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Separation of Enzymes from their Aqueous System by using Novel Concept of Unidirectional Freezing

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Authors' Contribution
MWM and JA designed the study and wrote the article. MWY and SB performed the experiments. SMA helped in editing and final analysis of the data.

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α-amylase, Cellulase, Pectinase, Radial freezing, Desizing and Biopolishing test.

ABSTRACT

In our present work, the separation and extraction of three different enzymes (alpha-amylase, cellulase and pectinase) from aqueous system has been studied by applying novel concept of unidirectional freezing. To separate the enzymes from their aqueous system, directional freezing was applied horizontally through synthetic enzyme contaminated water samples, called radial freezing. After complete freezing, the samples were collected from various sections of the frozen mass and concentration of enzymes was determined. It was found that more than ~85% mass of enzymes, migrated along with freezing front and concentrated in the centre in case of radial freezing. To examine the effect of unidirectional freezing over extracted enzymes, the working activity of separated enzymes was checked by performing desizing test for alpha-amylase and biopolishing test for cellulase. The noteworthy results of desizing and biopolishing test has promoted the significance of unidirectional freezing technique (UFT).

INTRODUCTION

Enzymes have very significant role in various industries, especially in paper, detergent, bio-fuels, food, textile and pharmaceutical industry. Enzymes are complex organic compounds, used as biocatalysts to speed the chemical reaction and enhances the efficiency of industrial processes. Amylase, cellulase and pectinase are very momentous enzymes of textile, food and paper industry. However, the excessive use of industrial grade enzymes and finally the drainage of used enzymes into fresh water due to inappropriate management, is enhancing the costs of end products and also polluting the fresh water to some extent. So an inexpensive, eco-friendly technique is required to recycle the enzymes from their aqueous system or industrial batches to make their use economical and to protect water from pollution.

With respect to current infrastructure of water treatment, various traditional materials and treatment technologies such as activated carbon, oxidation, activated sludge, nanofiltration (NF), and reverse osmosis (RO) are using to separate the industrial effluents and additives from their aqueous system (Falconer and Humpage, 2005; Leonard *et al*., 2003). Hence, water treatment technology featured with high efficiency and low cost is urgently required. There is actually a need for eco-friendly method of water purification (Wang *et al*., 2019).

Unidirectional freezing is an emerging, low cost, and easy to perform technique used for the purification of wastewater, now days. Various environmental, chemicals and biological aspects of this process have been explored and the phenomenon is found suitable for concentration, separation and desalination. A number of researchers have employed this phenomenon for the purification of water. Robinson *et al*. (2006) investigated a number of processes which take place at the interface of ice and water. Li *et al*. (2007) measured pH and electrical conductivity of polar ice cores in connection with air pollution in past centuries. Freeze induced pH changes in foods, drugs and tissues have also been studied by several workers (Elford and Walter, 1972; Eriksson *et al*., 2003; Yamamoto and Harris, 2001). Shafique *et al*. (2011) studied migration of ions and gases by directional freezing of water. Shafique *et al*. (2011) also studied forced migration of soluble and suspended materials by freezing front in aqueous systems. Mushtaq *et al*. (2018) worked on migration of bacterial contaminations in aqueous systems by employing directional freezing. The phenomenon of solute redistribution during the freezing of water has gained significant attention in recent years. Shafique *et al*. (2012) studied the forced migration of

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soluble and suspended materials by applying vertical as well as horizontal freezing. The same group investigated the migration of hydrogen, hydroxyl, carbonate and bicarbonate ions and dissolved molecular gasses during freezing (Shafique *et al*., 2011).

In the present work, purification of water was successfully performed by applying the unidirectional freezing technique (UFT) over unpurified water contaminated with enzyme and vitamins. To evaluate the authentication of UFT, the extraction of three enzymes (amylase, cellulase and pectinase) were studied. In case of all samples, a very fine extraction of enzymes and vitamins were noticed from their aqueous solutions. The extracted vitamins and enzymes also showed the positive tests of their activity.

MATERIALS AND METHODS

All the chemicals and reagents including cellulase, amylase, pectinase, and iodine solution were of maximum purity and directly purchased from BDH. Doubly deionized and sterilized water was used throughout the experimental work.

