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Influence of physicochemical conditions on the production of lipase by Psychrophilic Bacteria Isolated from Batura Glacier, Hunza Valley, Pakistan

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Abstract

Psychrophilic lipases have great biotechnological potential and are more preferable as compared to mesophilic or thermophilic lipases, due to their high activities at lower temperatures. In the present study, four bacterial isolates (HTB1, HTB2, LTB3, and HTB6), collected from different glaciers of Pakistan, screened for their lipase producing ability. On the basis of qualitative and quantitative analysis, out of four isolates, HTB2 isolate was selected for further production and optimization of lipase. Optimization of different culture conditions (incubation time, pH, temperature, carbon and nitrogen sources and various substrates) for the maximum production of lipase from HTB2isolate, were studied in shake flask fermentation. Maximum growth of HTB2isolate and lipase production (7.6 and 25.9U/mg), was observed at pH 9 and 10°C, after 72hrs of incubation, respectively. Tween80, was found to be the best substrate for lipase production (20.8 U/mg), in liquid medium with pH 9, at low temperature (10°C). Addition of glucose (19.4 U/mg) and casein (15.5U/mg) enhanced lipase production and were considered as good carbon and nitrogen sources for the production of lipase.

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Introduction

Psychrophilic organisms and their enzymes have been adapted to work efficiently in cold environment. Most of the metabolic reactions are catalyzed by microbial enzymes (Van Dyke *et al.*, 1991). Among, different microbial enzymes, lipases have a major role in the hydrolysis, mobilization and transfer of these water-insoluble compounds (Beisson *et al.*, 2000). Hence, large part of the Earth's biomass consists of lipids. Previously, microorganisms have been found that produce emulsifying agents or bio-surfactants to assist the dissolution of the *lipids* (Van Dyke *et al.*, 1991). Enzymes produced by microorganisms have gained special industrial importance because of the availability of great number of catalytic activities, the high possible yield production, easy genetic manipulation, and constant supply due to absence of seasonal fluctuation and high growth rate of microorganisms, in the presence of cheap growth media (Wiseman, 1995). Approximately 2% of microorganisms in the world, have been studied as enzymes producers. *Aspergillus niger*, *Aspergillus oryzae* and *Bacillus subtilis* are the most interesting and well- studied enzyme producing microorganisms (Hasan, 2006).

Lipases are very versatile and can work in the presence of wide range of substrates, temperatures and pH (Kademi *et al.*,2005). Therefore, generally, they exhibit good chemo-selectivity, regio-selectivity and enantio-selectivity. Lipases have gained high interest in the last few years due to their higher industrial applications e.g. in medicines, food, cosmetics and detergents, organic synthesis, and in pharmaceutical industries (Park *et al.*, 2005; Gupta *et al.*, 2007; Grbavcic *et al.*,2007). Naturally, a number of microbes can produce lipases in higher amounts with multifunctional properties (Jaeger *et al.*, 1994). Cold active lipases are isolated from cold adapted microorganisms.

These enzymes work efficiently at low temperatures with high rates of catalysis as compared to the lipases isolated from mesophiles or thermophiles as these show little or no activity at low temperature. Cold-enzymes, are used as additives in detergents for cold washing, additives in food industries, environmental bioremediations and having several applications in the field of molecular biology (Feller *et al.*, 1996). In biomedical field, cold active lipases have been appeared as a significant biocatalyst.

In spite of extended applications of cold active lipases, number of research studies related to the optimization of various conditions to get the maximum production of cold active lipases, are still limited. Therefore, screening of new microorganisms for the production of lipolytic enzymes will provide more ways to solve various environmental problems and also to carry out different industrial processes.

On the basis of above mentioned aspects and industrial needs, the present study was designed to screen the psychrophilic bacterial isolates (HTB1, HTB2, LTB3, and HTB6), for the production and optimization of lipases.

Materials and methods

In the present study, four bacterial isolates (HTB1, HTB2, LTB3, and HTB6), were screened for the production of extracellular lipase. These were obtained from sediment and surface ice samples previously collected from Batura glacier (36°30.302N to 074°51.138E), Karakoram range, Pakistan. The Batura glacier is 57 km long and ends at around 3000 m altitude in Hunza Valley.

The pH of all the samples was 7, whereas, temperatures of glacial sediments and water was 1°C while that of glacial ice was -2°C. All the collected samples were transported to Microbiology Research Lab, Department of Microbiology, Quaid-i-Azam University, Islamabad, in ice box and stored at -20°C.

