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Full Length Research Paper

Effective extraction of cephalosporin C from whole fermentation broth of *Acremonium chrysogenum* utilizing aqueous two phase systems

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The downstream processing of biotechnological products from fermentation broth is an important step of production and development of cost effective, efficient downstream processing of many biotechnological products. The present study was conducted by employing aqueous two phase systems (ATPSs) for the extraction of cephalosporin C (CPC) from whole fermentation broth of *Acremonium chrysogenum*. The biphasic system was prepared by mixing equal aliquots of 15% w/w polyethylene glycol (PEG) 3350 with 15% $(NH_4)_2SO_4$. The effects of pH, neutral salts, temperature and centrifugal force on partitioning in ATPS to develop efficient extraction system for recovery of CPC from fermentation broth were also examined. The extraction efficiency was improved by enhancing the centrifugal force. Similarly centrifugation for 12.5 min also gave the maximum extraction. Improvement in the recovery yield was also observed by the addition of 0.1% NaCl. The concentration of CPC was determined by high performance liquid chromatography (HPLC). Slight modifications in the mobile phase from 10 to 5% MeOH improved CPC resolution. Further development of more inexpensive systems for extraction can be the future target of research.

Key words: Cephalosporin C, *Acremonium chrysogenum*, fermentation, aqueous two phase system (ATPS).

INTRODUCTION

The development of cost effective and efficient down-stream process for production of a purified biomolecule is the key concern of pharmaceutical industry due to the increased demand of pure biochemicals in the last decades. However, the technology for separating biological products from the fermentation broth in which they are produced has not reserved rapidity with the advances in synthesis and production of these naturally produced metabolites (Ratnapongleka, 2010; Naganagouda and Mulimani, 2008). Commercially, significant antibiotics are produced naturally by several microorganisms like bacilli and species of fungi like *Cephalosporium* and *Penicillium*. Fermentation broth contains many metabolites and by-products. Product recovery from a mixed broth is usually done with multi-step purification techniques. Antibiotics thus obtained are modified chemically or enzymatically and used for either commercial purposes or basic studies (Fayerman, 1986).

The industrial separation of cephalosporin C (CPC), a bata-lactam antibiotic from fermentation broths, is usually achieved by liquid chromatography, adsorption on active carbon or more frequently a combination of these.

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Abbreviations: ATPSs, Aqueous two phase systems; **PEG,** polyethylene glycol; **HPLC,** high performance liquid chromatography; **CPC,** cephalosporin C.

Unfortunately, the efficiency is low. Moreover, the application of organic solvent whole broth extraction toward recovery of CPC is inadequate for hydrophilic compounds such as CPC due to its amphoteric nature (Hano et al., 1992; Yang et al., 1993) besides deleterious effects of organic solvents (Yan et al., 2001). Neither CPC nor desacetyl CPC (DAC, the major contaminant in the fermentation broth) is soluble in organic solvents.

The application of aqueous two-phase system (ATPS) for recovery of CPC has recently been projected and gained considerable attention since the last decade (Yang et al., 1994). ATPS after its introduction in 1954 by Albertsson for the recovery of chloroplasts from plant extracts (Albertsson and Leyon, 1954), is widely used for purification of proteins (Shinomiya et al., 2003; Balasubramaniam et al., 2003; Waziri et al., 2004), enzymes (Pan et al., 2001; Bim and Franco, 2000; Xu et al., 2005), amino acids (Li et al., 2002), antibiotics (Yang et al., 1994) and aroma compounds (Macro et al., 2000). The eminent advantages of ATPS include reduced volume, high capacity, fast separations, easy scale-up (Yan, 2001; Xu et al., 2005). In addition, it constitutes gentle environmental conditions containing high water content in both liquid phases (Ratnapongleka, 2010). Beside other advantages, It is also considered to be an attractive and economically workable technology for downstream processing (Naganagouda and Mulimani, 2008). These systems are formed when water soluble polymers and certain inorganic salts are mixed together at specific concentrations. In a bio-separation process, it is significant to elevate the differences of partition co-efficient (K) between target molecule and contaminants. This can be done by changing physical and chemical parameters of the separation system resulting in better separation (Drouin and Cooper, 1992).

