CHAPTER 6

Partitioning Studies of L-Glutaminase in Different PEG-Salt/Dextran System

6.1. Introduction

Aqueous two-phase system (ATPS) is commonly used for extraction of biomolecules by preferentially partitioning product biomolecules in one phase and interfering substances into other phase (Benavides and Rito-Palomares, 2008). The partitioning of product in one phase during extractive fermentation broth gets improved with careful adjustment of the phase composition and other physicochemical parameters. The biocompatibility of aqueous two phase systems provides a very low interfacial tension between phases, which results high mass transfer and ease of scale up. This extraction process is used for recovery of a variety of enzymes, biomolecules, and interferon (Kuboi et al., 1995; Rito-Palomares, 2004; Saravanan et al., 2008; Guan et al., 1996). The recovery of protease (Lee and Chang, 1990; Hotha and Banik, 1997), α-amylase (Kim and Yoo, 1991; Stredansky et al., 1993), β-galactosidase (Kuboi et al., 1995), chitinase (Chen and Lee, 1995), endoglucanase (Sinha et al., 2000), alkaline phosphatase (Pandey and Banik, 2011), glucose oxidase (Singh and Verma, 2010) was studied in polyethylene glycol (PEG)/salt system. There was no report for study of extraction process of glutaminase from *Bacillus cereus* MTCC 1305 using different aqueous two phase system.

In this study, two phase system was developed using different PEGX (X=2000, 4000, and 6000) with salts (magnesium sulfate, sodium sulfate, and sodium citrate) and PEGX with polymers (dextran40, dextranT500). The partition coefficient of L-glutaminase from *Bacillus cereus* MTCC 1305 in these aqueous two phase systems was determined and ATPS with maximum coefficient was selected as suitable system. The effect of fermentation time, pH and temperature on the extractive production of L-glutaminase in selected ATPS was further studied.

6.2. Materials and Methods

6.2.1. Materials

PEG2000, PEG4000, and PEG6000 were purchased from Sisco Research Laboratories, Mumbai, (India). Dextran40 and dextranT500 were purchased from Hi Media, Bombay (India). Salts like magnesium sulfate, sodium sulfate and sodium citrate were purchased from Qualigens, Bombay (India). All these reagents were of analytical grade.

6.2.2. Physicochemical parameters for aqueous two phase system

Binodal curve for aqueous two phase system was constructed by stepwise titration of 25% (w/w) dextran/salts with fixed amounts of 20% (w/w) PEG (2000, 4000, and 6000) and diluted with water to the point of disappearance of turbidity. 1ml cell extract was added to each phase system, mixed thoroughly in orbital shaker for 20-30 minutes and allowed to separate for 40-50 minutes at rest. Samples for each phase were collected using disposable syringe and analyzed for L-glutaminase activity in each system. The composition of top and bottom phases was determined gravimetrically in term of phase volume ratios (V_t/V_b). The tie-line length (%) was determined as the distance between the composition of top and bottom phases in the phase diagram. The partition coefficient (k) for L-glutaminase was determined from ratio of L-glutaminase activity in the top (C_t) and bottom phase (C_b). Percent yield of L-glutaminase in top phase was determined using the following formula:

$$Y\ (\%) = \frac{100 V_t K}{V_t K + V_b}$$

Where, V_t = Volume of top phase, and V_b = Volume of bottom phase

6.2.3. Extractive batch fermentation in shake culture

L-glutaminase fermentation by *Bacillus cereus* MTCC 1305 was carried out in production media with phase components of 8% (w/w) PEG4000 and 8.5% (w/w) dextranT500. The fermentation process of L-glutaminase carried out in production media without phase components was considered as homogeneous fermentation. The aqueous two phase fermentation medium was prepared by mixing separately sterilized stock solutions of PEG4000 and dextranT500 with sterilized culture medium under aseptic conditions. Fermentations were carried out by adding 2% inoculum to 100ml production medium and incubating in orbital shaker at 35˚C, 180rpm for 40 hrs. Samples from aqueous two-phase fermentation were withdrawn at regular intervals and allowed to settle in graduated tubes for 30 minutes. The activity of L-glutaminase was estimated in both phase and dry cell weight was estimated in bottom phase. Similarly, samples from homogeneous fermentation were withdrawn at regular interval, centrifuged and culture filtrate was measured for L-glutaminase activity. Effect of pH and temperature on glutaminase production in media of PEG 4000/dextran T500 was studied by varying pH from 5-8.5 and temperature 25-45˚C.

6.3. Results and Discussion

Aqueous two phase systems was developed using PEGX (X=2000, 4000, and 6000) with salts (magnesium sulfate, sodium sulfate, and tri-sodium citrate) or PEGX with polymer (dextran T500, and dextran 40). In these phase systems, top phase was found to be continuous and rich in PEG while the bottom phase was rich in salt/dextran. Microbial cells were retained in the dispersed bottom phase and enzyme was transferred to a continuous top phase in order to increase the overall

productivity. The partition coefficient of L-glutaminase was determined for each PEG/salt and PEG/dextran system and results are summarized in Table 6.1, 6.2, and 6.3. These results showed that seventy to eighty percent L-glutaminase was partitioned toward bottom phase in case of PEG/salt system, whereas, sixty to eighty percent L-glutaminase was partitioned towards the top phase in PEG/dextran system. The partition coefficient of L-glutaminase in PEG/dextran was increased with increase of molecular weight of PEG from 2000 to 4000 and then decreased at high molecular weight of PEG6000. The large molecular size of PEG6000 results high hydrophobicity in top phase which causes L-glutaminase to interact with opposite dextran phase and resulting low partition coefficient in top phase. The maximum partition coefficient of L-glutaminase (1.31) was obtained in PEG 4000/dextran T500 and hence this system was selected as most suitable systems for partitioning studies of L-glutaminase.