Preparation of enzyme contaminated samples

To determine the effect of UFT, standard alpha-amylase solution was prepared by mixing 20 ml of α-amylase (~90%) and 500 ml of distilled water in glass bowl whose inner bottom side was insulated with hot melt glue. The top and bottom of glass bowl was insulated with 2-inch thick shield of polystyrene. The prepared solution was subjected for the unidirectional freezing in the domestic refrigerator. After 24 h of continuous freezing, the sample was freezed and enzyme was centralized in centre of bowl. Four samples were extracted from centre to the sides of bowl and labelled them as 1, 2, 3 and 4, respectively. Samples were shifted into test tubes and covered with lid to protect the samples from the atmospheric contaminants. The concentration of α-amylase in samples (1-4) was determined spectrophotometrically (λmax=480 nm), by performing iodine titration test.

The same procedure and technique was repeated to extract enzymes (cellulase and pectinase) from their prepared standard samples. To check the concentration and activity of cellulase enzyme extracted from freezed bowl was determined by performing digestion and biopolishing test, respectively.

Radial freezing

The extraction of α-amylase, cellulase and pectinase from their aqueous contaminated samples was carried out by performing radial freezing. Directional freezing was performed in a circular glass tank having inner diameter of 30 cm and 15 cm depth. The wall of the glass tank was thick enough (about 1cm) to retard rapid cooling. After placing water sample inside the glass tank, the opening and bottom of the glass tank was insulated with one-inch thick polystyrene shield. This covering of tank ensured gradual cooling only from sides. After 24 h of continuous cooling in a freezer of domestic refrigerator, it was observed that contaminants are concentrated in centre of bowl as shown in Figure 1. The frozen mass was taken out from the tank, softly in the form of ice. To monitor the effect of freezing on movement of contaminants quantitatively, aliquots from the centre, middle and side sections of the frozen mass was taken out with the help of a cork borer and transferred into various test tubes for the testification.

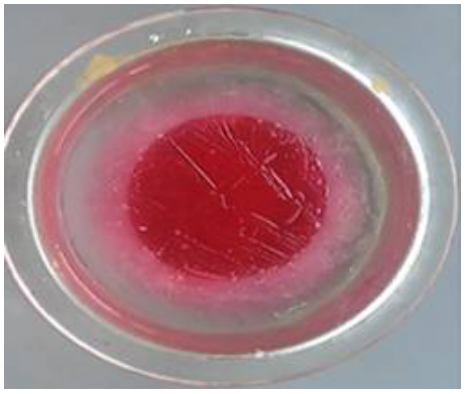

Fig. 1. Effect of radial freezing over centralization of pectinase enzyme.

Iodine titration test

This test was performed to check the quantity of α-amylase extracted from various sections of glass tank. The quantity was checked with the help of calibration curve. From sample 1 (extracted from centre of bowl), 2 ml of α-amylase extract was taken in new test tube having 1 ml of iodine solution. Its concentration was determined by checking absorbance at 480 nm using spectrophotometer. Same procedure was repeated for the samples 2, 3 and 4 to determine the concentration of amylase in aliquots.

Desizing test

To check the effect unidirectional freezing separation technique (UFT) over working activity of extracted α-amylase, desizing test was performed. First of all, a buffer solution of pH 5-6 was prepared using acetic acid. 10 mL of sample 1 (extracted from centre of bowl after after applying UFT), Alkapol (2g/L) was added in the buffer solution. Alkapol was used as wetting agent to decrease the surface tension b/w cotton fabric and solution.

The above mixed solution was heated about 60-70°C for 15 min. A piece of cotton fiber having weight (~10 g) and size approximately 10×10 inches, was impregnated in this hot solution for about 20 min. After complete wetting, the piece of cotton was passed through padder machine. The pad cotton fabric was wrapped in polyethene sheet to protect from external impurities. After 10 h of stay time, the cotton fabric was washed with hot water, dried in oven and finally the rating of enzyme, α-amylase was checked on Tegewa scale.

