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Production of Extracellular Cold Active Lipase by *Curtobacterium* sp. using Cell Immobilization

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Abstract

A cold adapted lipolytic bacterium *Curtobacterium* sp. was isolated from the soil of Gangotri glaciers, Western Himalayas. The optimum temperature and pH for cold active lipase activity was 15 ^{0}C and 8.0 respectively. The effect of different carbon sources, organic and inorganic nitrogen sources on production of cold active lipase were studied. Glucose served as a good source of carbon for the production of cold active lipase. Soyabean oil used as an additional carbon source induced the production of cold active lipase by two fold. Beef extract and ammonium nitrate was found to be a potential source of organic and inorganic nitrogen respectively. *Curtobacterium* cells immobilized in various concentrations of agar (1-4 %) and alginate (3-6 %) showed an enhanced production of cold active lipase of 9.00 U ml^{-1} (in agar) and 6.00 U ml^{-1} (in alginate) over the free cell fermentations. A decrease in total viable count of beads with increasing concentration of agar and alginate matrices were observed. However, the effect of dehydration on beads showed decrease in enzyme activity with increasing time duration. A consistent production of cold active lipase from cells immobilized in agar was observed in repeated batch culture.

Keywords: Agar, calcium alginate, cold active lipase, *Curtobacterium* sp., immobilization.

Introduction

Cold adapted enzymes catalyze reactions effectively at low temperature (Feller & Gerday 2003, Cavicchioli & Siddiqui 2004). The inherently high specific activity of

this enzyme generally accompanies a low thermostability. The source of cold adapted enzymes are the psychrophiles having optimum growth at less than or equal to 15 ^{0}C and a limit of growth at about 20 ^{0}C (Morita 1975). The permanently cold regions such as glaciers and mountains are the source for psychrophilic lipase producing microorganisms and they possess the tendency to grow and produce extracellular cold active lipases at low temperature range (nearly 5-15 ^{0}C). Gangotri, the second largest Himalayan glacier, situated in the Uttarkashi of Uttaranchal is an efficient source of lipolytic psychrophiles and psychrotrophs.

Cold active lipases represent an extremely versatile group of bacterial extracellular enzymes that are capable of performing a variety of important reactions. These offer economic benefits through energy savings by overcoming the requirements for expensive heating, functioning in cold environments, providing increased reaction yields, accommodating high levels of stereospecificity, minimizing undesirable chemical reactions that could occur at high temperatures and facilitating rapid and easy inactivation of the enzyme when required. These enzymes are found to have unique flexible structures for biocatalysts at low temperatures (Cavicchioli *et al.* 2002). These structural features include less hydrophobic groups, more charged groups on their surface, less substitution of proline residues by glycine in surface loops, less arginine/lysine ratio, longer surface loops, fewer aromatic interactions and less ionic interactions and hydrogen bonds. Due to these properties, the cold active lipases cover a broad spectrum of biotechnological applications (Joseph *et al.* 2007) such as additives in detergents and food industries, in environmental bioremediations, in biotransformations, etc.

In spite of the practical use of cold active lipases in various systems, there occur difficulties as instability of the enzymes due to harsh conditions and the reuse of enzymes. So, in order to increase their industrial application and to increase enzyme stability, an efficient method of cell immobilization is needed because it promotes cells reuse and reduces overall process costs.

Immobilization is a process of physical or chemical fixation of cells, organelles, enzymes or other proteins onto a solid support, into a solid matrix or retained by a membrane, which limits its free movement so that it can be retained there and reused in successive process runs. The immobilization of whole microbial cells and their application has been of interest for nearly 30 years (Chibata & Tosa, 1980, Yun *et al*. 1990, Carvalho *et al*. 2002). It is a potential method for utilization of the microbial enzymes without an enzymatic extraction procedure. Lipases from Antarctic cold adapted bacteria have been the subject of several studies, all of them failing to obtain purified forms due to the difficulty of eliminating lipopolysaccharides produced by Antarctic microorganisms (Gerday *et al*. 1997). However, the use of immobilized cells as an enzyme source will generally eliminate the need for the succeeding purification steps. Among the various matrices, the most widely used ones are agar and alginate due to its cheap cost and strong gel strength.

In comparison with free suspended cells, immobilized cells exhibit tolerance to toxic substrates (Lee *et al*. 1998), enhance fermentation productivity (Kim & Chang 1990), can adapt to a wide range of pH environments (Buzas *et al*. 1989) and high process temperature (Bajpai & Margaritis 1987, Tan & Day 1998) and are reusable

(Paik & Glatz 1994).