Table6.1. Partitioning of L-glutaminase from *Bacillus cereus* in different PEG2000/salt and PEG2000/dextran system

Systems	%PEG	%Salt or %Dextran	Phase Volume ratio (V_t/V_b)	Enzyme activity (U/ml)		Partition Coefficient (K)
				Top Phase	Bottom Phase	
PEG2000/ Magnesium sulfate	23.600	20.500	1.000	0.146	1.980	0.073
PEG2000/ Sodium sulfate	16.800	10.600	1.300	0.253	1.760	0.144
PEG2000/ Tri-sodium citrate	14.600	10.400	1.000	0.456	1.860	0.245
PEG 2000/ Dextran 40	12.200	14.000	1.500	1.290	1.590	0.811
PEG2000/ DextranT500	9.000	8.000	1.500	1.590	1.250	1.275

Table6.2. Partitioning of L-glutaminase from *Bacillus cereus* in PEG4000/salt and PEG4000/dextran system

Systems	%PEG	%Salt or %Dextran	Phase Volume ratio (V_t/V_b)	Enzyme activity (U/ml)		Partition Coefficient (K)
				Top Phase	Bottom Phase	
PEG4000/ Magnesium sulfate	20	20.54	1.0	1.39	1.46	0.95
PEG4000/ Sodium sulfate	14.8	11.6	1.3	1.28	1.71	0.75
PEG4000/ Tri-sodium citrate	12.6	10.0	1.0	1.19	1.72	0.69
PEG 4000/ Dextran 40	8.0	14.0	1.0	1.79	1.45	1.23
PEG4000/ DextranT500	8.5	9.5	1.5	2.09	1.59	1.31

Table6.3. Partitioning of L-Glutaminase from *Bacillus cereus* in PEG6000/salt and PEG6000/dextran system

Systems	%PEG	%Salt or %Dextran	Phase Volume ratio (V_t/V_b)	Enzyme activity (U/ml)		Partition Coefficient (K)
				Top Phase	Bottom Phase	
PEG6000/ Magnesium sulfate	12.0	20.6	0.67	0.078	1.54	0.051
PEG6000/ Sodium sulfate	12.6	10.0	1.3	0.123	1.67	0.074
PEG6000/ Tri-sodium citrate	10.52	12.9	1.0	0.386	1.49	0.26
PEG6000/ Dextran 40	7.5	12.0	1.0	1.53	1.59	0.96
PEG6000/ DextranT500	8.0	8.5	1.0	1.89	1.78	1.06

Binodal curve for partitioning of L-glutaminase in PEG 4000/dextran T500 separated the heterogeneous area from homogeneous and showed the exact composition of top and bottom phases (Fig6.1).

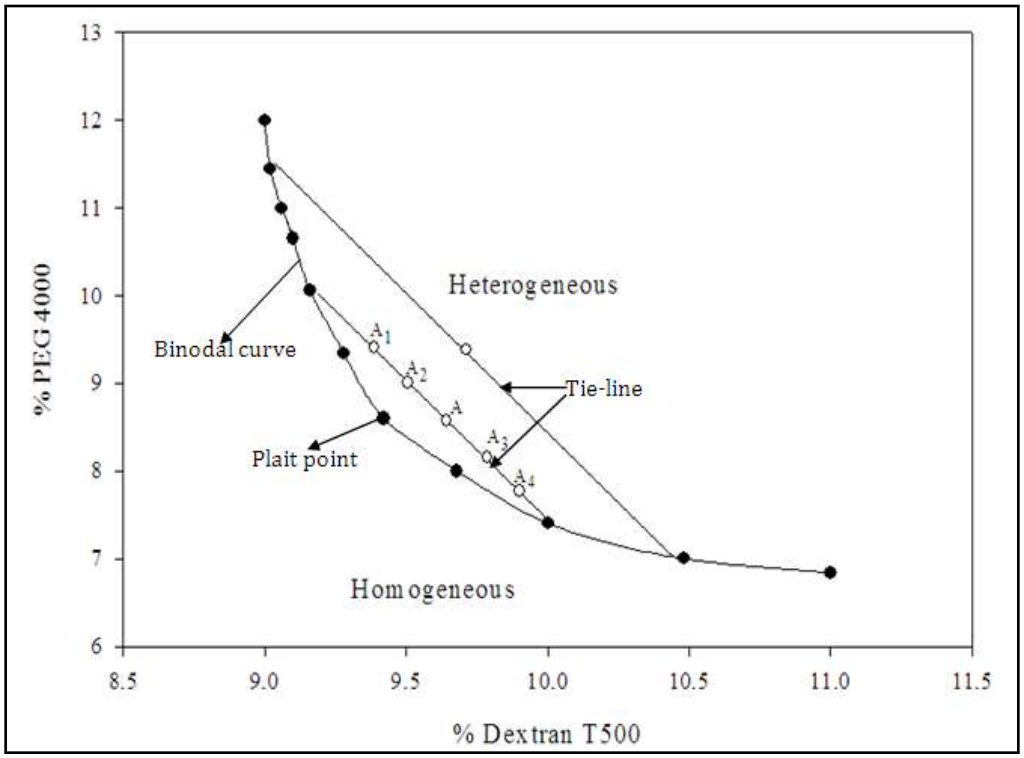

Fig.6.1. Binodal curve of PEG4000-dextranT500-water system separating heterogeneous region from homogeneous region

The phase composition becomes more and more sensitive with shortening of tie-line. The plait point (P) represents the composition of 8.5% (w/w) PEG 4000 and 9.5% (w/w) dextran T500 and tie-line near plait point favor the partitioning of L-glutaminase into upper phase. L-glutaminase transferred more toward top phase due to least polarity difference between PEG and dextran. The volume ratio (V_t/V_b) corresponding to various tie-line length of different concentrations of dextran T500 was also estimated in order to supplement the exact phase system compositions at those points (Table6.4).