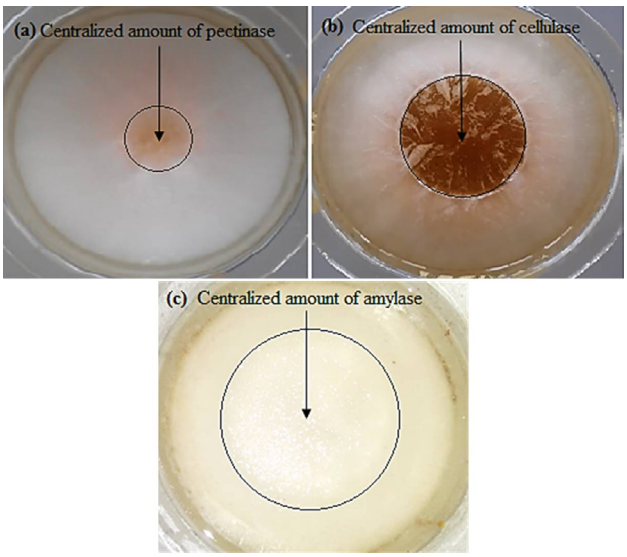

Fig. 2. Comparison of centralization efficiency of three enzymes (pectinase, cellulase and α-amylase) after applying UFT.

Digestion test

A small piece of filter paper was completely dried in oven at 120°C for about 6 h. After absolute removal of all moisture, dried filter paper was divided into four equal sized pieces in such a way that the weight of each piece was 0.007 g. 6 mL of sample 1 was added in titration flask having 14 mL of water. A piece of filter paper (0.007 g) was added in the solution of titration flask and its pH (~4.5) was adjusted by acetic acid. The whole mixture was heated at 70°C and magnetically stirred for about 20 min. After continuous stirring and heating, the filter paper dried and weighed again. Finally, the difference in weight of paper was noted before and after applying digestion test. Same procedure was repeated to measure the concentration of cellulase enzyme in samples 2, 3 and 4.

Biopolishing test

To check the effect unidirectional freezing separation technique (UFT) over working activity of extracted cellulase enzyme (sample 1), biopolishing test was performed. First of all, a piece of fiber (100% cotton) having weight 5g and size approximately 10 × 10 inches was taken in the H-T machine pots. After maintaining a material to liquor ratio of 1:10, mechanical agitation was enhanced by addition of five steel discs (each weighing 350 g). The pH of bath was maintained at 4.5–5.5 with acetic acid. After continuous stirring of bath for about 30-40 min (60 rpm) at 60-70°C in H-T machine, the filter paper dried in oven and the amount of removed fluffed by cellulase enzyme was noted. The amount of fluff removed from the surface of fabric is directly related with the activity and efficiency of extracted cellulase enzyme using UFT.

RESULTS AND DISCUSSION

After applying unidirectional radial freezing technique over enzyme contaminated samples, it was observed that all enzymes (α-amylase, cellulase and pectinase) moved along with freezing front and concentrated in the centre of bowl showing the significance of UFT. Figure 1 is showing a qualitative analysis that in case of all enzyme contaminated samples, approximately more than 85% of the enzyme was concentrated in the center of the bowl, a little contamination was found in the samples taken from midway of the radius of the bowl and almost negligible quantity of enzyme was found along the walls of the glass bowl. In fact, during unidirectional radial freezing, ice crystals start to form from corner to centre of the bowl. As crystallization of water starts through strong H-bonding in the form of ice at the sides of bowl, the additives such as enzymes are pushed towards the centre of bowl containing liquid medium. So, enzymes move along with the freezing front and concentrated at the centre of bowl as cooling starts at sides of bowl. From Figures 2 and 3, it is obvious that in case of pectinase the sepration efficiency is high, as compared to cellulase and amylase. The reason is that cellulase and amylase have hydrogen bonding affinity with water solvent while in case of pectinase there is no hydrgen bonding present, due to which pectinase show better separation.

By performing the experiments with different kind of enzymes, it was observed that under similar conditions different enzymes moved along with freezing front with different speeds and efficiency. The order of centralization of all the three employed enzymes was found as follows:

Pectinase > Cellulase > Amylase

The difference in movement of enzymes can be explained on the basis of hydrogen bonding and the arrangements which they constitute. In case of pectinase, there is no hydrogen bonding between water and enzyme. So easy to move for pectinase with solvent front and

showed well centralization. Cellulase are found to containing hydrogen bonding, therefore showed lesser efficiency for centralization. Amylase containing max hydrogen bonding as compared to pectinase and cellulase. As a result more amount of amylase (20 ml) has been used in experiment as compared to pectinase and cellulase (5 ml). So, the high concentration amylase and relative strong hydrogen bonding potential between enzyme and solvent, giving less efficiency of centralization (Shafique *et al.*, 2012). The relative quantitative efficiency of centralization for the three enzymes along with real sample has been shown in Figure 2.

