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Isolation and Characterization of Psychrotrophic Antarctic Bacteria from Blue-green Algal Mats and Their Hydrolytic Enzymes

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Abstract

Blue-green algal mat samples were collected from various locations around Schirmacher Oasis, Antarctica. Psychrotrophic bacteria have been isolated and were studied for the production of hydrolytic enzymes. The bacteria have been categorised into proteolytic, lipolytic and amylolytic strains. Their optimum growth temperature was determined as 25-28°C, The optimum carbon and nitrogenous substrates were determined for two isolates that belong to the genera of *Pseudomonas* sp and *Bacillus* sp showing high protease and lipase activity. Preliminary characterization of the enzymes has been carried out. The bacteria were able to produce acidic proteases and esterases with an optimum temperature of 40°C. The proteases were able to hydrolyse only denatured protein substrates like gelatin and azocasein. The molecular weights of the proteases ranged from 46-80 kDa. Lipases have shown negligible activity in the presence of oils. Water soluble monomelic lipase substrates were hydrolysed at much higher rates.

Introduction

Bacteria which grow at low temperatures are known as psychrophiles and psychrotrophs. Psychrophiles are defined as having optimum growth temperature below 15°C and upper growth temperature below 20°C. Whereas psychrotrophs have optimum growth temperature around 20°C and sometimes they show an upper growth-temperature of 40°C. Most of the investigations in the past have been dealt with proteases and lipases produced by food-spoiling psychrotrophs mainly from *Pseudomonas fluorescens* (Patel, 1986). The Antarctic habitat has its characteristic extreme low temperatures and dry weather conditions. Despite the hostile conditions of the Antarctic continent various forms of algae, fungi, lichens and bacteria have been known to thrive exuberantly (Shivaji, 1988 & 1989). The microflora of this continent was investigated to some extent but most of the microflora has not been completely explored for application in different fields (Feller, 1990). The substrate utilization and

nutrient requirements of cold bacteria of Antarctic have been very sparse and limited to understand the phenomenon of enzyme induction and their importance in biodegradation (Juffs, 1976; Mc Keller, 1982;Fairbarn and Law, 1987; Reichadt, 1989).

In this investigation the aim has been the isolation of cold- active bacteria from Antarctic samples, their substrate utilization, carbon and nitrogen source preference for enzyme production and characterization of their hydrolytic enzymes to facilitate biodegradation at low temperatures.

Materials and Methods

Sample collection: Samples of blue-green algal mats were collected from various ponds, lakes and water bodies of Schirmacher Oasis in sterilized polythene bags. The samples were transported to Gwalior at 4°C.

Bacterial counting and isolation: Ten fold serial dilutions of the samples were prepared in nutrient broth and 0.1ml of each were spreaded on the surface of nutrient agar, skim milk agar, tributyrin agar and starch agar. The plates were incubated for one week at 15°C. The bacterial colonies appearing on nutrient agar were recorded as total bacterial counts. The proteolytic and lipolytic bacteria were recognised by observing the zone of hydrolysis on skim milk agar and tributyrin agar, respectively. The amylolytic bacteria were identified by fuming the plates of starch agar with iodine crystals and observing halo as a result of starch hydrolysis around the colonies. The proteolytic and lipolytic bacteria were isolated and maintained in nutrient agar slants at 4°C.

Screening for protease and lipase production: The medium for quantitative screening of enzyme production was consisted of yeast extract 0.02%, casein/tributyrin 0,05%, K_2HPO_4 1.0% and KH_2PO_4 0.5% with *p*H adjustment to 7.4. A loopful of freshly grown nutrient broth culture was used as inoculum for enzyme production in 250 ml conical flask containing 100 ml of broth. The broth was incubated at 15°C for 10 days and the supernatant was used for enzyme estimation.

Optimization of growth and enzyme production: For finding out the suitable carbon source, glucose, maltose, lactose, starch and oils comprising corn oil, olive oil, tributyrin and tween 80 were used independently in liquid medium. Gelatin, peptone, casein, bovine serum albumin (BSA), soybean meal and egg albumin were used for testing the optimum nitrogenous substrate. The optimum temperature and *p*H were determined by incubating the flasks at different temperatures and *p*H. Growth was monitored by measuring the optical density of culture broth at 540 nm.

Quantitation of enzyme activities: Protease activity was quantified using culture supernatants by taking azocasein as substrate according to the method of Razak *et al.* (1994). Lipase activity has been measured by using α- and β-naphthyl acetate, butyrate, caprylate, palmitate and myristate according to the method of Lambrechts and Galzy (1995). Tributyrin and olive oil were used as triglyceride substrates. Protease activity was expressed as Azocasein Digestion Units (ADU). One unit of ADU was defined as the increase in absorbance of 0.001 units per. hour at 425 nm. One unit of lipase was defined as the amount, which releases 1 μmole of fatty acid per min under the specified conditions. As says were performed in triplicates and the average was indicated.