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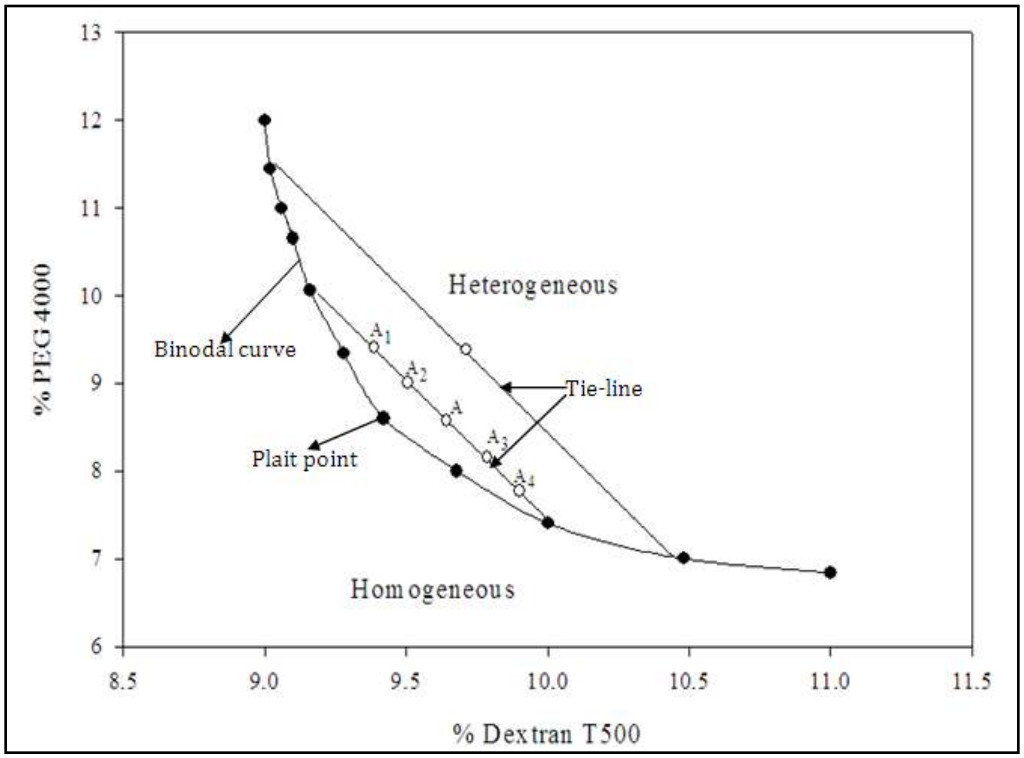

Fig.6.1. Binodal curve of PEG4000-dextranT500-water system separating heterogeneous region from homogeneous region

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Table6.4. Effect of phase volume ratio (V_t/V_b) on partitioning of L-glutaminase in PEG4000/DextranT500 system

System	% PEG 4000 (w/v)	%Dextran T 500 (w/v)	Phase volume ratio (V_t/V_b)	Enzyme in Control (U/ml)	Enzyme in top phase (U/ml)	Enzyme in bottom phase (U/ml)	K = C_t/C_b	Yield (Y%)
A_1	9.200	9.350	1.450	1.580	1.850	0.732	2.530	74.090
A_2	9.100	9.450	1.420	1.580	1.920	0.741	2.590	72.150
A	8.500	9.600	1.260	1.580	1.960	0.761	2.570	69.580
A_3	8.100	9.650	1.190	1.580	1.890	0.764	2.470	68.240
A_4	7.600	0.800	1.050	1.580	1.780	0.774	2.300	65.180
Error value of 5% was considered for enzyme production in top phase and bottom phase at 40h fermentation time								

Total estimated yield of enzyme from entire phase system was approximately same to the enzyme assayed from the same amount of the culture filtrate used for forming the phase system. The effect of the phase forming polymers on cell growth and enzyme production is necessary to complete the evaluation of the suitability of the phase system for enzyme fermentation. Fig6.2 shows the growth profile of *Bacillus cereus* MTCC 1305 in homogeneous fermentation media and PEG4000/dextran T500 ATPS system. The maximum cell biomass (X_{max}) was found as 1.212g/l in aqueous two-phase medium during 18h fermentation time which was less compared to the homogeneous fermentation (X_{max}=1.242g/l). The inhibition in cell growth might be due to the toxicity of PEG molecules arising from interaction of these molecules with cell wall of the organism (Sinha et al., 2000). The production profile in Fig6.3 shows higher production of L-glutaminase in ATPS (2.09U/ml in top phase & 1.91U/ml in bottom phase) than that in homogeneous fermentation (1.61U/ml). This might be due

to simultaneous effect of PEG on the permeability of the microbial cell wall and the stabilizing effect on the enzyme in the ATPS. Catabolic repression will be reduced due to the partitioning of substrate and product in the two-phase system.