Aqueous two-phase extraction system was investigated as a powerful technique for separation, concentration and purification of biomolecules and pharmaceutical by studying the partitioning of ciprofloxacin in aqueous two-phase system of polyethylene glycol (PEG)–Na_2SO_4–water. The results of the model show that the ciprofloxacin partitioning is highly dependent on salt concentration. However, temperature and PEG concentration were shown to have moderate effects on partitioning but PEG molecular weight has no significant effect on the antibiotic partitioning (Mokhtarani et al., 2008). The application of aqueous two-phase systems in the purification, characterization and study of biomaterials was reviewed by Xu et al. (2001). The applications of aqueous two-phase systems in the separation and study of various pharmaceuticals, including recombinant proteins, antibodies and antigens, antibiotics, amino acids and oligopeptides, lactic acid, enzymes were conversed. New developments of the aqueous two-phase systems and the prospects of this technology were also discussed.

The addition of salts, a change in pH or the addition of affinity ligands are often used to alter the partitioning of the biomolecules and improve the selectivity of the two phase concentration. Although such interactions in ATPS are not well understood yet, it has been suggested to involve molecular forces (Huddleston et al., 1991). The present study was undertaken for effective extraction of CPC from whole fermentation broth of *Acremonium chrysogenum* utilizing aqueous two phase systems. The effects of pH, neutral salts, temperature, and centrifugal force on partitioning in ATPS to develop efficient extraction system for recovery of CPC from the fermentation broth were also examined.

MATERIALS AND METHODS

Microorganism, media and culture conditions

A. chrysogenum IM-1.1 strain was used in the study. The strain was cultured and maintained on potato dextrose agar slants. Potato dextrose broth containing 2% dextrose in 100 ml potato extract was used for inoculum's development.

Antibiotic production medium

Fermentation was carried out in a defined media developed with slight modifications (Zanaca and Martin, 1983). It contained 2.7% glucose, 3.6% sucrose, 0.7% DL-methionine, 13.5% salt solution A (composed of 115 g KH_2PO_4, 156 g K_2HPO_4, 1.6 g Na_2SO_4, 1.3 g $MgSO_4.7H_2O$, 0.22 g $ZnSo_4.7H_2O$, 0.22 g $MnSO_4$.H2O and 0.055 g $CuSO_4$ per liter of distilled water) and 0.75% salt solution B made up of 2% ferrous ammonium sulfate.

Batch culturing in shaker incubator

Batch fermentations were performed in 250 ml Erlenmeyer flasks with 100 ml production media, initially at 25°C. Parameters such as pH, agitation and time of incubation were optimized for maximum antibiotic production in another experiment. Finally, optimum fermentation conditions considered for maximum antibiotic yield were pH 6.5, 200 rpm and incubation of culture for 72 h, as determined in our previous experiment.

Assay of cephalosporin C

CPC concentration in broth was determined by a spectro-photometric method utilizing hydroxylamine-nickel reagent as described by Mays et al. (1975). After extraction in PEG-rich phase, CPC concentration was determined by high performance liquid chromatography (HPLC) (9A-Shimazzu, Japan). A reversed phase micro-Bondapak C18 (3.9 × 300 mm, Waters, USA) column was used. Mobile phase consisted of 0.02 M phosphate buffer containing 5% methanol and pH 3.5 at flow rate of 0.8 ml/min. CPC was detected at 254 nm.

Extraction of CPC from whole broth

Different organic solvents were used to extract CPC from whole broth. It was observed from quantitative analysis using HPLC that none of the organic solvents including ethyl acetate, methyl-Isobutyl ketone, isopropyle alcohol and acetone. ATPS was developed by