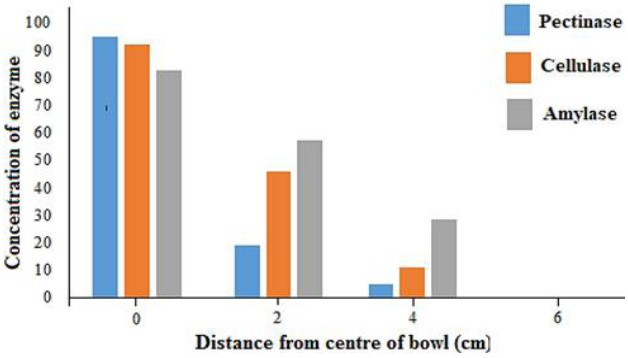

Fig. 3. Relative quantitative comparison of centralization of enzymes.

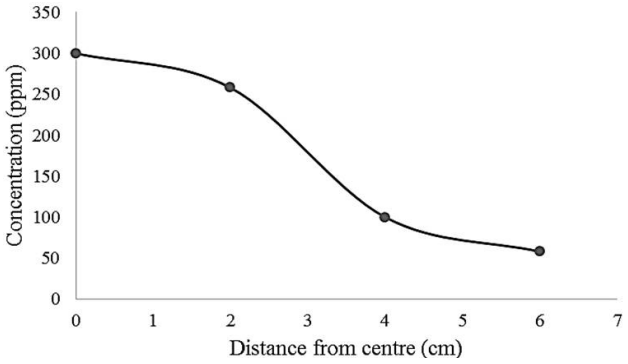

Fig. 4. Distance travelled by amylase and its relative concentration after applying UFT.

From sample 1, 2 ml was taken in a test tube having 1 ml of iodine solution. Its absorbance was checked at 480 nm with the help of spectrophotometer. Same procedure was repeated for the samples 2, 3 and 4 (Fig. 4). It is clear that the sample 1 (which is taken from centre of bowl) is showing highest concentration of enzyme which is due to its maximum movement of enzyme along with freezing front from sides to centre of bowl during process of directional. As we move from centre to sides of glass bowl, the value of absorbance decreases from sample 1 to 4, respectively, which shows the concentration of amylase decreases as we move from centre to sides. Sample 4 shows very minute absorbance which indicates very less concentration of amylase at corners of bowl. It means unidirectional freezing efficiently helps in the movement and centralization of amylase enzyme.

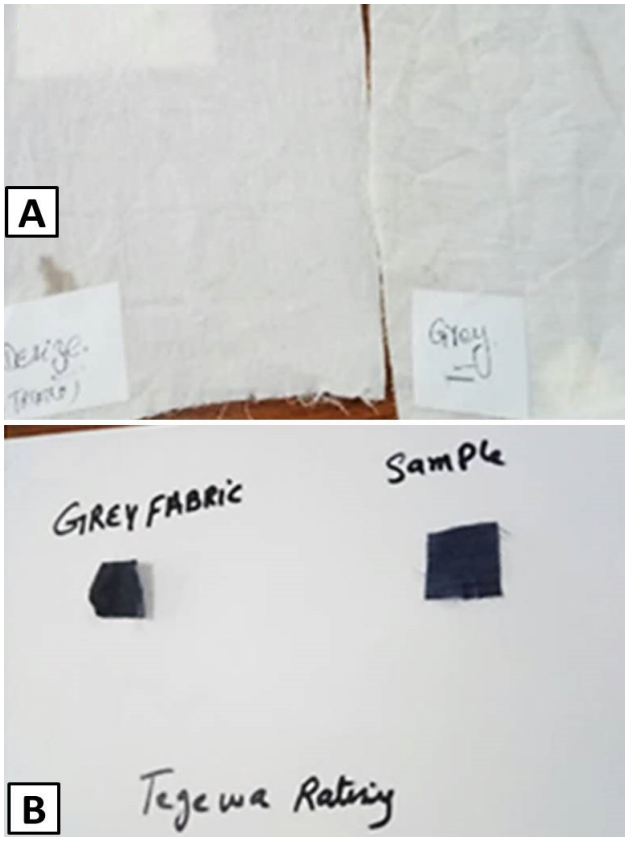

Fig. 5. Activity of extracted α-amylase over grey (A) and treated (B) fabric.