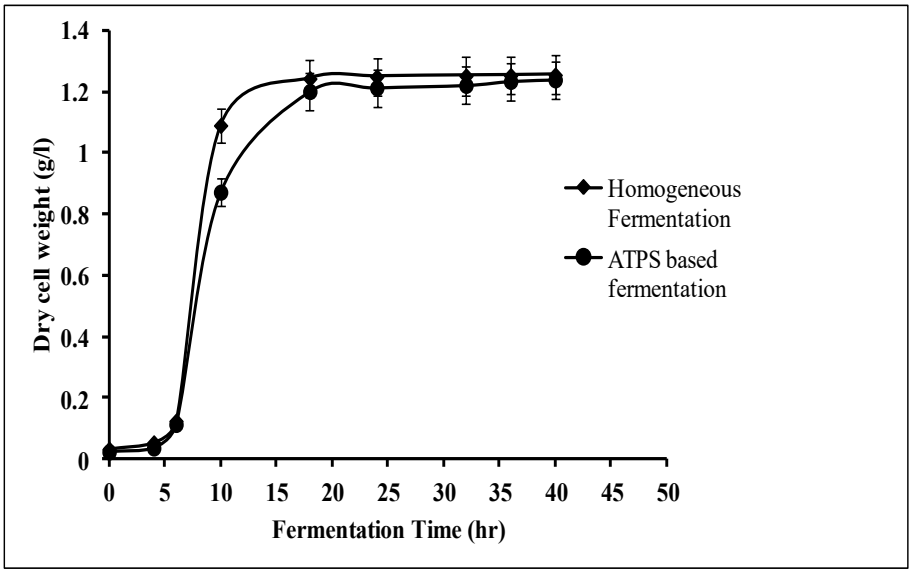

Fig.6.2. Growth profile of *Bacillus cereus* MTCC1305 in homogeneous fermentation and media supplemented with phase component polymer

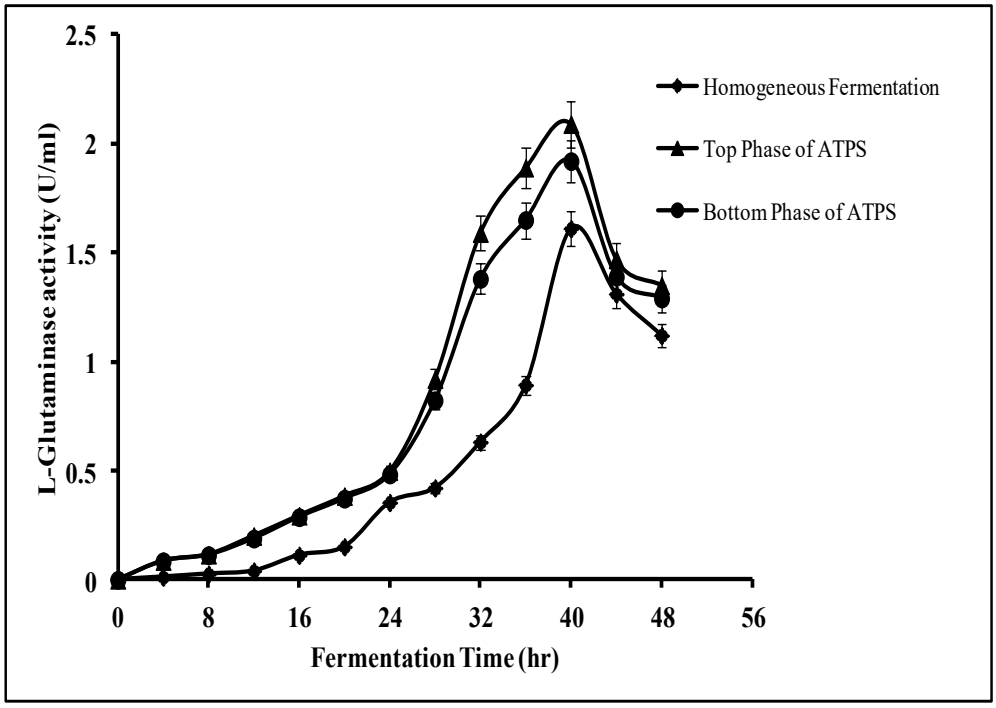

Fig.6.3. Effect of fermentation time on production of L-glutaminase from *Bacillus cereus* MTCC 1305 in PEG4000/DextranT500 system

The extractive fermentative production of L-glutaminase was studied in 100ml media supplemented with aqueous two phase components (Fig6.4). The activity of L-glutaminase was obtained as 121.22U in 58ml of top phase and 80.22U in 42ml of bottom phase as shown in Table4.5. The overall production of enzyme in aqueous two-phase medium refers to the enzyme production based on the entire volume of the phase system. Overall L-glutaminase production was obtained as 201.44U for 100 ml aqueous two-phase medium, which was higher than that produced in homogeneous fermentation (Table6.5). The overall volumetric productivity was obtained as 47.87U/l/h in ATPS media which was 1.35 times higher than that in homogeneous fermentation. These results confirm that ATPS supports cell growth and production of L-glutaminase.