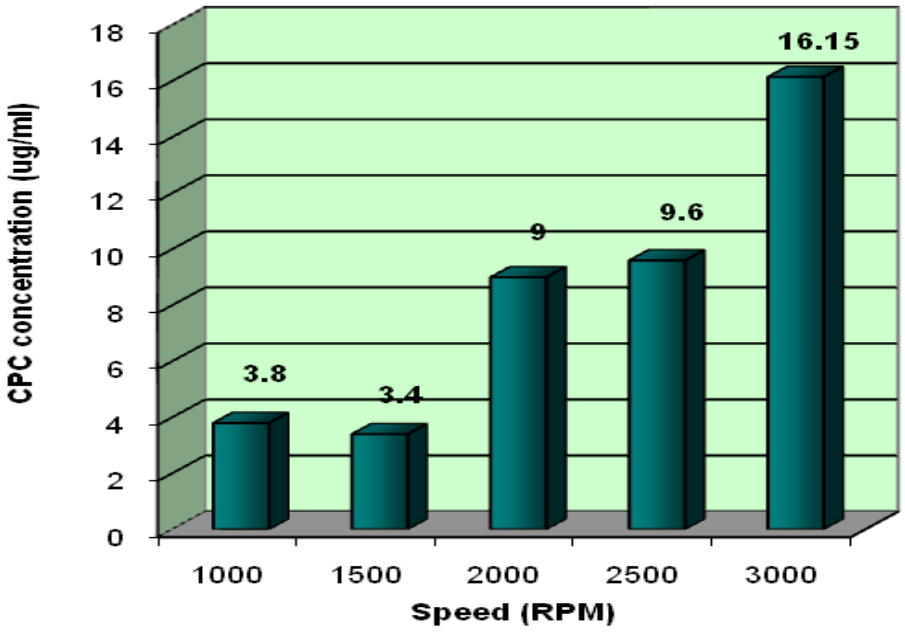

Figure 1. Effect of centrifugal force on extraction efficiency of cephalosporin C.

mixing 15% PEG 3350 and 15% ammonium sulfate and placed on magnetic stirrer for 5 min. Subsequently, 0.5 ml of broth was mixed with 10 ml of PEG-ammonium sulfate system and was then centrifuged at different speeds, for different time periods (Beckman, USA). Different neutral salts were also added in concentration of 0.1% in different systems to improve the recovery yield. All the operations were conducted at room temperature (25°C). The pH of stock solutions was adjusted to desired pH during those experiments, which explored the effect of pH. Otherwise pH was not adjusted and final pH was between the ranges of 2.8 to 6.2. Temperature pretreatment for 15 min was also done in order to study the effect of temperature on extraction of CPC.

RESULTS AND DISCUSSION

The effect of $(NH_4)_2SO_4$ concentration, time, speed of centrifugation and neutral salt addition were investigated to find a suitable extraction system for recovery of CPC antibiotic from whole fermentation broth. As earlier reported, changing the system properties like pH value, addition of salt and concentration of phase forming salt interfere with the surface properties of the partitioning molecules. Although these interactions are not well understood, it is suggested that certain molecular forces must be involved (Huddleston et al., 1991). Moreover, slight modification of mobile phase from 10% MeOH in 0.02 M buffer to 5% MeOH in 0.02 M improved HPLC resolution such that retention time of CPC increased. This made the CPC separation from its impurities easy.

pH effects

It is observed from results (Figure 5) that maximum extraction was achieved at neutral pH 7. The change in the yield was not pronounced between pH 6 to 7. However, extraction efficiency dropped by further increases in pH (pH 8). Similar results were obtained by Yang et al. *(*1994) in their experiments where partition co-efficient remained ineffective by changing pH from 4.5 to 7. Lee and Sandler (1990) also studied pH effects on vancomycin partitioning in ATPS. They explained that since pH determines net electric charge on a bio-molecule, it can play an important role in determining its partitioning behavior. As reported in their experiment, partitioning co-efficient (K) was much higher at pH 4.0 and 7.0 than at 9.3. Newton and Abraham (1955) stated that CPC is rapidly inactivated at pH 12, while it is stable at pH 3.5. Similar pH induced changes are also observed in whole broth extraction of antibiotics using organic solvents. pH increase resulted in emulsion formation in such cases, hence causing reduced degree of extraction (Chaung et al., 1989).

The effect of centrifugal force on extraction

Several centrifugal forces were tried for different time periods and their effects on extraction were measured. As shown in Figure 1, the degree of extraction increases with increase in centrifugal force. This is in line with findings reported by Yang et al. (1994) that the recovery yield of CPC in whole broth extraction is observed to be a function of centrifugal forces used in phase separation. Similarly, it was reported that for whole broth extraction, higher centrifugation speeds are applied than for diluted or filtered broths.