Qualitative test

Screening on Tween80 containing medium

For the screening of lipase, Tween80 was, used as the lipid substrate. Tween80 (T-80) and rest of medium was autoclaved separately. About 1ml of T-80 was added per 100ml in the medium. After inoculation of

bacterial isolates on sterilized medium, these plates were incubated at 10˚C, and appearance of clear zones was observed after 24hours.

Rhodamine B assay

Above mentioned isolates were further screened for lipases on Rhoda mine B (0.1% w/v), and olive oil containing medium. Bacterial colonies were visualized under UV-trans illuminator.

Production of lipase

Four isolates (HTB1, HTB2, LTB3, and HTB6), were further screened for the production of lipase in liquid medium. Inoculum of four isolates was prepared separately in olive oil containing production medium (KH_2PO_4, 1.0, K_2HPO_4, 0.1, NH_4Cl, 1.0, $MgSO_4$, 0.25, $CaCl_2$, 0.025, Fructose, 2%, casein hydrosylate, 2.5% and olive oil1%)..

Batch-Shake flask fermentation

For shake-flask fermentation, 50ml of (sterilized production medium) for each bacterial isolate, was poured into 250ml Erlenmeyer flasks and inoculated with (2%), inoculum from isolates HTB1, HTB2, LTB3 and HTB6.Incubation was performed in shaking incubator at 150 rpm at a temperature of 10°Cfor 120hrs. Samples were collected after regular interval of 24hrs. The supernatants or crude enzymes were separated from cells by centrifugation at 10000 rpm for 15 minutes. The supernatants were stored at -20°C for further study. Lipase assay and protein estimation, was carried out to determine the lipolytic activity.

The best producer strain HTB2 was selected for study of various factors that affect production of lipase. The isolate HTB2 was Gram positive, slender, non- spore forming rods, which were mostly straight or slightly curved, present singly or in pairs joined end-to-end at an angle of 45-50° in the shape of 'V' and produced round, slightly yellowish and convex colonies on Nutrient agar.

Optimization of lipase production

For the regulation of maximum production of lipase in shake flask fermentation, effect of various parameters (incubation period, pH, temperature, carbon source, nitrogen source and substrates) on lipase production was determined.

Effect of incubation time

Effect of incubation time on lipase production was investigated through shake flask fermentation that was carried out for 120hrs at 10°C, samples were collected after regular interval of 24hrsfor further lipase activity analysis.

Effect of pH and temperature

The lipase production from isolateHTB6, was observed at different temperatures 5, 10, 15, 20, 25, 30 and 35°C. Optimum pH for lipase production was also carried out at various pH (5, 7, 9), hence medium was adjusted to various pH by 1N HCl or 1N NaOH.

Effect of various substrates

Effect of different substrates or inducers on the production of lipase, for example, olive oil, mustard oil and Tween80 (1%),by adding these substrates in production medium, was studied at pH 9, temperature and agitation speed, at 10°C and 150 rpm respectively.

Effect of carbon sources

Effect of different carbon sources on lipase production was studied. Different carbon sources including; glucose, galactose and fructose were used as 1% w/v solution at pH and temperature of 9 and 10°C, respectively.

Effect of nitrogen sources

Effect of various nitrogen sources on lipase production was studied by providing different nitrogen sources like casein, peptone and yeast extract (2.5%) in production media. The production was carried out at pH 9, temperature 10°C, and at 150 rpm agitation speed.

Lipase assay and protein estimation

Samples of crude enzyme (lipase), were collected after regular interval of 24 hrs from all above mentioned types of fermentation for different parameters.

These crude lipase samples were used for lipase assay and protein estimation to determine the lipase activity. For lipase assay, method of (Lesuisse *et al.*,1993) was used to determine lipase activity. In this method, a chromogenic substrates *p*- nitropheny l laurate (*p*NPL) was used as substrate, lipase break substrate intolauric acid and *p*-nitrophenol. Enzyme activity is defined as one unit of enzyme is the amount of enzyme that can hydrolyzes 1 µl/ mol substrate in one minute. Protein estimation of all crude lipase samples was done by Lowry method (Lowry *et al.*, 1951). BSA (bovine serum albumin), was used as standard. The four types of the solutions including, solution.

