Production and optimization of Lipase Enzyme by Thermo-alkalophilic *Bacillus* sp.

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ABSTRACT

Microbial lipases catalyzing the hydrolysis and synthesis of esters from glycerol and fatty acids are commercially important in food, dairy, detergent and leather industries. In the present study 15 thermoalkalophilic bacterial strains isolated from a compost soil sample were screened for lipase production in beef extract nitrate medium containing 1% tributyrin. The strain 8C giving maximum lipolytic activity was morphologically and biochemically identified as *Bacillus* sp. Lipase production under static condition was maximum after 7 days of incubation at 50˚C and pH 9.0 and when sesame oil was used as a substrate. Supplementation of ribose or raffinose and ammonium sulphate (1%) as sole sources of carbon and nitrogen also stimulated lipase production over control. Of the different thiols tested, methionine greatly increased lipase production whereas $FeCl_3$ (50mM) found to be most suitable metal salt for optimal lipase production. Under optimized conditions, lipase production by thermoalkalophilic *Bacillus* sp 8C was increased by 6.36 fold compared to unoptimized conditions.

KEY WORDS: Thermoalkalophilic, *Bacillus*, lipase, methionine, sesame oil.

INTRODUCTION

Lipases (triglycerol hydrolases E.C.3.1.1.3) are the hydrolytic enzymes belonging to serine hydrolase family and catalyze conversion and synthesis of esters from glycerol and fatty acids. Lipolytic reactions are highly chemo-, regio- and or enantioselective and operate at the interface of oil and water (Nguyen *et al*., 2010). High quality and low energy consumption of lipase catalyzed reactions make them superior over conventional steam splitting methods operating at high temperature and pressure (Treichei *et al*., 2010). These characteristics of lipases increased their demand in different industries including food and dairy, detergent, cosmetics, leather, paper and pulp, biodiesel and pharmaceutical (Hasan *et al*., 2007).

Thermophilic lipases are of special interest for industrial applications due to their stability and high activity at higher temperatures and being stable in presence of chemicals (Messias *et al*., 2009, Uttatrei *et al*., 2010). Lipases being third largest selling group of enzymes with more than 1000 tonnes of annual production and approaching to a billion dollar business. Lipases active at alkaline pH are of great importance in detergent industry where these enzymes are used for removal of fat stains and dirt (Bora *et al*., 2003). The use of alkaline lipases in detergent formulation substantially acts as a substitute for the phosphate builders in chemical detergent which are considered as major pollutant from detergent industry (Jellouli *et al*., 2011). The extremozymes working at high temperatures and alkaline pH decrease the need of added steps in the process, reduce the cost and increase the process

efficiency (Myers *et al*., 2000). Lipases from different thermoalkalophilic *Bacillus* sp including *Bacillus stearothermophilus* (Kim *et al*., 2000) *Bacillus licheniformis* (Sangeetha *et al*., 2010) and *Bacillus thermocatenulatus* BTL-2 (Schmidt *et al.*, 2004) were reported earlier. The research on identification and isolation of new lipase produced in microorganisms has been intensified in recent years (Hasan *et al*., 2006, Shu *et al*., 2010, Treicheil *et al*., 2010).

Enzyme production process is closely controlled in microorganisms and for improving its productivity. It is necessary to mitigate these controlling factors on production medium. This can be achieved by manipulating growth conditions to optimal level that generally improves the lipase productivity as evidenced in earlier studies (Gupta *et al*., 2007, Treichel *et al*., 2010, Rabbani *et al*., 2013). The most important parameters that critically affect the lipase production are carbon and nitrogen sources (Kumari *et al*., 2009 and Wang *et al*., 2008). However, optimization of other nutritional and growth parameters affecting lipase production will be the added advantage for improvement studies. Knowing suitable conditions required for higher lipase production will reduce the production cost, minimize production time and other critical conditions required for production process. In view of this the present research work deals with the isolation of lipase producing thermoalkalophilic *Bacillus* sp from compost soil sample and optimization of conditions required for lipase production.

MATERIALS AND METHODS

Sample collection and isolation of lipolytic bacteria

A compost soil sample was collected from Trikut, Nanded M.S. (India). One gram of air dried soil was suspended in 100 ml beef extract nitrate broth (peptone 3%, beef extract 5% and KNO_3 0.1%) supplemented with 1% tween-80 for enrichment of lipolytic bacteria. The pH of medium was adjusted to 9.0 by using 0.1 N NaOH. The flask was kept for incubation at 50˚C under static conditions for 7 days. After incubation, 0.1 ml of enriched broth sample was spread on the surface of sterile beef extract nitrate agar plates containing 1% Tween-80 in triplicates. The plates were incubated at 50˚C for 7 days and observed for colonies showing zone of lipolysis. The lipolytic activity of selected isolates were further confirmed on tributyrin agar plates (peptone 3%, beef extract 5%, KNO_3 0.1% and tributyrin 1%) by spot inoculation and incubating the plates under previously mentioned conditions.

Quantitative determination of enzyme activity

The selected isolates were grown in tributyrin broth (peptone 3%, beef extract 5%, KNO_3 0.1% and tributyrin 1%) with pH 9.0 as mentioned before. After incubation, the culture broths were centrifuged at 10,000 rpm for 20min at 4˚C and supernatant was used as a source of crude enzyme and used for estimating lipase activity

Lipase assay

The assay was performed by using the method described by Selvam *et al.*, (2011), on the basis of hydrolysis of olive oil. To the reaction mixture containing 1ml of Tris- HCl buffer of pH 9.0, 2.5 ml of deionized water and 3ml of olive oil emulsion (10% gum Arabic and 5%

olive oil in deionized water), 1ml of culture supernatant for test and 1ml of deionized water for blank were added. The reaction mixture was mixed thoroughly by swirling and incubated at 50˚C for 30 min. After incubation, enzyme substrate reaction was terminated by addition of 3ml of 95% ethanol and mixed by swirling. The amount of fatty acid liberated due to lipase activity was estimated by titrating the contents of assay mixture against 0.05 M NaOH using thymolophthalein as an indicator. The end point observed was from colorless to light blue.