The aim of the study was to investigate the effect of cell immobilization on the production of cold active lipase using *Curtobacterium* sp., a plant associated pathogen. The effect of incubation time, temperature, pH, carbon source, nitrogen source, agar and alginate concentration, dehydration and repeated batch culture on the production of cold active lipase was studied.

Materials and Methods

Bacterial Strain

Five lipolytic psychrotrophic bacterial strains were isolated from the soil samples of Gangotri glaciers on tributyrin agar plates and observed for zone of clearance around the colonies (Farrell *et al*. 1993). The bacterial strain which produced larger zone was isolated and identified by morphological and biochemical characteristics based on Bergey's manual of Determinative Bacteriology (Holt *et al*. 1989).

Optimization of culture conditions

The physical and chemical cultural conditions like incubation time (12-72 h), temperature (10, 15, 25 and 35 ^{0}C), pH (5-10) and effects on addition of various carbon sources and nitrogen sources were evaluated in relation to enzyme yield. The effect of one parameter was evaluated at a time and it was taken as a standard condition before optimizing the next parameter.

Production of cold active lipase

The liquid medium used for the production of cold active lipase was composed (g/L) of: yeast extract, 1.0; NaCl, 2.0; $MgSO_4.7H_2O$, 0.4; $(NH_4)_2SO_4$, 0.5; K_2HPO_4, 0.3; KH_2PO_4, 0.3; $CaCl_2.2H_2O$, 0.002 %; gum acacia, 0.02 % and glycerol tributyrate, 0.5 %. The pH of the medium was adjusted to 7.0. The medium was inoculated at 5 % (v/v) with a 48 h old culture and incubated at 15±2 ^{0}C in a shaker (130 rpm) for 48 h. The culture medium was centrifuged at 10,000 rpm for 10 min at 4 ^{0}C. The supernatant obtained was used as crude enzyme for further studies.

Lipase Assay

Lipase activity was determined using *p*-nitrophenyl palmitate (*p*NPP) as a substrate (Winkler *et al.* 1979). The substrate mixture containing phosphate buffer (90 ml) with gum arabic (100 mg) and sodium deoxycholate (207 mg) along with 30 mg of *p*NPP in 10 ml of isopropanol was measured spectrophotometrically at 405 nm. One unit of lipase activity is defined as the amount of enzyme releasing 1 µmol *p*-nitrophenol (*p*NP) per min under the assay conditions.

Immobilization of bacterial cells

The culture of cells were grown in nutrient broth (1 l) at 15±2 ^{0}C for 48 h and harvested by centrifugation at 8000 rpm for 10 min at 4 ^{0}C. After washing thoroughly with 0.85 % saline solution, the cells were immobilized in matrices like agar and alginate at a cell concentration of 0.8 %. For immobilization of cells in agar, the cells

were dropped in a hydrophobic phase (Joseph *et al*. 2006) while the cells were allowed to enter dropwise in $CaCl_2$ solution to prepare insoluble calcium alginate beads (Srinivasulu *et al*. 2003).

Optimization of the matrix concentration

In order to determine the optimum concentration of agar and alginate matrix for the production of cold active lipase, various concentrations of agar (1-4 %) and alginate (3-6 %) were used to prepare the beads. These beads were employed for the production of cold active lipase and the fermentation was conducted for 60 h at 15±2 ^{0}C. The production of cold active lipase was assayed at different time duration of fermentation with an interval of 12 h.

Determination of total viable count in beads

Immobilized cells in beads were counted after dissolution of beads in phosphate buffer (0.1 mol l^{-1}, pH 7.0), serially diluting and plating on nutrient agar. The number of viable cells were counted after incubation for 48 h at 15±2 ^{0}C.

Effect of dehydration on beads

The beads were placed at 4 ^{0}C for a period of 30 days to induce freeze dehydration. This resulted in shrinkage of beads due to water removal.

Determination of cell washout from beads

In order to determine the cell washout from the beads during fermentation, the beads were inoculated in phosphate buffer solution (pH 7.0) and kept in the shaker incubator for 1 h. The phosphate buffer was sampled on nutrient agar and total bacterial count present in the buffer was analyzed.