Purification of enzymes: Enzymes were fractionated by adding solid ammonium sulfate to the extent of 70%. The precipitate was collected by centrifugation at 10000 rpm for 10 minutes and were dialysed prior to gel filtration using Sephadex G-100. The purified fractions were subjected to native - polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulphate potyacrylamide gel electrophoresis (SDS-PAGE) (Blackshear, 1984).

Results and Discussion

Seventy bacterial strains were isolated and ten of them were found to be *Pseudomonas* sp and fifteen as *Bacillus* sp. They were isolated from fresh and decaying blue-green algal mat samples. All the isolates were screened for their ability to elaborate hydrolytic enzymes such as protease, lipase, cellulase and amylase. It has been reported that cyanobacteria, algae, lichens and moss are the phototrophs that contribute for the enrichment of both aerobic and anaerobic bacterial growth in the Antarctic ecosystem. The decomposition of phototrophic biomass is known to proceed both by the active role of aerobic and anaerobic bacteria.

Bacterial counts: In our investigations a plethora of bacterial strains were found in active and decomposing blue-green algal mats of Antarctica. The total count of bacteria in algal mats varied from $21x10^2$ to $96x10^3$ /g of sample. The amylolytic, proteolytic and lipolytic counts ranged from $25\text{-}30x10^2$, $36\text{-}50x10^1$ and $35\text{-}40x10^1$, respectively. The dominance of a particular class of hydrolytic bacteria differed from sample to sample probably depending on the extent of decomposition. The existence of particular hydrolytic bacteria reflects the availability of respective substrates in the decaying samples (Table 1).

Screening of bacterial isolates for protease and lipase production: Seventy bacterial isolates from nutrient agar were subcultured on skim milk agar and tributyrin agar. The colonies showing hydrolytic zones were subjected to quantitative enzyme production in liquid culture media. The protease produc-

Table 1: Quantitative studies on bacterial numbers from decaying blue-green algal mat samples at 10°C (Bacterial count/gm of sample)

Sample no.	Total count	Amylolytic	Proteolytic	Lipolytic
BGAS1	$21x10^2$	25	$16x10^1$	$40x10^1$
BGAS2	$23x10^2$	$17x10^2$	$36x10^1$	$11x10^1$
BGAS3	$96x10^3$	$30X10^3$	51	35
BGAS4	$30x10^3$	$39x10^1$	80	91___
BGAS5	$16x10^4$	$44x10^1$	$24x10^1$	$20x10^1$
BGAS6	$15x10^4$	$28x10^1$	$25x10^1$	$24x10^1$

tion by different isolates varied from 10-90 units/ml and lipase from 0.3-0.9 units/ml.

Protease production has been recorded during log phase of bacterial growth curve while lipase production occurred during the start of stationary phase or at the transition period from logarithmic to stationary phase of growth. During our screening of the isolates it has been observed that all of the *Pseudomonas* and *Bacillus* strains were able to produce proteases and lipases. Out of the fifteen *Bacillus* strains two have shown the presence of extracellular amylases in very low quantities.

Carbon and nitrogen source preference of the isolates for the production of proteases and esterases: The effect of carbon and nitrogen sources on growth and enzyme production by *Bacillus* sp is shown in Table 2. Different carbon

Table 2: Evaluation of optimum carbon and nitrogen source for growth and enzyme production of *Bacillus* sp

	Optical density at 520nm	Protease production μ/ml	Lipase production μ/ml
Carbon substrate			
Starch	1.04	160.0	1.7
Lactose	0.88	180.0	1.6
Glucose	0.82	150.0	1.6
Maltose	0.70	190.0	1.3
Tributyrin	0.75	110.0	1.1
Nitrogen source			
Egg Albumin	0.97	110.0	1.9
Gelatin	0.88	270.0	1.9
Casein	0.44	100.0	1.7
BSA	0.49	50.0	1.4

sources like starch, lactose, glucose, maltose and tributyrin as well as nitrogen sources like egg albumin, gelatin, casein and bovine serum albumin (BSA) supported the bacterial growth as well as induction of protease and lipase. The maximum growth of *Bacillus* sp has occurred in the presence of starch along with lipase production. However, protease was induced maximally in the presence of maltose. It is evident from the table data that tributyrin induces lower levels of enzymes. Among the nitrogen sources tested egg albumin has yielded maximum growth and lipase activity. In comparison, gelatin was able to induce both protease and lipase enzymes.

The *Pseudomonas* sp showed better growth in the presence of glucose and maltose than other carbon sources. Although, starch was showing poor growth supporting property, it has induced highest amounts of protease and lipase in the medium. Gelatin was found to be the most suitable nitrogen source for growth as well as for production of enzymes by the *Pseudomonas* sp (Table 3).