A (Na_2CO_3, 1.0g, Na Ktartarate, 1.0g, NaOH, 0.4g), solution B ($CuSO_4$,0.5g), solution C (S.A,50% and S.B,50%) and solution D (Folin phenol, 1:1). All solutions were separately prepared in 100ml distilled water for the estimation of protein.

Results

Qualitative and quantitative analysis

Isolate HTB2 was found effective producer of lipase, on the basis of qualitative analysis. Among four bacterial isolates (HTB1, HTB2, LTB3, HTB6), zone of hydrolysis was observed around the colonies of HTB2 isolate at 10°C after 72hours of incubation (Fig. 1A).

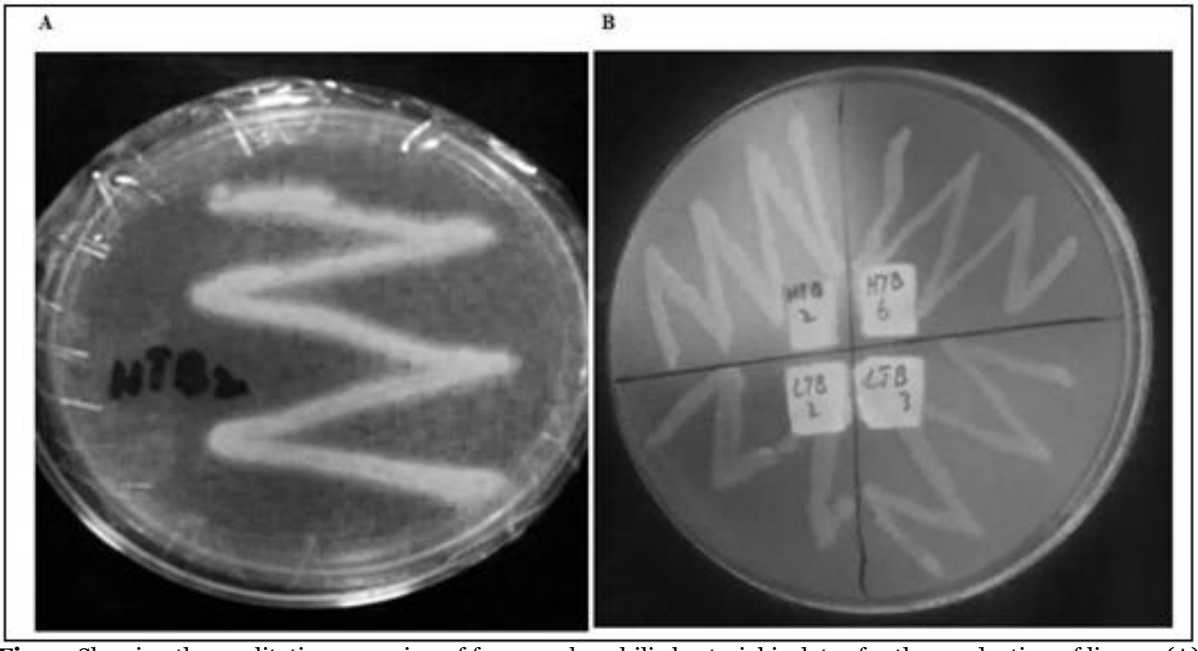

Fig. 1. Showing the qualitative screening of four psychrophilic bacterial isolates for the production of lipases (A) hydrolysis of Tween80 (B) Rhodamine B plate assay.

The lipase activity was further confirmed by growing the HTB2 isolate on Rhodamine B and olive oil containing agar media and visualizing the HTB2 isolate under UV transilluminator (Fig. 1B).Four isolates were further screened for lipase production in liquid medium. Isolate HTB2was also found potent for lipase production in liquid medium, as compared to other three (HTB1, LTB3, HTB6), bacterial isolates (Fig. 2).

Optimization of lipase production

For the maximum production of lipase various conditions (incubation period, pH, temperature, carbon source, nitrogen source and substrates), were optimized to produce lipase through shake flask fermentation.

Effect of incubation time

Four bacterial isolates (HTB1, HTB2, LTB3 and HTB6), were incubated for 120hours at10˚C to

analyze lipase production in liquid medium. Growth of four isolates was observed regularly intervals of 24hrs and samples from all four isolates were collected.

Lipase production in liquid medium was confirmed by lipase activity, determined through lipase assay. The maximum lipase production from (HTB2, LTB3, HTB6), was found after 72 hrsand from HTB1isolate, after 96 hours of incubation. Among four bacterial isolates, highest lipase production (specific activity, 3.85 U/mg) was found from HTB2 isolate, after 72hrs of incubation period (Fig. 2).