Repeated batch culture of cells immobilized in agar and alginate beads

The efficacy of beads in repeated batch culture was determined by inoculating the beads in production medium and removing it after fermentation. The same beads were inoculated to the successive broths. Samples were withdrawn at an interval of 48 and 60 h for agar and alginate respectively and assayed for lipase activity.

Results

Till date no attempt has been undertaken to immobilize cold adapted microorganism for the enhanced production of extracellular cold active lipase. Keeping this point in view, the investigation was made in which the production of cold active lipase from a cold adapted microorganism *Cutobacterium* sp. was optimized and enhanced.

The bacterial strain which showed the formation of maximum clear zone on tributyrin agar was selected and identified as *Curtobacterium* sp. The characteristics shown by the organism is summarized in Table 1.

Table 1 Identification of the potential bacterial strain by morphological and biochemical tests

Tests	**Results**
Colony Characteristics	
Configuration	Round
Margin	Regular
Elevation	Convex
Surface	Smooth moist
Density	Opaque
Pigment	Yellow
Gram's Reaction	+
Shape	Rod
Size	Short
Arrangement	Single
Spore	-
Motility	+
Growth on Mac Conkey Agar	-
Biochemical Characteristics	
Indole	-
Methyl Red	+
Voges-Proskauer	-
Citrate	-
Gelatin hydrolysis	+
Casein hydrolysis	+
Starch hydrolysis	+
Urea hydrolysis	-
Nitrate reduction	-
H_2S production	-
Oxidase	+
Catalase	+
Hugh Leifson's Test	
a) Aerobic	+
b) Anaerobic	-
Acid Production from Carbohydrates	
a) Glucose	+
b) Sucrose	-
c) Xylose	-
d) Maltose	-
e) Lactose	-
f) Mannitol	-

Effect of physical and chemical parameters on production of cold active lipase

The physical and chemical parameters for the production of cold active lipase by the

organism are shown in Table 2. The maximum lipase activity was obtained at 60 h with an activity of 1.33 U ml^{-1}. A temperature of 15 ^{0}C was found to be the optimum for the production of cold active lipase by *Curtobacterium* sp. considering that the organism was a psychrotroph in accordance with Morita (1975) who stated that psychrotrophs are essentially mesophilic organisms that can also grow under cold conditions below 15 ^{0}C. In *Psychrobacter* sp., an Anatarctic bacterium, maximum lipase activity was at 10 ^{0}C (Pascale *et al*. 2005). The activity of the cold enzyme presents an apparent optimal activity around 35 ^{0}C and retains about 20 % of its activity at 0 ^{0}C, whereas the activity of the mesophilic lipases is close to zero at temperatures below 20 ^{0}C and increases at temperatures above 60 ^{0}C (Gerday *et al*. 1997). So, we suggest that the lipase of *Curtobacterium* sp. has the characteristic of a cold adapted enzyme and lipase may be better in application to low temperature and a more stable alternative to other lipases.

Table 2 Effect of various physical parameters on production of cold active lipase

S.No.		Parameters	Lipase activity (U ml^{-1})
1.	Incubation time (h)	12	0.33
		24	0.33
		36	0.66
		48	1.00
		60	1.33
		72	0.33
2.	Temperature (^{0}C)	10	1.33
		15	1.66
		25	0.66
		35	1.00
3.	pH	5	0.66
		6	1.00
		7	1.00
		8	1.33
		9	0.66
		10	0.6

The optimum pH of 8.0 was reported for the production of cold active lipase from the organism. This was in agreement with the results of Choo *et al.* (1998) who also obtained the enzymatic hydrolysis of tributyrin at an optimal pH of 8.0. The effect of various carbon sources on the production of cold active lipase is illustrated in Figure 1 and Figure 2. Addition of glucose which is a cheap and easily available carbon source showed the maximum lipase activity of 1.66 U ml^{-1} in comparison to sucrose and mannitol. Lactose and maltose did not show any significant role in the production of cold active lipase.

