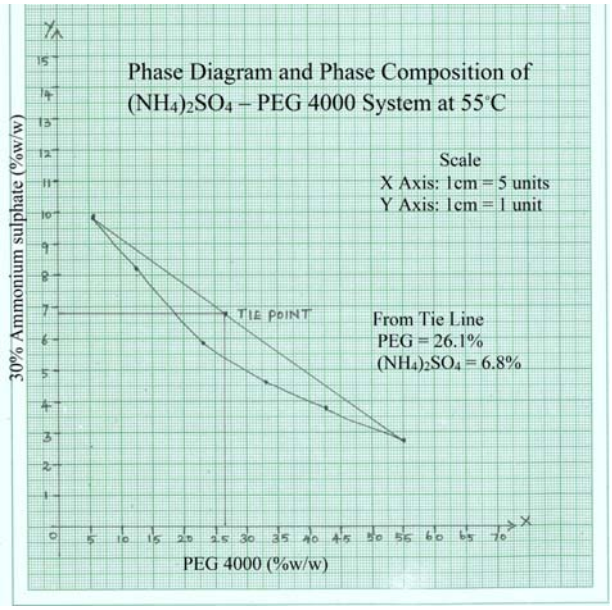


Figure A4.8 Phase Diagram and Phase Composition of $(NH_4)_2SO_4$ – PEG 4000 System at 50°C



FigureA4.10 Phase Diagram and Phase Composition of $(NH_4)_2SO_4$ – PEG 4000 System at 55°C

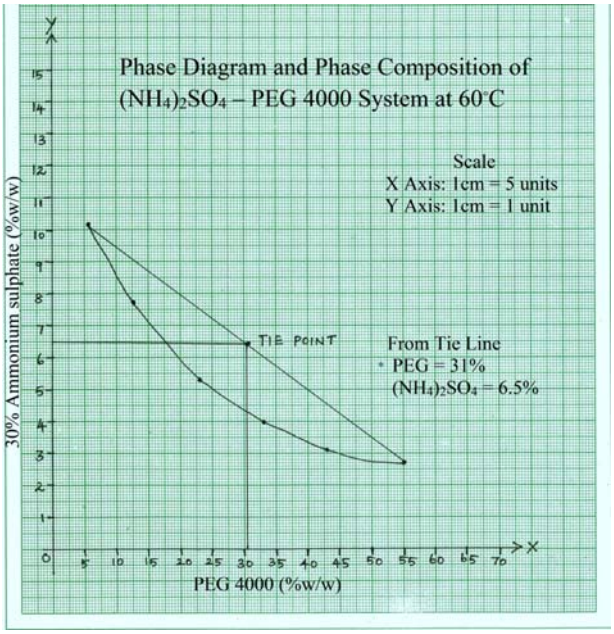


Figure A4.11 Phase Diagram and Phase Composition of $(NH_4)_2SO_4$ – PEG 4000 System at 60°C

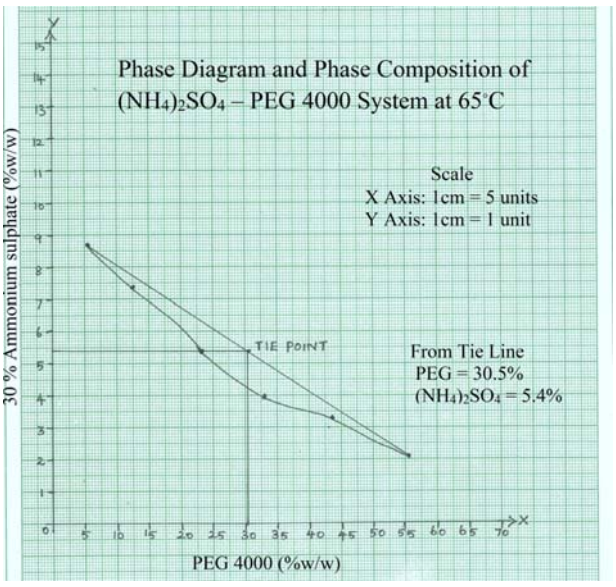
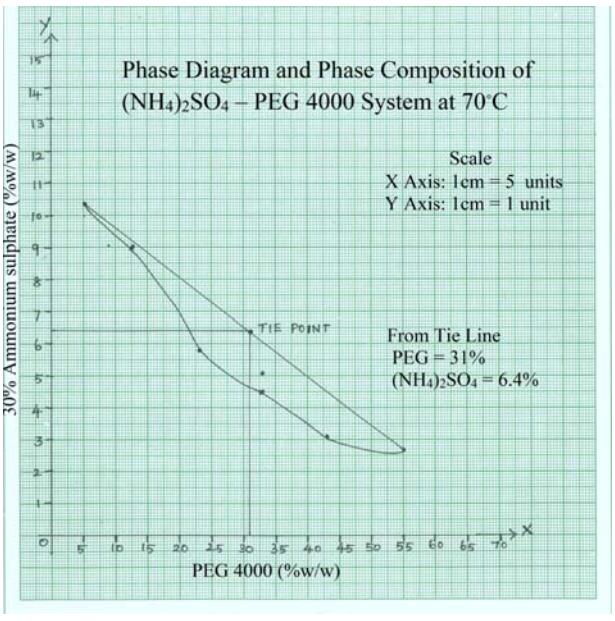


Figure A4.12 Phase Diagram and Phase Composition of $(NH_4)_2SO_4$ – PEG 4000 System at 65°C



FigureA4.12 Phase Diagram and Phase Composition of $(NH_4)_2SO_4$ – PEG 4000 System at 70°C