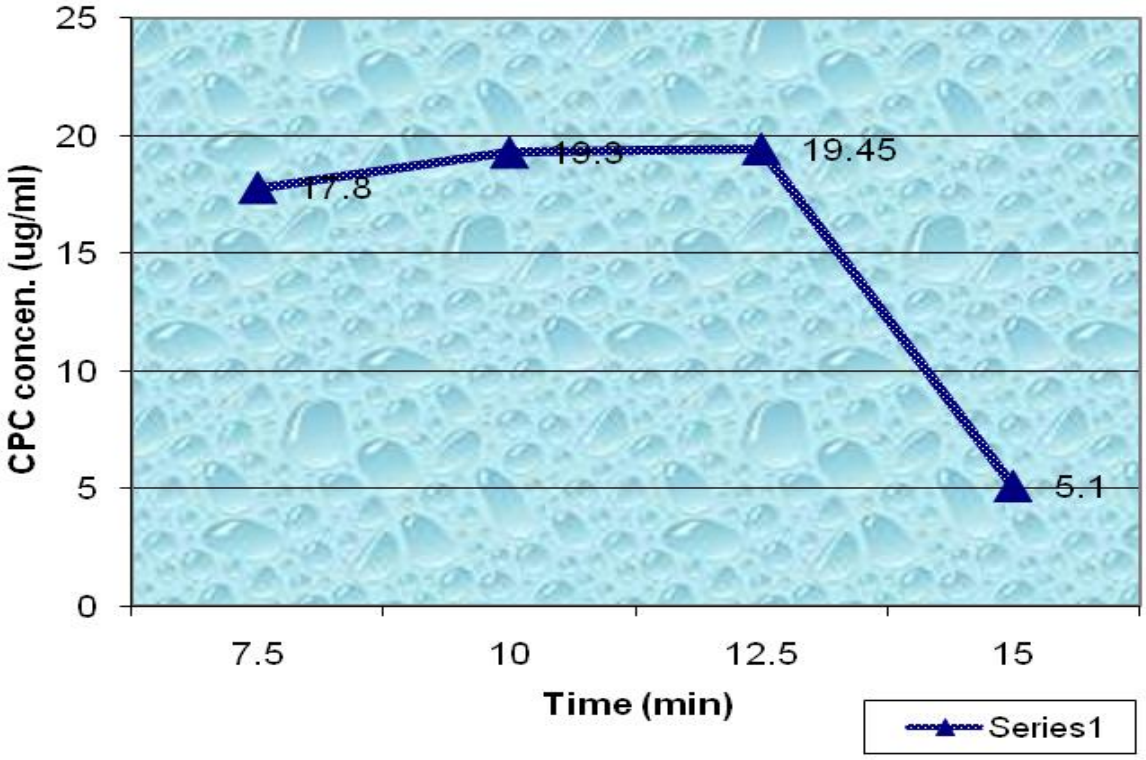

Figure 2. Effect of time of centrifugation on extraction of cephalosporin C.

Time of centrifugation

This was also optimized in our study to find suitable extraction condition for the antibiotic, and it was observed that centrifugation for 12.5 min gave best results (Figure 2). Beside all other advantages of PEG-ATPS, the system has a reasonable phase separation characteristics and can even be used with traditional solvent extraction equipment (Walter et al., 1985).

Salt addition

Enhancement of separation factor by addition of neutral salts into ATPS was also investigated. Results show that additions of salts that may disrupt water structure were found to be effective, and the effectiveness of salts were in the following sequence NaCl = KCl >KSCN > Na_2SO_4 = KI as shown in Figure 3. The addition of neutral salts in PEG-salts systems was reported to change both phase composition and differences of hydrophobicities between the two phases, which consequently changed the partition co-efficient (K) of target compound (Kuboi et al., 1991). Moreover, different salts had been found to produce different potential differences according to type and concentration. Therefore, in most commonly used PEG-salt ATPS, solute partitioning is the most significantly effected by type and concentration of biphasic forming anions.