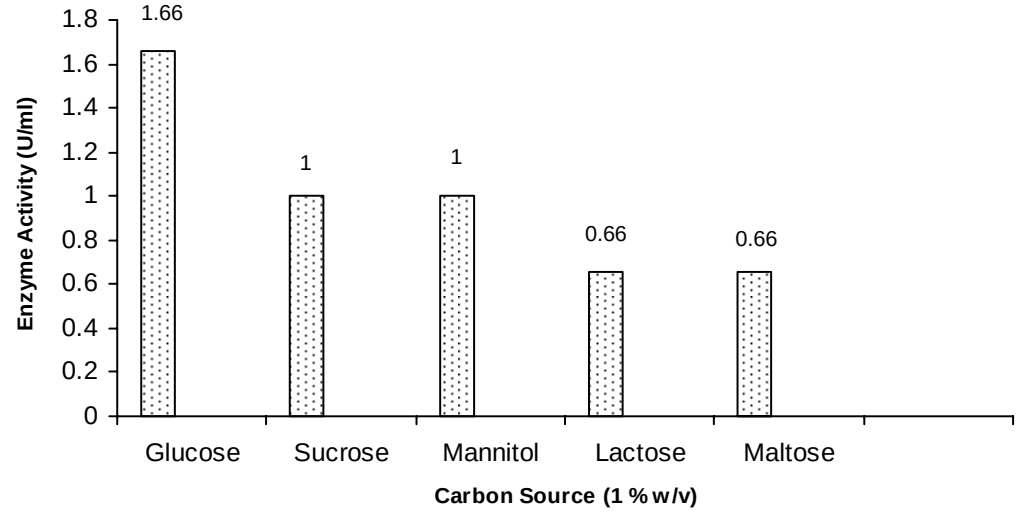

Figure 1 Effect of supplementation of different carbon sources on production of cold active lipase

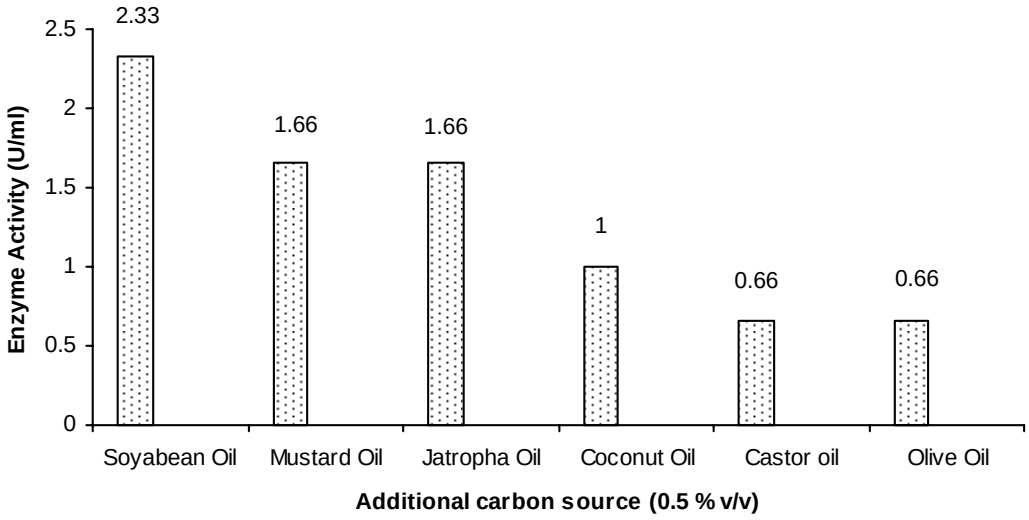

Figure 2 Effect of supplementation of different additional carbon sources on production of cold active lipase

Among the additional carbon sources used like soyabean, mustard, jatropha, coconut, castor and olive oil, 0.5 % soyabean oil when added to the production media, the production of cold active lipase was increased by two fold. This result agreed well with the results of Joshi *et al.* (2006) in which soyabean oil induced the production of cold active lipase in *Corynebacterium paurometabolum*. Soyabean oil being a cheap commercial commodity and easily available can be used for the increased production of cold active lipase from the organism.

The effect of various organic and inorganic nitrogen sources on production of cold active lipase is shown in Figure 3. Beef extract was found to be the best organic nitrogen source with an activity of 1.66 U ml^{-1}. However, peptone served as a poor organic nitrogen source for the production of cold active lipase. This was contrary to the result of Saxena *et al.* (2003) who obtained peptone as the best organic nitrogen source. Ammonium nitrate served as a good inorganic nitrogen sources with an activity of 1.66 U ml^{-1} to increase the production of cold active lipase while compared to sodium and potassium nitrate in agreement with the result of Gopinath *et al.* (2003) in which ammonium nitrate when supplemented to the production medium increased the production of cold active lipase in comparison to sodium and potassium nitrate.