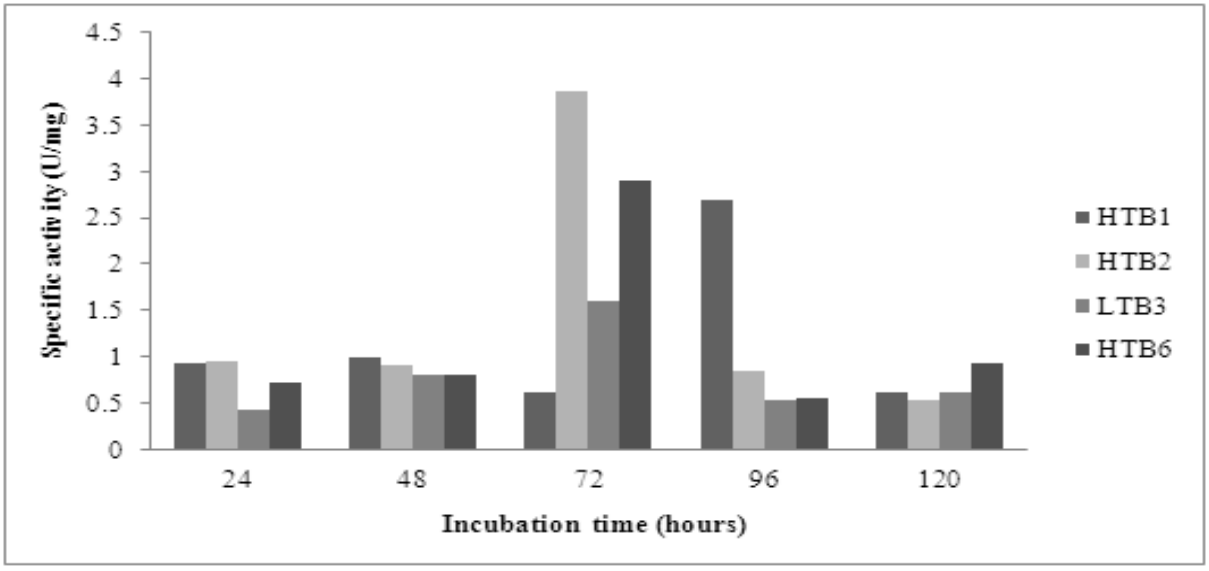

Fig. 2. Effect of incubation time on the production of lipases from bacterial isolates, HTB1, HTB2, LTB3 and HTB6.

Effect of pH and temperature: The effect of pH and temperature on the production of lipase from HTB2 isolate was analyzed. Maximum lipase specific activities (7.6 U/mg), were observed for pH 9 and (25.9 U/mg), for optimum temperature(10˚C), after 72hrs of incubation. Production of lipase (5.7, 16.9, 12.6, 11.11 and 12.1 U/mg), from isolateHTB2, was also found at other temperatures (5, 20, 25, 30 and 40°C), respectively (Fig. 3, 4).