Effect of $(NH_4)_2SO_4$ concentration

At 15% PEG 3350, the effects of $(NH_4)_2SO_4$ concentration are illustrated in Figure 4. Out of different concentrations tested, a system containing 17% $(NH_4)_2SO_4$ as phase forming salt was found to be the best for maximum CPC extraction. $(NH_4)_2SO_4$ are more familiar systems and it was reported earlier that bivalent and multivalent salts are better for salting out PEG than monovalents. Yang et al. (1994) used 15% PEG and tried different salt concentration e.g. 12, 17 and 25%. In their case 20% PEG and 20% salt system+ 1% KSCN was found to be most suitable. Similarly Lin and Chu (1995) used 15% PEG and 17.5% $(NH_4)_2SO_4$ along with 1.45% KSCN for CPC extraction in ATPS. The distribution ratio for different solutes increases with phase forming salt concentration to a certain limit, beyond which no further improvement in resolution takes place (Rogers et al., 1998).

Effect of temperature

Temperature has been found to have role in promoting extraction as shown in Figure 6. Maximum extraction was achieved when the system was heated at 30°C for 15 min in a water bath. No further increase in the recovery yield was observed by increasing or decreasing the temperature. Temperature like pH is an important

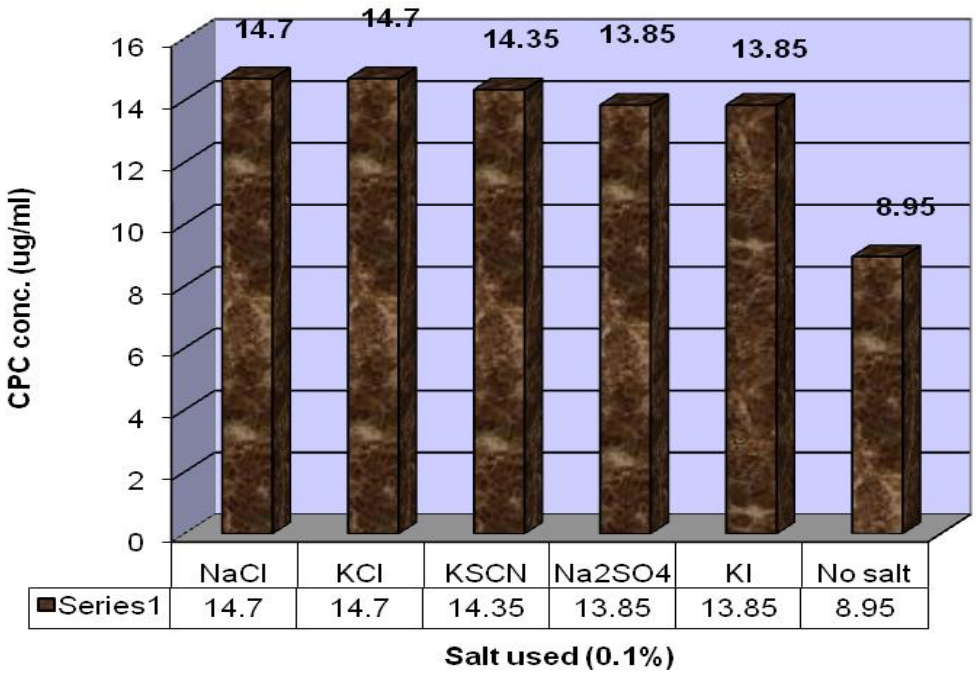

Figure 3. Effect of the addition neutral salts on partitioning of cephalosporin C.