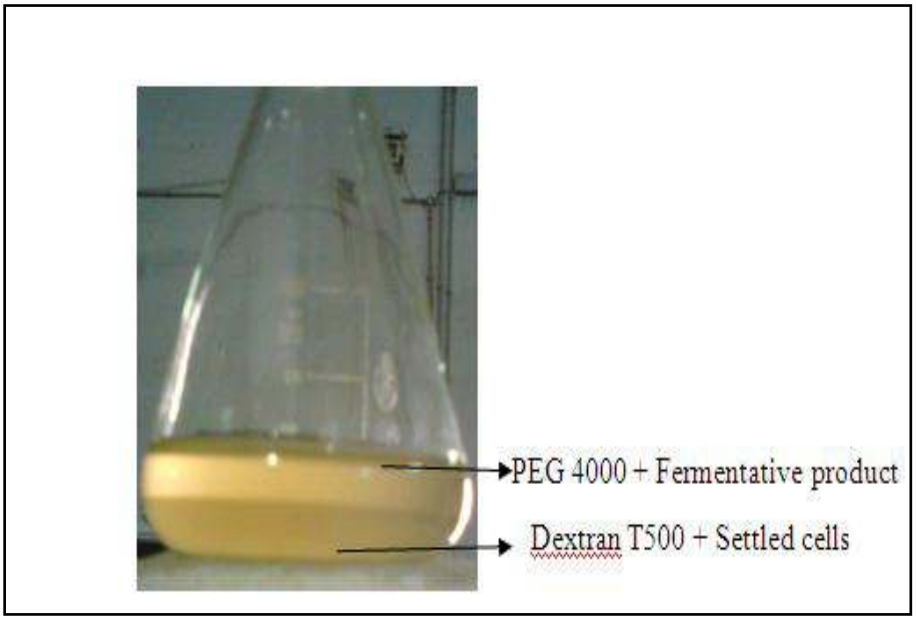

Fig.6.4. Extractive fermentative production of L-glutaminase from *Bacillus cereus* MTCC 1305 in media supplemented with PEG4000/dextranT500 system

Table6.5. Comparative analysis for production of L-glutaminase from *Bacillus cereus* MTCC 1305 in homogeneous fermentation and PEG4000/dextranT500

PEG4000/dextranT500 system				Homogeneous fermentation	
L-glutaminase activity (U)				Maximum activity of L-glutaminase in 100ml media (U)	Overall volumetric Productivity (U/l/h)
Top phase (58ml)	Bottom phase (42ml)	Overall production (100ml)	Overall volumetric productivity (U/l/h)		
121.22	80.22	201.44	47.87	161	35.5

The surface of globular protein has generally different types of amino acid containing both polar and non-polar in nature which cause different hydrophobicity and hydrophilicity. The change in pH influences the ionizable groups of molecules, which in turn alter the surface charge of the molecule and hence its partition coefficient (Banik et al., 2003). At the isoelectric point of the protein, the sum of all the charges on the protein is zero. Thus, partitioning of a protein in a two-phase system frequently depends on the net biomolecule charge, which is a function of the solution. The change in pH may induce conformational changes in the structure of the protein, causing also a change in protein partitioning behavior. In this chapter, effect of pH (5.0–8.5) on the production of L-glutaminase in PEG4000/DextranT500 was also studied (Fig6.5). The production of L-glutaminase in ATPS system was enhanced with change of pH of media upto 7.5 and then decreased at high pH. The maximum production of L-glutaminase was achieved in top phase of extractive fermentation process than bottom phase. Several researchers reported that at high pH, the negatively charged biomolecule prefers the top phase and partition coefficient

increases. It may be because of the electrostatic interactions between the biomolecule and PEG molecules (Rito-Palomares, 2004; Saravanan et al., 2008).

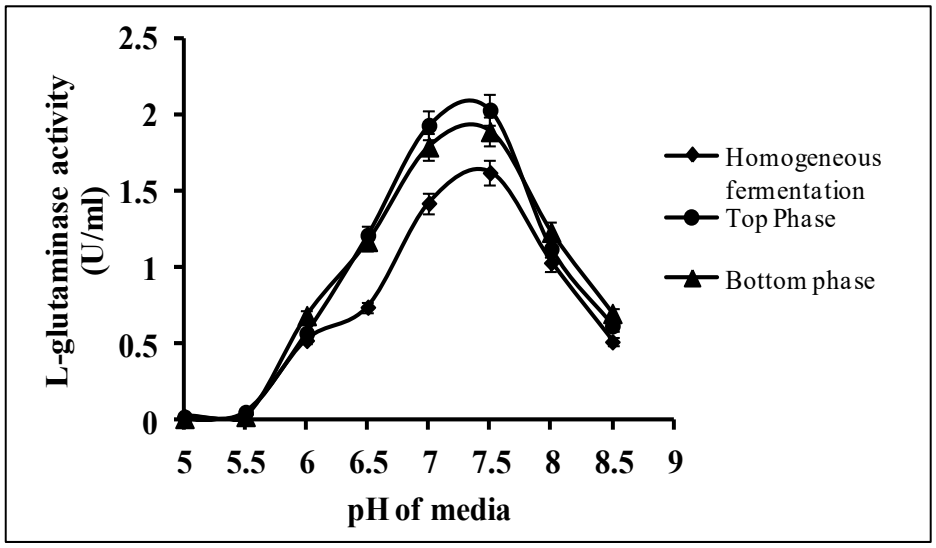

Fig.6.5. Effect of pH on the production of L-glutaminase from *Bacillus cereus* MTCC 1305 in homogeneous fermentation and PEG4000/Dextran T500 system

In a polymer-polymer system, lower temperature favors phase separation whereas higher temperature is required in a polymer/salt system (Banik et al., 2003). The binodal curve is generally moved down with increase of temperature of ATPS system and heterogeneous region above the binodal curve resulted in increased differences in the phase compositions. The partition coefficient of the biomolecules probably influences by this variation in the phase compositions (Gautam and Simon, 2006; Ratanapongleka, 2010). The effect of temperature on the extractive fermentation process of L-glutaminase was also studied by varying temperature from 20-45˚C (Fig6.6). The production of L-glutaminase in ATPS media increased with increase of temperature upto 30˚C and decreased beyond this temperature. The maximum activity of L-glutaminase was found in top phase of extractive fermentation process.