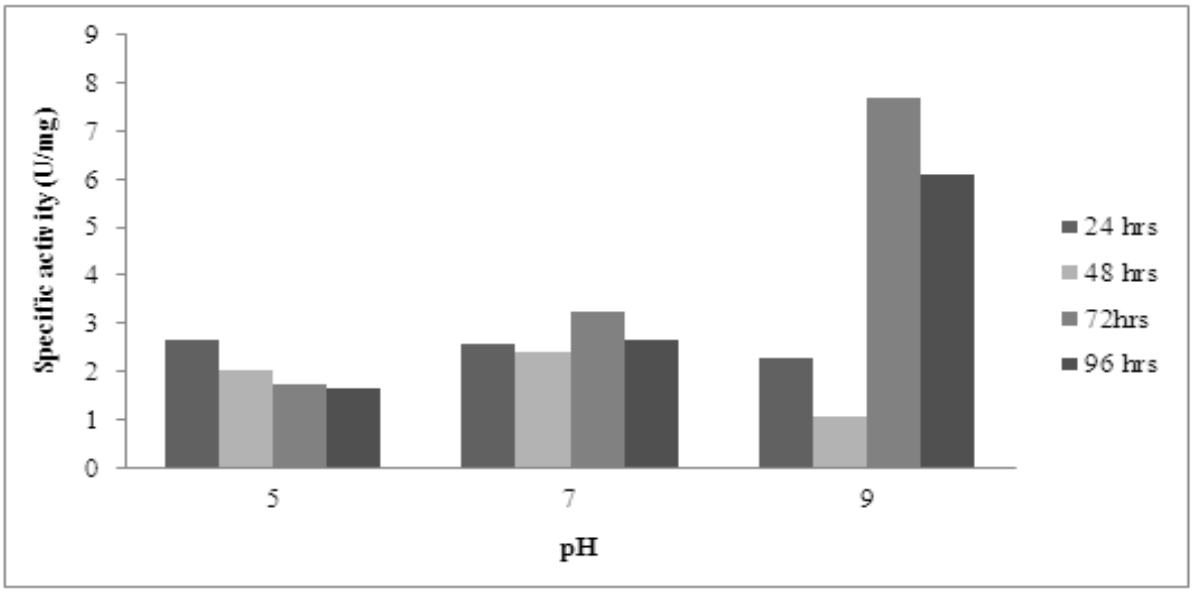

Fig. 3. Showing the production of lipase from isolate HTB2at various range of pH.

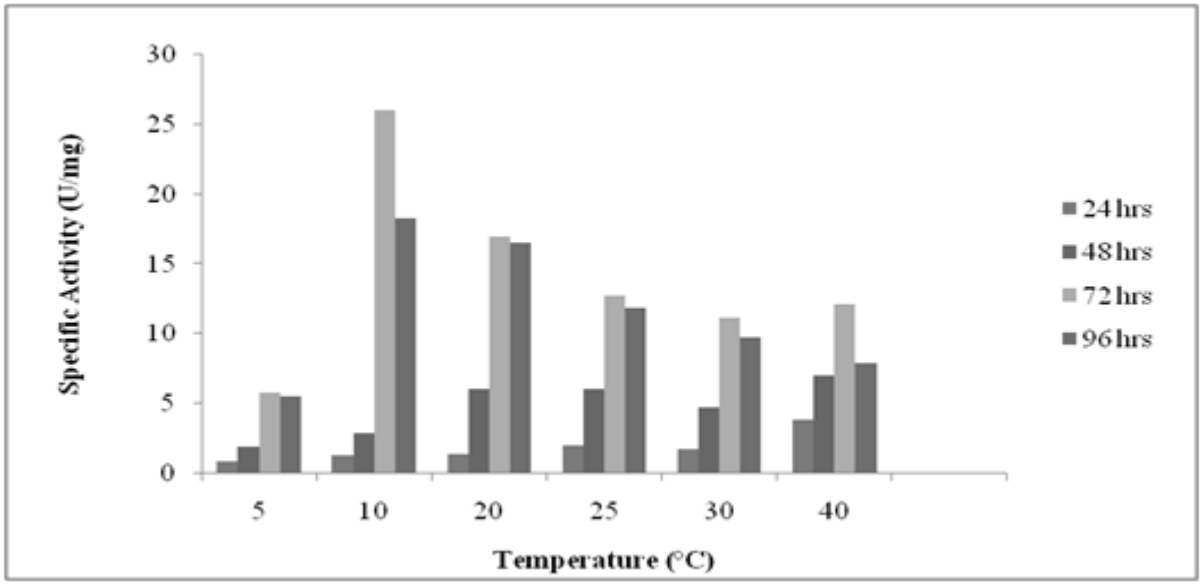

Fig. 4. Showing the production of lipase from isolate HTB2 at various range of temperature.

Effect of various substrates: Various substrates or inducers (olive oil, mustard oil, and Tween80 (1%v/v), were studied for lipase production. It was observed that maximum lipase production (20.8 U/mg) was achieved, when Tween80 was added in lipase production medium. Olive oil was also found effective for lipase production after 24, 48 and 72hrs of incubation (Fig. 5).