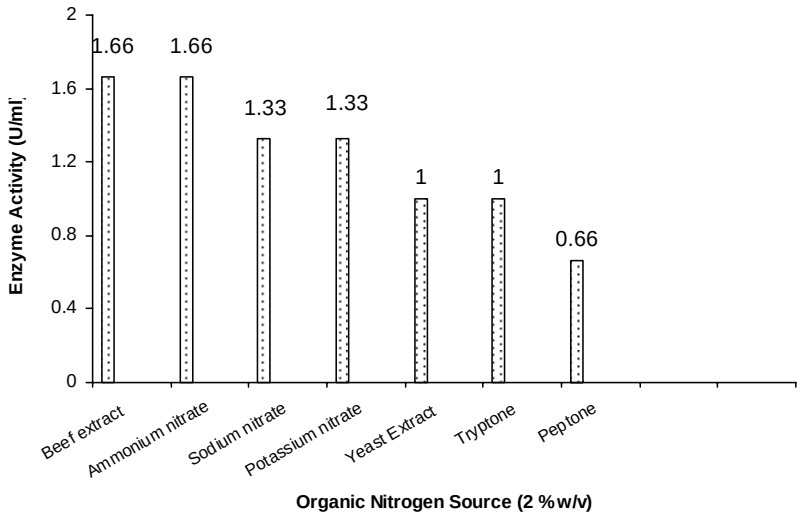

Figure 3 Effect of supplementation of different nitrogen sources on production of cold active lipase

Effect of different concentrations of agar and alginate on production of cold active lipase

Rosevear (1984) studied different cell immobilization technique and found entrapment as one of the most effective method of immobilization, calcium alginate being the most used matrix due to its simplicity and non-toxic character. Agar at a concentration of 1 % (9.00 U ml^{-1}) and alginate at a concentration of 3 % (6.00 U ml^{-1}) was found to be the optimum for formulation of stable beads with better production of cold active lipase (Table 3 and 4). However, it was observed that the enzyme titre reduced with increasing alginate and agar concentration, which may be due to the reduced porosity of the beads limiting the nutrient supply and oxygen diffusion.

Table 3 Effect of different concentrations of agar on production of cold active lipase

	Enzyme activity (U ml^{-1})					
	12 h	24 h	36 h	48 h	60 h	72 h
1.0	6.00	4.66	7.33	9.00	7.33	4.00
1.5	6.00	4.00	5.33	7.33	7.00	3.33
2.0	5.33	2.66	3.33	6.66	6.00	2.66
2.5	5.33	2.66	3.33	6.66	6.00	2.00
3.0	4.00	2.00	4.66	6.00	5.33	2.00
3.5	4.66	2.66	4.00	5.33	4.66	2.00
4.0	4.66	2.66	4.66	4.66	4.00	1.33

Table 4 Effect of different concentrations of alginate on production of cold active lipase

	Enzyme activity (U ml^{-1})					
	12 h	24 h	36 h	48 h	60 h	72 h
3.0	4.33	5.33	5.33	5.33	6.00	5.33
3.5	2.66	4.00	2.66	3.00	5.33	5.00
4.0	2.00	3.00	2.00	3.00	5.33	5.00
4.5	2.00	3.00	2.00	2.33	4.66	5.00
5.0	2.00	3.00	2.00	2.33	5.00	4.66
5.5	1.33	2.66	2.00	2.33	5.00	4.66
6.0	1.33	2.66	2.00	2.33	2.00	4.33

Effect of different concentrations of agar and alginate on total viable count

The higher concentration of matrix resulted in decrease in TVC due to the low porosity of matrix (Figure 4 and 5). However, the concentration of 3.5 % agar was found to be optimum for the viability of cells with TVC of 11.2×10^5 cfu/g and the lipase activity of only 5.33 U ml^{-1}. Maximum lipase activity of 9.00 U ml^{-1} was shown by 1 % of agar beads while the TVC in these beads was less (8.1×10^5 cfu/g) as compared to 3.5 % agar. Similarly, maximum TVC of 15.0×10^5 cfu/g was found in 4 % alginate beads, its activity being only 5.33 U ml^{-1} while compared to 3 % alginate (11.2×10^5 cfu/g) having activity of 6.00 U ml^{-1}. Thus, the TVC in 1 % agar and 3 % alginate, although being low, is capable of producing maximum enzyme yield which may be due to the utilization of all the available nutrients for the maximum production of cold active lipase.