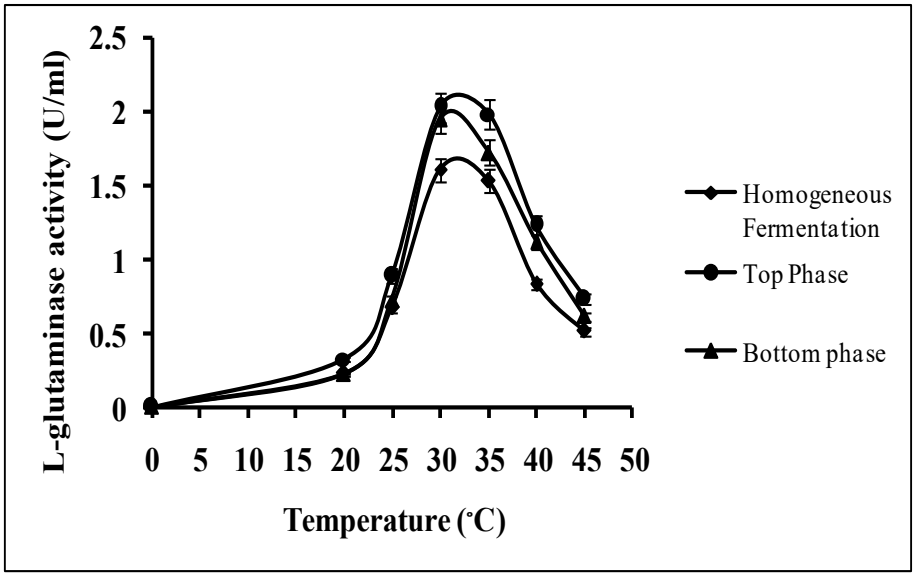

Fig.6.6. Effect of temperature on the production of L-glutaminase from *Bacillus cereus* MTCC1305 in homogeneous fermentation and PEG4000/Dextran T500 system

The activity of L-glutaminase in ATPS system was decreased beyond this optimum temperature. The water molecules present in two phase system may interact with the functional group of the bio-molecules and denature the native structure by weakening of nearby hydrogen bonds (Ratanapongleka, 2010).

6.4. Conclusion

ATPS containing phase components of PEG4000 (8.5%) and dextranT500 (9.5%) was selected as suitable system for partitioning studies of L-glutaminase produced from *Bacillus cereus* MTCC 1305. The overall volumetric productivity of L-glutaminase was enhanced by 1.35 times after extractive fermentative production of L-glutaminase in PEG4000/ dextranT500 system. The optimum pH and temperature for extractive fermentation process was obtained as 7.5 and 30˚C respectively.

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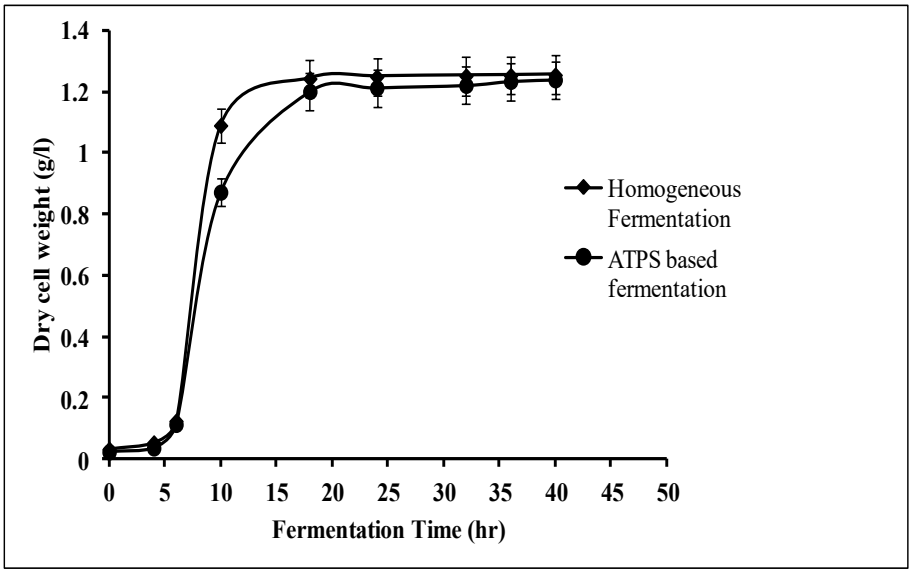