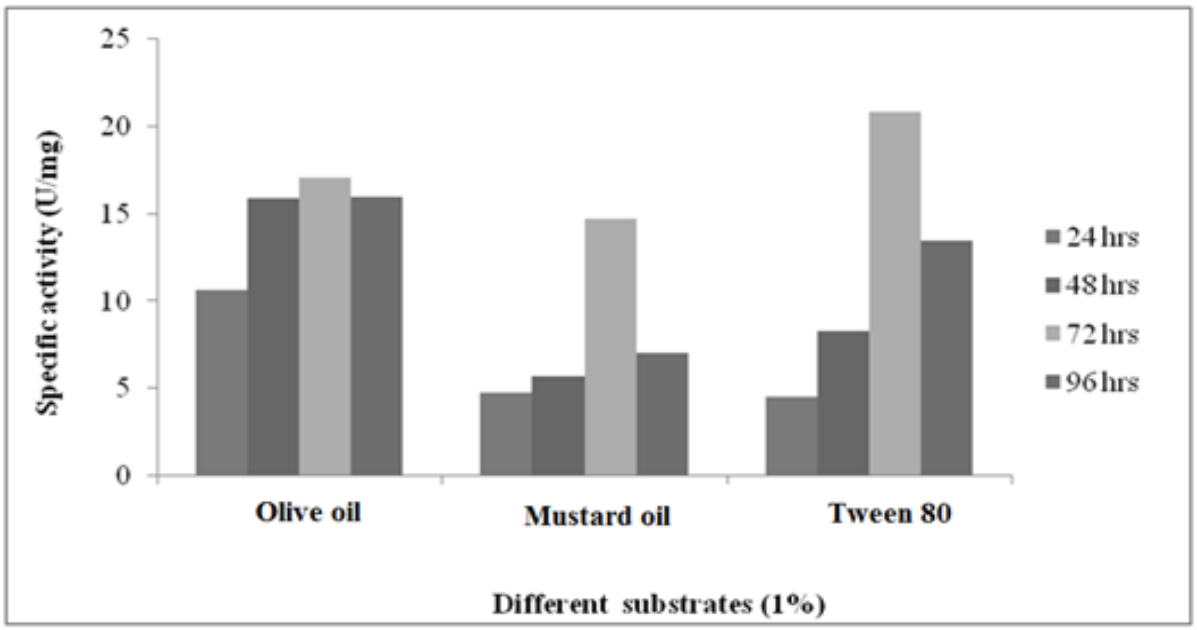

Fig. 5. Effect of different substrates on the production of lipase from isolateHTB2.

Effect of various carbon sources: Various carbon sources (2% w/v), were used to determine the maximum lipase production from HTB2 isolate. Glucose (specific activity, 19.4 U/mg) and galactose (10.8 U/mg), were found more effective carbon sources for lipase production as compared to fructose (Fig. 6).

Effect of nitrogen sources: Casein, peptone and yeast extract as nitrogen sources (2.5%) were used to analyze the production of lipase. It was observed that maximum lipase production (15.5 U/mg), was attained in the presence of casein as compared to peptone and yeast extract (Fig. 7).

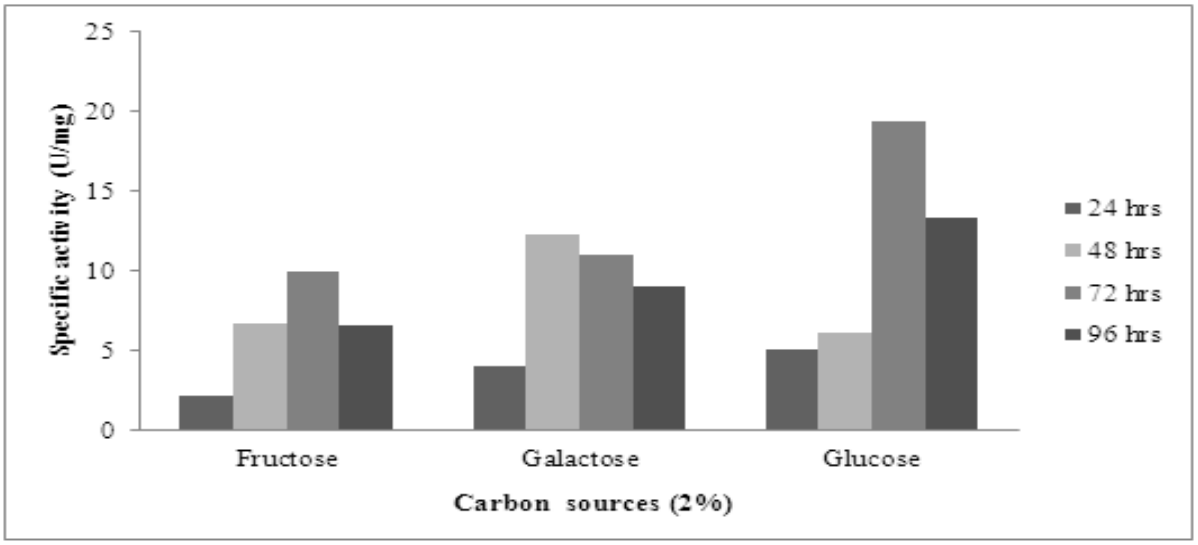

Fig. 6. Effect of various carbon sources on the production of lipase from isolateHTB2.