Some of the other compounds that have been used as nitrogen source by *Pseudomonas* sp and *Bacillus* sp were glutamic acid, peptone and soybean meal (data not shown). Soybean meal and peptone has increased the enzyme production almost 2 fold than others but at higher concentrations (2%). This is in agreement with the observations of Margesin (1992b) who reported proportional increase in enzyme production with the substrate concentration. Ammonium sulphate, known to be a common nitrogen source for bacteria has shown drastic inhibition of enzyme production by both *Pseudomonas* and *Bacillus* sp isolates.

Table 3: Evaluation of optimum carbon and nitrogen source for growth and enzyme production by *Pseudomonas* strain

	Optical density at 520 nm	Protease production μ/ml	Lipase production μ/ml
Carbon source			
Starch	0.9	450.0	1.0
Lactose	1.0	390.0	1.7
Glucose	1.12	250.0	1.3
Maltose	1.12	310.0	1.2
Tributyrin	0.86	160.0	1.0
Nitrogen source			
Egg albumin	0.9	350.0	1.5
Gelatin	0.95	480.0	1.9
Casein	0.39	100.0	1.4
BSA	0.55	160.0	1.5

Proteases isolated from these bacteria were highly active on denatured proteins like azocasein and gelatin. They have shown negligible hydrolysing activity when substrate was casein. Although the bacterial proteases and lipases are not highly active on casein and triglyceride substrates they were able to utilize them during growth on solid agar medium as well as in liquid cultures. Slow emulsification of oils was observed after about 72 hours of growth even at suboptimal temperatures of 5 - 15°C. The bacterial strains seem to be requiring higher oxygen partial pressures for exuberant growth and enzyme production. The preferable hydrolysis property of proteases isolated from *Bacillus* sp and *Pseudomonas* sp is in agreement with other reports. Protease produced by a strain of psychrophilic *Pseudomonas fluorescens* had exhibited similar substrate preference. In contrast psychrophilic *Xanthomonas maltophila* protease preferred high molecular weight substrates like casein and dimethyl casein, gelatin hydrolysis was less than 50% (Margesin, 1992). Nevertheless, during growth the isolates were able to utilize casein as nitrogen source. Similarly lipases have shown increased activity on α- and β- naphthyl substrates and on Tweens which are water soluble monomeric esterase substrates in comparison with triglyceride substrates.

In our study two proteases were detected in *pseudomonas* sp oi molecular weight 68kDa and 46kDa. However, in the case of *Bacillus* sp only one protease of molecular weight 80 kDa was detected in the extracellular medium after analysis by SDS-PAGE. It has been evident that in the case of *Bacillus* sp the amount of protease production is inversely proportional to total growth of the organism in liquid broths, whereas esterase production has increased proportionately with growth. The esterases have shown maximum activity in the presence of α-naphthyl caprylate and β-naphthyl caprylate in comparison with α-naphthyl acetate a-naphthyl butyrate, palmitate and myrislate. Both the isolates have shown optimum growth temperature below 30°C. In case of *Bacillus* sp maximum protease production was noticed at 20°C but the optimum

Table 4: Growth and enzyme properties of *Bacillus* sp and *Psendomonas* sp

	Bacillus sp	*Pseudomonas* sp
Optimum growth temperature	25°C	28°C
Optimum temperature for protease production	20°C	30°C
Protease optimum temperature	35°C	35°C
Lipase optimum temperature	40°C	40°C
*p*H range for growth	5.5-9.0	5.5-9.0
Optimum *p*H for growth	7.5	6.5-7.5
Optimum *p*H for protease	6.0	7.0
Optimum *p*H for lipase	8.5	7.5

temperature for the enzyme activity was 35°C, which is 10°C higher than optimum growth temperature. From the same isolate, lipase exhibited an optimum temperature of 40°C. Similarly, in *Pseudomonas* sp maximum protease production was observed at 30°C showing its optimum activity at a temperature of 35°C, as shown in the Table 4. The enzyme, protease of these isolates can be classified as acidic proteases with an optimum *p*H of 6.0. The enzymes were active even in distilled water which represents a poor buffering capacity. Another characteristic feature of the isolates was their ability to grow over a wide *p*H range of 5.5-9.0.

Furthermore, *the pseudomonas* isolate could grow in the nutrient broth in the presence of 5% KC1 and its protease and esterase have shown stability at a concentration of 2% NaCl and KC1 in the reaction mixture. Almost 95% of the activity was retained in the presence of these salts. However, *Bacillus* sp could not grow in the presence of high salt concentrations in the medium but its enzymes were stable in 2% NaCl and KC1 reaction mixtures retaining 85% of their original activity. The bacterial isolates will be useful for bioaugmenting organic waste disposal at low temperatures and saline conditions. The two common hydrolytic enzymes (protease and lipase) can be utilized in basic research as well as for commercial exploitation.

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