To check the efficiency of extracted enzyme amylase, desizing test was also performed. In this test the enzyme alpha amylase converted the starch on the fabric into the glucose which is water soluble. More the starch removed by the enzyme more will be the enzyme active and efficient and consequently shows more ratting on the tegewa scale. Tegewa scale is a general scale which tells us about the efficiency of enzyme. Its range is from 1-9 the maximum activity which an enzyme can show on tegewa scale is 9 while minimum is 1.

The ratting value of our extracted enzyme (amylase) on tegewa scale is 4 which show the better working capacity of extracted enzyme from unidirectional method. It means the extraction or separation of enzyme using unidirectional

freezing method, does not affect the efficiency of amylase and enzymes can be reused. The efficiency of separated enzyme is shown in Figure 5.

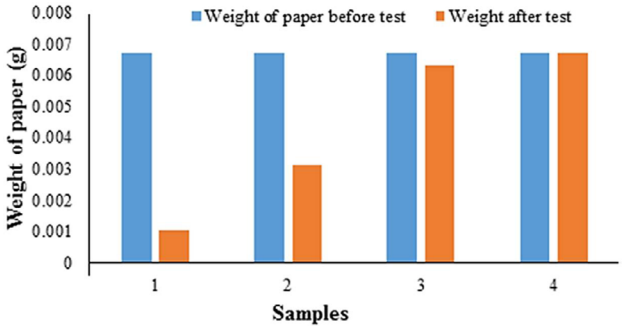

Fig. 6. Digestion test for cellulose.

Centralization of cellulase by directional freezing

The results of radial freezing for centralization of cellulase enzyme are shown in Figure 2. Approximately 91% of the cellulase was concentrated in the center of the bowl, a little contamination was found in the samples taken from midway of the radius of the bowl and almost negligible quantity of cellulase was found along the walls of the container. The quantity of cellulase was determined by performing digestion test. Figure 6 is showing the weight of paper digested by cellulase during performing digestion test. The max paper weight 0.006 g was reduced in sample 1 which is showing max concentration of cellulase in sample 1. The decrease in weight of paper for sample 1, 2, 3 and 4 was noted as 0.006 g, 0.004 g, 0.0005 g and 0.00 g, respectively. Results shows that the sample 1 which is taken from Centre shows max concentration of cellulase due to maximum weight lost. As we move from centre to sides of glass bowl, paper weight loss decreases from sample 1 to 4, respectively, which shows that the concentration of cellulase decreases as we move from centre to corner. In sample 4, no paper weight loss was observed which indicates very less concentration of cellulase at corners of bowl.

The weight of paper decreases because the enzyme cellulase removed the cellulose present in the paper. The decrease in the weight of paper depends upon the conc. of cellulase. More the conc. more will be the weight lost. All result shows that the concentration of cellulase was maximum in centre and minimum at the sides of bowl.

To check the efficiency of extracted enzyme cellulase, biopolishing test was performed. The amount of the fluff removed from the fabric is directly related with the activity and efficiency of cellulase enzyme. The efficiency of our extracted enzyme is 1.2% while standard Alkazym acidic have the efficiency of 2.5%. This shows the better working capacity of extracted enzyme from unidirectional freezing method. The efficiency of separated enzyme is shown in Figure 7.