Discussion

Screening of bacterial isolates for lipase production

In present work, four bacterial isolates (HTB1, HTB2, LTB3 and HTB6), present in Applied, Environmental & Geomicrobiology Laboratory (Quaid-i-Azam University, Islamabad), were used for the production of lipases, above isolates were previously isolated from Batura glacier of Pakistan. These isolates (HTB1, HTB2, LTB3 and HTB6), were cultured on Tween80 agar plates at 10°C for 72hrs of incubation. Lipase producing isolates primarily were detected by producing clear zones around the bacterial colonies. Isolation and lipase production from different microbes through these methods were also reported by (Seinthi and Selvakumar, 2008).

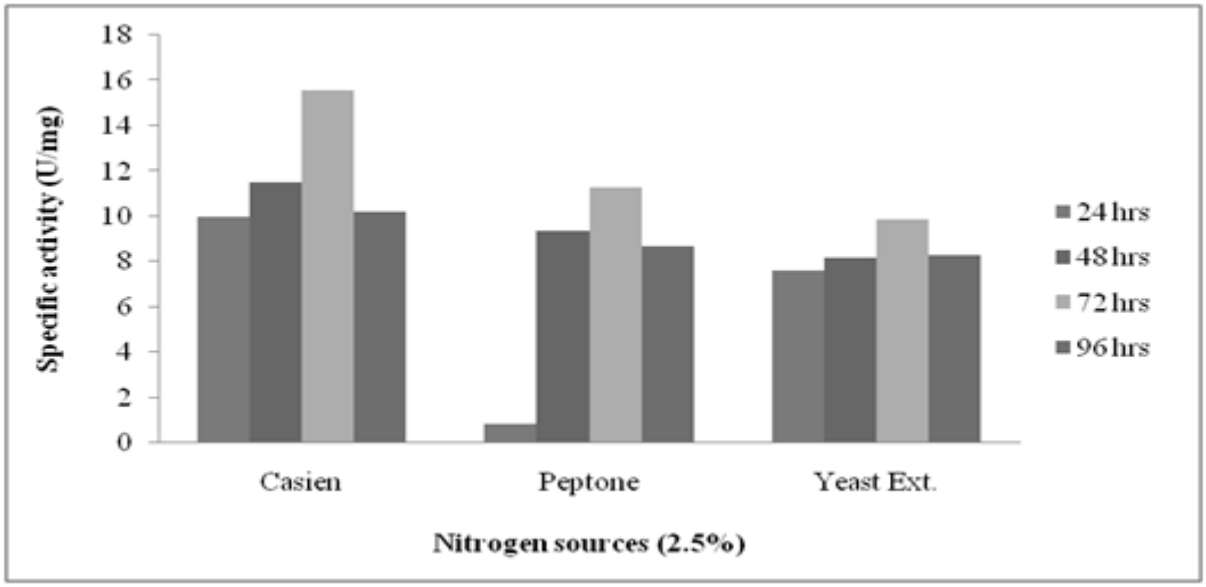

Fig. 7. Effects of various nitrogen sources on the production of lipase from isolate HTB2.

In present study, HTB2 isolate was further confirmed for lipase production through Rhodamine B test. However, sometime, false positive result are observed in case of Tween80, because, Tween 80 is easily hydrolyzed by esterase enzyme. This confusion has been removed by using fluorescence dye method (Rhodamine B).Olive oil in the presence of Rhodamine B, cause the development of fluorescence with the free fatty acids (Thomson *et al.*, 1999). Therefore, orange fluorescence has been observed, in

the case of lipase positive isolates. Bacteria especially *Bacillus* strains, capable of lipase producing, were identified on Rhodamine B and olive oil agar medium (Sebdani *et al*., 2011).