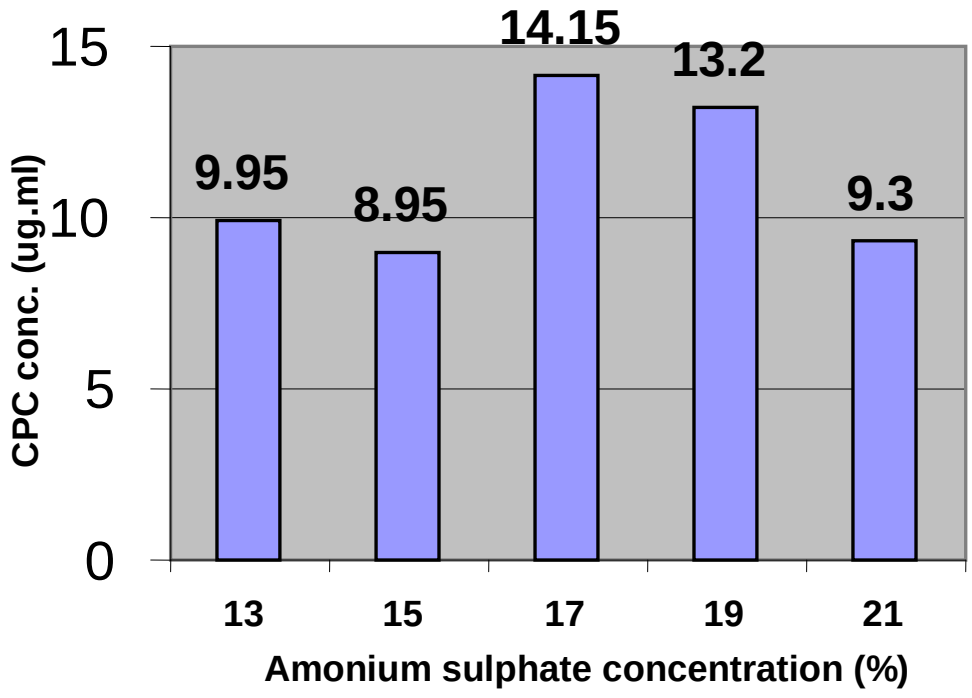

Figure 4. Effect of ammonium sulphate concentration on CPC partitioning.

variable which effects partitioning of solutes in ATPS, as such variables modify the partitioning character (Rogers et al., 1996). Experimental observations by Johansson (1986) also shows the influence of different water structuring factors such as temperature change, additives of urea and different inorganic salts on phase separation in ATPS. Temperature is also found to be an important factor in whole broth extractions of antibiotics involving organic solvents. Broth temperature pre-treatment had successfully been applied in such cases to reduce the

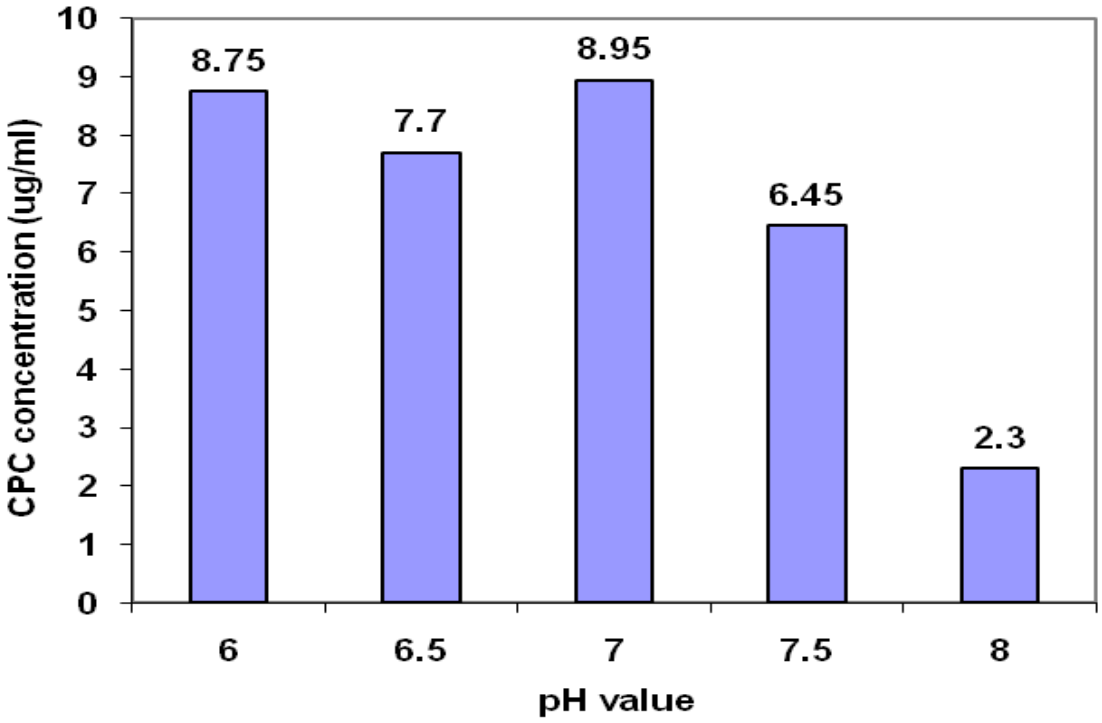

Figure 5. Effect of pH value on recovery yield of cephalosporin C.