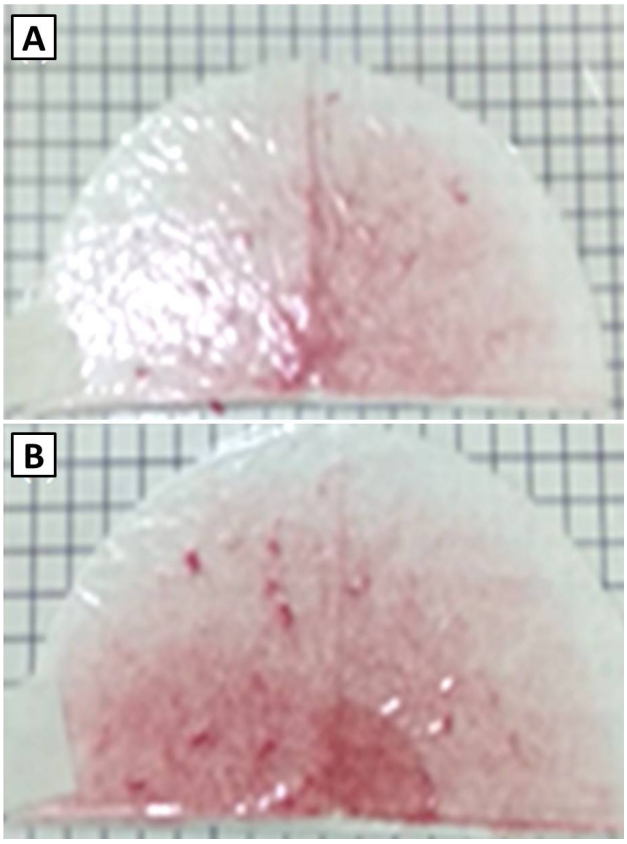

Fig. 7. Biopolishing test for our extracted enzyme cellulase, sample-1 (A) and standard sample (B).

Centralization of pectinase by directional freezing

The results of radial freezing for centralization of pectinase enzyme are shown in Figure 2. Approximately 95% of the pectinase was concentrated in the center of the bowl, a little contamination was found in the samples taken from midway of the radius of the bowl and almost negligible quantity of pectinase was found along the walls of the container. From Figure 8, it is clear that the sample 1 (which is taken from centre of bowl) shows max concentration of pectinase due to maximum absorbance. As we move from centre to sides of glass bowl, the value of absorbance decreases for sample 1 to 4, respectively, which shows that the concentration of pectinase decreases as we move from centre to corner of glass bowl. Sample 4 (which is taken from centre of bowl) shows very minute absorbance which indicates very less concentration of pectinase at corners of bowl. All result show that the concentration of cellulase is maximum in centre and

minimum at the sides of bowl. The reason is that cellulase and amylase show hydrogen bonding with aqueous system while in case of pectinase there is no such like bonding between pectinase and aqueous medium, due to which pectinase show movement along with freezing front, resulting maximum centralization.

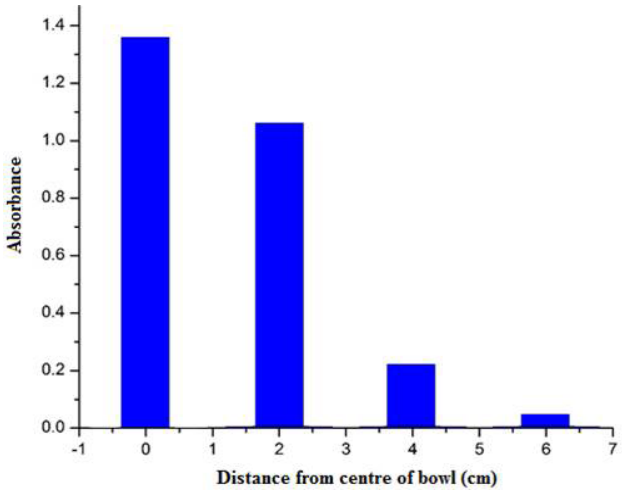

Fig. 8. Concentration of pectinase in various sections of glass bowl after applying UFT.

Here, we can mention that the concentration of enzymes, mode of arrangement, hydrogen bonding, and the rate of cooling influence the movement of enzymes significantly. It was observed that more the concentration of enzymes as in the case of amylase sample, there will be less efficacy to centralize. In addition, it was noted that faster cooling rates do not allow the ice front to push the contaminants so efficiently rather imprison them in solidifying water (Mushtaq *et al*., 2018). In all cases, it was observed that better separation was carried out efficiently at low concentration of enzymes. This is because when ice is crystallized, the crystals built up by pure water leaving the foreign species in the remaining liquid phase. Many research workers employed freezing process for centralizing the different foreign component of solutions (Shafique *et al*., 2011). Ice growing from sides has pushed the particles or any other ingredients to the centre because of strong interactions between water molecules themselves in ice.

CONCLUSION

From these studies it can be safely concluded that enzymes contamination in aqueous systems can be significantly reduced by slow radial freezing technique. Especially, unidirectional freezing technique is more useful in cold icy areas. However, the performance of centralization of enzymes depends upon the rate of cooling and concentration of the contaminants. By using this technique, we can protect our environment from these effluents. The appreciable working activity of enzymes extracted from aqueous system using unidirectional freezing, is promoting the significance of this technique. As a result, this extraction technique is not suitable only for environment protection but also good to control the cost of industrial processes due to reuse of enzymes.

Statement of conflicts of interest

The authors declare no conflict of interest.

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