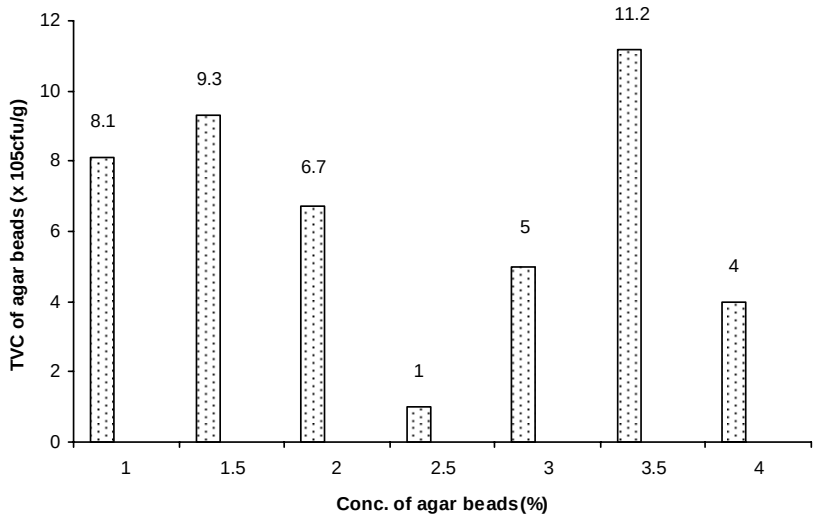

Figure 4 Effect of different concentrations of agar on total viable count

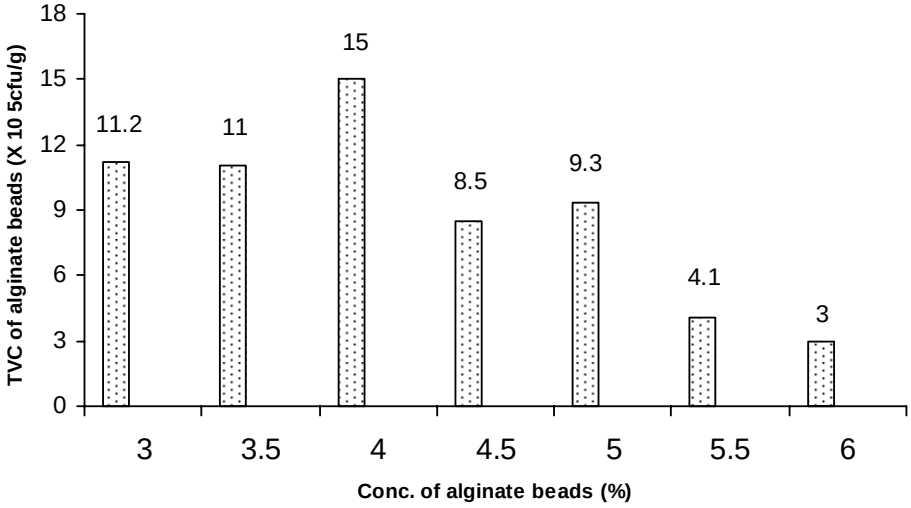

Figure 5 Effect of different concentrations of alginate on total viable count

Effect of dehydration on agar and alginate beads

Maximum activity of 9.00 U ml^{-1} (in agar) and 6.00 U ml^{-1} (in alginate) was found on 0 day i.e. from the hydrated beads after an incubation of 48 and 60 h respectively. The TVC obtained on the 0 day was 6.1×10^5 cfu/g and 9.2×10^5 cfu/g in agar and alginate beads respectively. However, there was observed a decrease in activity as well as TVC on the 15 th and 30 th day i.e. from the dehydrated beads (Table 5). The reason behind this may be attributed to the fact that during the dehydration, ice formed outside the agar and alginate beads which lowered the vapour pressure and hence pulled water from inside the beads, as a result of which some cells may be lost

during the process. This was in agreement with the results of Shin *et al.* (2004) who also obtained a decrease in enzyme activity with the dehydrated beads compared with the hydrated beads. The results indicated that increased activity was obtained in case of dehydrated agar beads in comparison to the dehydrated alginate beads. Although the enzyme activity with the dehydrated beads is less, it is possible to pack considerably more into a fixed volume bioreactor as the volume of the former gets reduced due to water removal.