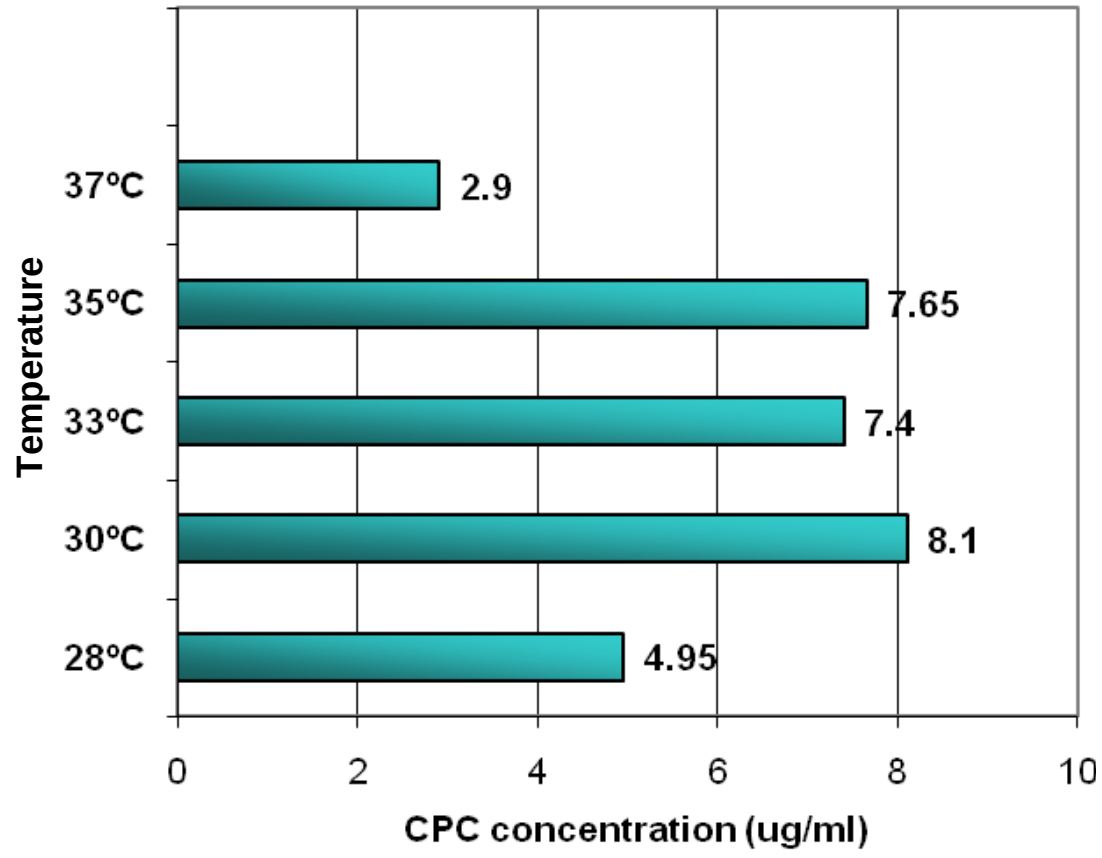

Figure 6. Temperature pre-treatment effect on extraction of cephalosporin C.

emulsion formation, which results in improved extraction (Chaung et al., 1989).

Conclusion

In the pharmaceutical sector, demand of pure bio-chemicals has increased largely since the last few decades. In the present study, ATPS was applied for the recovery of CPC antibiotic from crude fermentation broth. Parameters like pH, temperature, concentration of ammonium sulphate, time and speed of centrifugation were optimized to find a suitable extraction system. The isolation of purified final product usually requires complicated steps that result in high manufacturing cost (Aragon, 2008). It is only very recent that new in-expensive systems utilizing ATPS have been developed for extraction of CPC from whole broth. Each phase in the biphasic systems is over 80% water. ATPS have been studied widely as gentle, non-denaturing systems for the separation of biomolecules, proteins and other cell matters (Albertsson, 1986; Rogers et al., 1996). Further development of more inexpensive bio-separation systems with improved downstream processing procedures can be the future target of research.

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