Effect of different substrates on the lipase production

Lipids or lipids related substrates as well as a variety of fatty acids can induce lipase production by microorganisms. Therefore, selection of production medium, with specific composition, to support microbial growth as well as secretion of microbial enzymes, has been considered very important aspect in enzymes production. Mostly, microbial lipases are extracellular and are secreted the culture medium through cell membrane (Rajendran *et al*., 2008; Wang *et al*., 2008). Generally, various culture media can be prepared for the optimization purposes by varying one or two components in the composition of relevant medium. Different types of production media with variety of composition has been tested for lipase production from different organismse. g. *Pseudomonas fragi, Aspergilluss wantii, Staphylococcus* spp. and *Mucor* species. Generally, a higher yield of enzymes has been achieved by optimizing culture media (Nashif and Nelson, 1953; Chander *et al*., 1980).

In current study, for the production of lipase, four isolates (HTB1, HTB2, LTB3 and HTB6), were grown in minimal salt medium containing; KH_2PO_4, NaCl, K_2HPO_4, $CaCl_2$, $MgSO_4$and with following substrates (Tween80, mustard oil and olive oil).Maximum lipase production from HTB2 isolate in shake flask fermentation, was observed in the presence of (Tween80), as substrate in medium. Maximum lipase activity from various cold-loving bacteria including *Psychrobacter*sp.WP37, was attained in the presence of Tween80 or Tween20, substrates in the production media (Zheng *et al*.,2004). It was also identified that Tween80 and tributyrin were the best inducers for the production of lipase by *Pseudomonas* sp. strain B-119 (Choo *et al*., 1998).

Apart from lipids substrates, other factors like carbon, nitrogen sources, pH, temperature, agitation and oxygen availability also can effects on the production of lipase (Nashif and Nelson, 1953; Chander *et al*., 1980). In the current study, various production parameters like effect of incubation time, temperature, pH, carbon source, nitrogen source and different substrates were optimized for maximum lipase production. Isolate HTB2 produced maximum lipases after 72hrs of incubation. It indicates that maximum yield of the lipase enzyme occurred during late stages of the growth. Similar results were observed and reported (Pogaku *et al*., 2010). Maximum activity of psychrophilic alkaline lipase by *Pseudomonas* was obtained in late growth phase (Kiran *et al*., 2008). Lipase activity was found maximum at pH 7, after 72hrs of incubation from *Bacillus* spp. (Sebdani *et al*., 2011).

Effect of various range of pH on the lipase production

pH of the medium has a vital role in the lipase production. In present study, growth and lipase production from HTB2 isolate was carried out on wide range of pH, maximum lipase yield was obtained at pH 9, from HTB2 isolate. Lipase production from current HTB2isolate,may be valuable as detergents, because higher activity of lipase was observed at basic pH9.Many species of *Bacillus*, *Pseudomonas*, *Acinetobacter*, *Burkhulderia*, have been studied and reported for maximum lipase production at neutral pH, these differences in optimum pH may be due to the variations in origin of the microorganisms and their genetic potential(Sugihara *et al*., 1991;Barbaro*et al*.,2001;Rathi*et al*.,2001).However, lipase production from *Acinetobacter calcoaceticus*, was studied and reported at pH 7, while 10°C was the optimum temperature range (Pratuang dejkul and Dharmsthiti, 2000). While, lipase production was also reported in medium of pH 6.5, at 10°C, by *A. calcoaceticus* (Mahler *et al*., 2000).

Effect of various range of temperature on the lipase production

The effect of temperature on the lipase production by HTB2, was carried out by incubating the isolate at temperatures 5, 10, 20, 25, 30 and 40°C, at 120rpm.

Optimum lipase production fromisolate HTB2 was achieved at 10°C and at pH 9. A decrease in lipase production was observed by changing temperature at different degrees. Studies related to the role of temperature on production of various enzymes have been conducted by different researchers (Margesin *et al.*, 2003). However, it had observed that enzymes from psychotropic *Pseudomonas*, were active only at 25°C, while, no enzymatic activity was found at higher temperature ranges. Temperature affects the rate of chemical and biological reactions in microbial cells and ultimately causes variations in metabolic pathways (Ray *et al.*, 1999; Ruberto *et al.*, 2005).

Conclusion

On the basis of above findings, we conclude that isolate HTB2 has the potential for lipase production. It was observed that pH9, temperature 10°C, Tween80 as substrate and 72hrs of incubation period, are favorable conditions for the growth of HTB2 isolate as well as for the relevant lipase production. Isolate HTB2and its enzymes may be industrially important on the basis of above findings.

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