Determination of cell washout from agar and alginate beads

There was no loss of cells from the agar and alginate beads. This may be applicable to keep the cell concentration and productivity from immobilized cells stable for a long period of time during storage and furthermore, the enzyme can be recovered whenever required.

Repeated Batch Culture of Curtobacterium sp. immobilized in agar and alginate beads

The repeated batch production of cold active lipase with entrapped cells of *Curtobacterium* sp. in agar and alginate matrices were carried out for five cycles at optimal conditions (Figure 6). There was a loss in enzyme activity (after the first cycle) of 15, 42, 54 and 77 % in respective four cycles in case of agar, while in case of alginate, the loss in enzyme activity for the respective four cycles were 13, 25, 25 and 62 %. This was in agreement with the results of Nigam *et al.* (2007) who also obtained a loss in enzyme activity with repeated batch fermentation using cells of *Pseudomonas diminuta*.

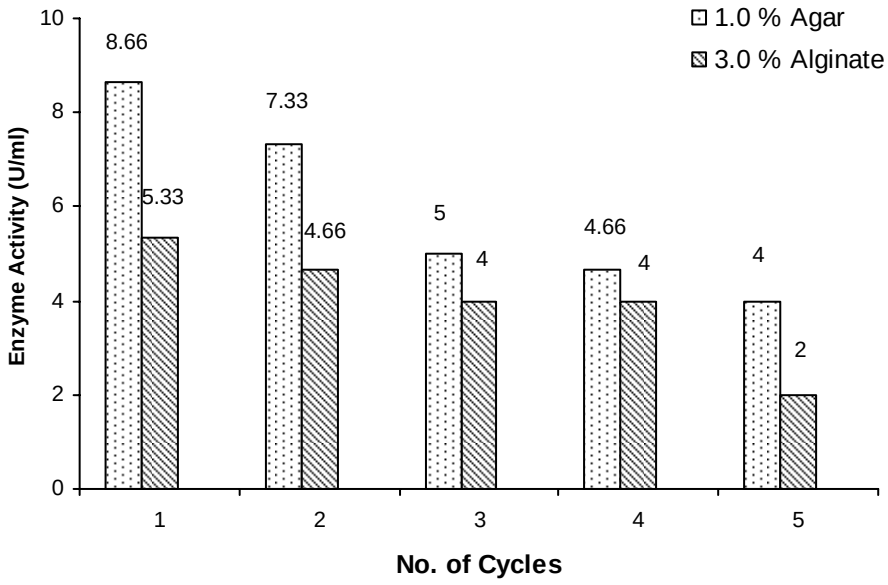

Figure 6 Repeated Batch Culture of *Curtobacterium* sp. immobilized in agar and alginate beads

Discussion

Gangotri glaciers provide a rich source to explore lipolytic and proteolytic psychrophiles and psychrotrophs. The organism *Curtobacterium* sp. Isolated from the soil samples of Gangotri glaciers was able to produce cold active lipase at low temperature (15 ^{0}C) and alkaline pH (8.0). The high activity of psychrophilic enzymes at low and moderate temperatures offers potential economic benefits (Cavicchioli *et al.* 2002). Microorganisms that can degrade lipids rapidly at low temperatures are necessary for the treatment of lipid-containing waste water because the temperature of such waste water drainage is relatively low (approximately 5-25 ^{0}C). Low temperature lipases can be added to detergents to hydrolyze oily stains at the temperature of tap water to reduce energy consumption and protect the colour of fabrics (Feller & Gerday 2003). Besides the alkalophilc nature of the organism can be used to improve the performance and stability of biological treatment systems designed for the bioremediation of water (contaminated with chlorinated solvents, hydrocarbons, nitrates and other biologically degradable compounds), detergent formulations, sewage treatment, leather processing which are carried out at moderately low temperatures. The study has proved that by optimizing the growth conditions i.e. by manipulating the nutritional and physical factors of the growth condition, the production of cold active lipase can be increased.

An enhancement in production of cold active lipase was observed with immobilized cells. Immobilized cells are preferable and more convenient to control and handle; it permit easy separation of product from the catalyst and it has been found to be less susceptible to microbial contamination. Industrialists have also confirmed that the use of immobilized cells enable the conditions for cell growth and a full optimization of feed for product formation (Jimoh 2004). It was found that whole cells in a gel matrix lived for a month under certain conditions. Thus, immobilized cells can be considered as a better alternative to free cells to increase cell concentration and productivity.

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