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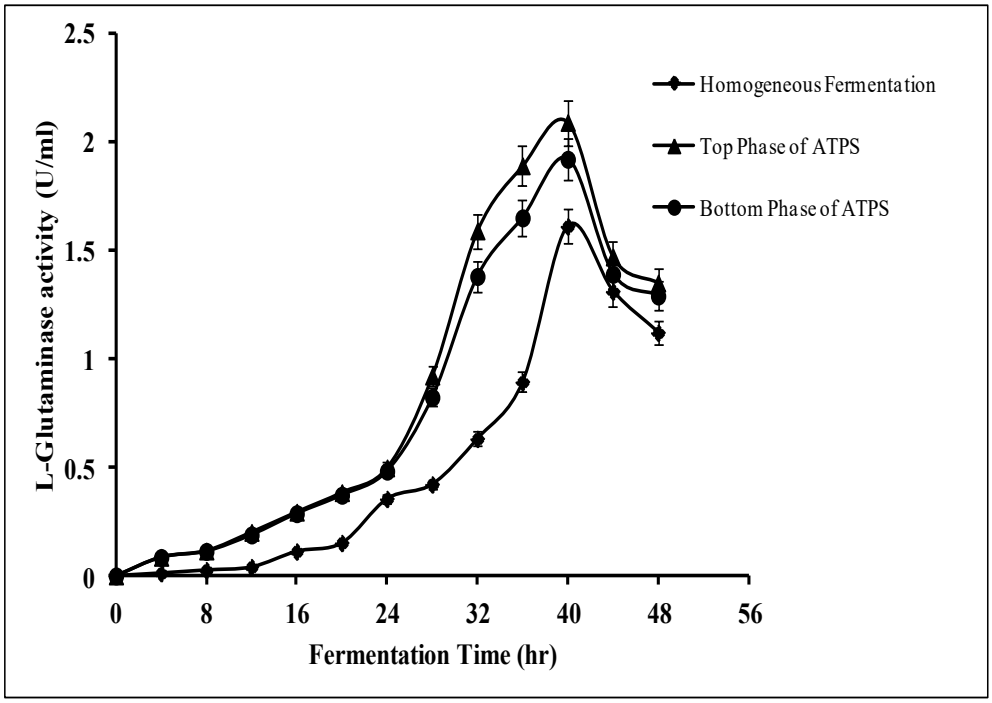

Fig.6.3. Effect of fermentation time on production of L-glutaminase from *Bacillus cereus* MTCC 1305 in PEG4000/DextranT500 system

The extractive fermentative production of L-glutaminase was studied in 100ml media supplemented with aqueous two phase components (Fig6.4). The activity of L-glutaminase was obtained as 121.22U in 58ml of top phase and 80.22U in 42ml of bottom phase as shown in Table4.5. The overall production of enzyme in aqueous two-phase medium refers to the enzyme production based on the entire volume of the phase system. Overall L-glutaminase production was obtained as 201.44U for 100 ml aqueous two-phase medium, which was higher than that produced in homogeneous fermentation (Table6.5). The overall volumetric productivity was obtained as 47.87U/l/h in ATPS media which was 1.35 times higher than that in homogeneous fermentation. These results confirm that ATPS supports cell growth and production of L-glutaminase.

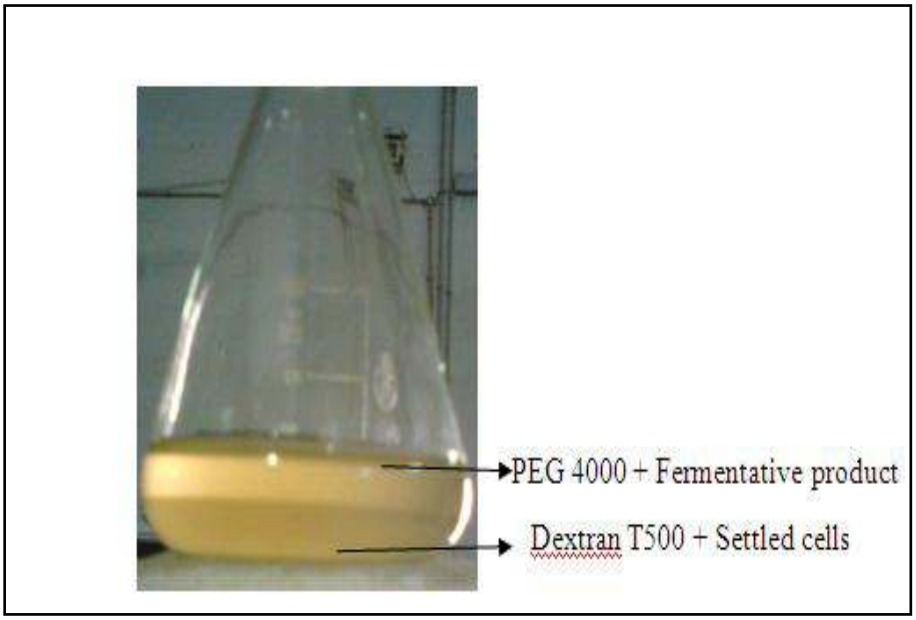

Fig.6.4. Extractive fermentative production of L-glutaminase from *Bacillus cereus* MTCC 1305 in media supplemented with PEG4000/dextranT500 system

Table6.5. Comparative analysis for production of L-glutaminase from *Bacillus cereus* MTCC 1305 in homogeneous fermentation and PEG4000/dextranT500

PEG4000/dextranT500 system				Homogeneous fermentation	
L-glutaminase activity (U)				Maximum activity of L-glutaminase in 100ml media (U)	Overall volumetric Productivity (U/l/h)
Top phase (58ml)	Bottom phase (42ml)	Overall production (100ml)	Overall volumetric productivity (U/l/h)		
121.22	80.22	201.44	47.87	161	35.5

The surface of globular protein has generally different types of amino acid containing both polar and non-polar in nature which cause different hydrophobicity and hydrophilicity. The change in pH influences the ionizable groups of molecules, which in turn alter the surface charge of the molecule and hence its partition coefficient (Banik et al., 2003). At the isoelectric point of the protein, the sum of all the charges on the protein is zero. Thus, partitioning of a protein in a two-phase system frequently depends on the net biomolecule charge, which is a function of the solution. The change in pH may induce conformational changes in the structure of the protein, causing also a change in protein partitioning behavior. In this chapter, effect of pH (5.0–8.5) on the production of L-glutaminase in PEG4000/DextranT500 was also studied (Fig6.5). The production of L-glutaminase in ATPS system was enhanced with change of pH of media upto 7.5 and then decreased at high pH. The maximum production of L-glutaminase was achieved in top phase of extractive fermentation process than bottom phase. Several researchers reported that at high pH, the negatively charged biomolecule prefers the top phase and partition coefficient

increases. It may be because of the electrostatic interactions between the biomolecule and PEG molecules (Rito-Palomares, 2004; Saravanan et al., 2008).

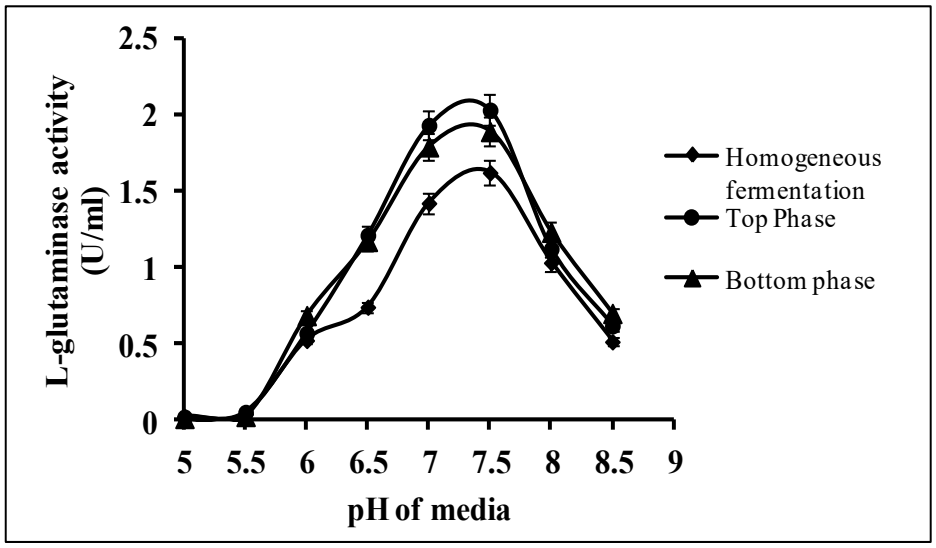

Fig.6.5. Effect of pH on the production of L-glutaminase from *Bacillus cereus* MTCC 1305 in homogeneous fermentation and PEG4000/Dextran T500 system

In a polymer-polymer system, lower temperature favors phase separation whereas higher temperature is required in a polymer/salt system (Banik et al., 2003). The binodal curve is generally moved down with increase of temperature of ATPS system and heterogeneous region above the binodal curve resulted in increased differences in the phase compositions. The partition coefficient of the biomolecules probably influences by this variation in the phase compositions (Gautam and Simon, 2006; Ratanapongleka, 2010). The effect of temperature on the extractive fermentation process of L-glutaminase was also studied by varying temperature from 20-45˚C (Fig6.6). The production of L-glutaminase in ATPS media increased with increase of temperature upto 30˚C and decreased beyond this temperature. The maximum activity of L-glutaminase was found in top phase of extractive fermentation process.

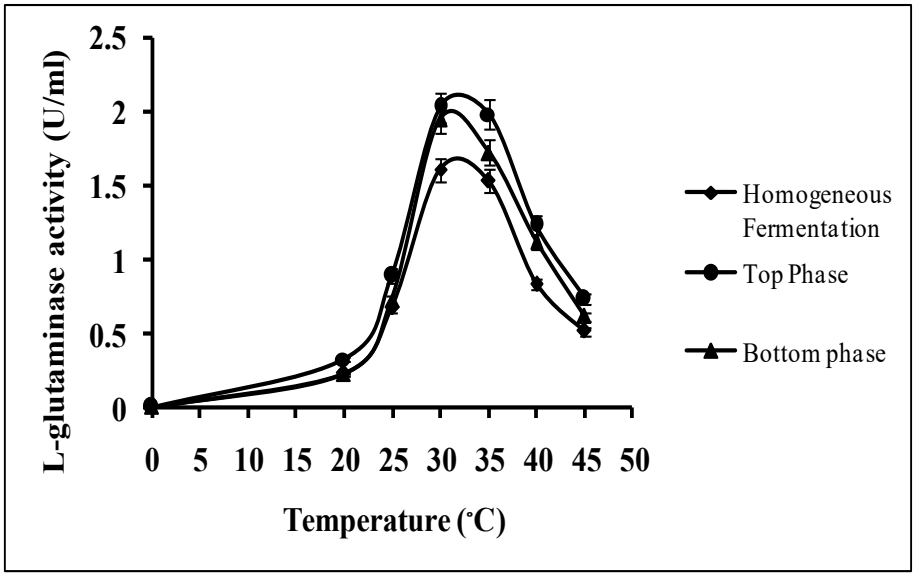

Fig.6.6. Effect of temperature on the production of L-glutaminase from *Bacillus cereus* MTCC1305 in homogeneous fermentation and PEG4000/Dextran T500 system

The activity of L-glutaminase in ATPS system was decreased beyond this optimum temperature. The water molecules present in two phase system may interact with the functional group of the bio-molecules and denature the native structure by weakening of nearby hydrogen bonds (Ratanapongleka, 2010).

6.4. Conclusion

ATPS containing phase components of PEG4000 (8.5%) and dextranT500 (9.5%) was selected as suitable system for partitioning studies of L-glutaminase produced from *Bacillus cereus* MTCC 1305. The overall volumetric productivity of L-glutaminase was enhanced by 1.35 times after extractive fermentative production of L-glutaminase in PEG4000/ dextranT500 system. The optimum pH and temperature for extractive fermentation process was obtained as 7.5 and 30˚C respectively.