One unit of lipase was defined as the amount of enzyme required to release 1 µmole of fatty acid per ml under assay conditions. The enzyme activity is expressed as Eu/ml.

Identification of lipase producing bacterial isolate:

The potential lipase producer strain 8C was selected for further studies and identified on the basis of morphological traits and biochemical pattern as per the standard criteria given in *Bergy's manual of systematic bacteriology* (9^{th} edn).

Optimization of process variables:

Effect of incubation period:

8C was inoculated in beef extract-nitrate broth of pH 9.0 containing 1% tributyrin as a substrate and incubated at 50˚C for 11days. Five ml culture media was removed periodically at an interval of 48hr and used for lipase assay.

Effect of initial pH of medium:

Active culture of 8C was inoculated in beef extract-nitrate media containing 1% tributyrin. pH of media were adjusted to different pH (7, 8, 9, 10, 11 and 12) with the help of 0.1N NaOH on a pH meter. The media were incubated at 50˚C for 7 days. Enzyme units were calculated by performing the lipase assay.

Effect of carbon sources:

To study the effect of carbon sources on lipase production by 8C, 11 different carbon sources such as xylose, fructose, dextrin, mannitol, galactose, sucrose, maltose, raffinose, ribose, lactose, and gum acacia at 1% concentration were added individually to the beef extract-nitrate media. The broths were incubated at 50˚C for 7 days at static condition. After incubation, broth was centrifuged and used as crude enzyme for lipase assay as mentioned earlier.

Effect of nitrogen sources:

For the selection of suitable nitrogen source for lipase production by 8C different organic and inorganic nitrogen sources such as yeast extract, pyruvate, bile salts, glycerol, ammonium sulphate and arginine were screened. By supplementing those at 1% concentration in beef extract-nitrate media of pH 9.0, the active culture was inoculated and incubated at 50˚C for 7 days and assayed to determine the enzyme units.

Effect of metal salts:

The effect of different metal salts (NaCl, KCl, $CaCl_2$, $MgCl_2$, $ZnCl_2$, NH_4Cl and $FeCl_3$ 1%) on lipase production was assessed by inoculating active culture of 8C in beef extract nitrate broth containing ribose and ammonium sulphate. The flasks were incubated at 50˚C for 7 days at static condition and used for lipase assay as mentioned before.

Effect of substrates:

Lipase production was accelerated by incorporation of suitable lipidic substrate. Therefore, effect of different lipidic substrates on lipase production by 8C was screened. Different substrates such as castor oil, mustard oil, eucalyptus oil, soya bean oil, coconut oil, sesame oil and clove oil at 1% concentration were supplemented individually in beef extract-nitrate media of pH 9.0. The flasks were incubated at 50˚C for 7 days at static conditions. After incubation enzyme activity was determined as mentioned above.

Effect of thiols:

To determine the effect of thiols on lipase production, different thiol containing compounds (methionine, dithiothreitol, β-mercaptoethanol, cysteine, sodium sulphite, and thiourea) were added individually to beef extract-nitrate medium at 50mM concentration. After 7 days of incubation at 50˚C under static conditions, the enzyme activity was determined as stated earlier.

RESULTS AND DISCUSSION

Isolation and identification of lipolytic bacteria

In the present study, 15 bacterial isolates obtained from compost soil were tested for lipase production potential. Out of the fifteen isolates, five (1C, 2C, 3C, 8C, 15C) showed good lipolytic activity on tributyrin agar and broth (Table-1). The isolate 8C showing higher activities (28mm, 55Eu/ml), was selected as a potent lipase producer strain for further studies.

The isolate 8C was characterized morphologically and biochemically as per the criteria given in *Bergy's manual of systematic bacteriology* (9^{th} edn). The isolate was gram positive, non-motile, catalase positive rod, with ability to ferment lactose, mannose, maltose, fructose and ribose while not utilizing salicin, mannitol and arabinose. The isolate was positive vogues-proskeur test, hydrolyzed starch and showed citrate utilization capacity.

Based on these characteristics the isolate 8C was identified as *Bacillus* sp and used further for optimization studies. Thermophilic *Bacillus* sp have previously shown the lipase production at 50-60˚C. However, the reports on lipase production by *Bacillus* sp under thermoalkalophilic conditions are scanty.

Optimization of process variables

Effect of incubation period:

Lipase production by *Bacillus* sp started after 72 hours at 50˚C and increased thereafter with the increase in incubation time. The maximum enzyme activity (125Eu/ml) was obtained by 7 days of incubation and then decreased gradually from 8th day to 11th day of incubation (fig.1). The high incubation time required for enzyme production might be due to the growth of microorganism at high temperature (50^{o}C) under alkaline (pH-9.0) and static conditions. Under these conditions the organism may have high lag period before its exponential growth. The gradual decrease in enzyme production after 7 days might be due to the accumulation of toxic end products of metabolism or exhaustion of available nutrients in medium. Mukesh *et al.,* (2012) have found that *Bacillus* sp gave the maximum yield of (1.2Eu/ml) lipase after 72 hours of incubation whereas Sharma *et al.,* (2002) showed the lipase production was highest after 12 hours of incubation.

Effect of initial pH of medium:

Changes in the initial pH of production medium may induce the production of new metabolites that affect the biosynthesis of desired products. Hence, we examined the effect of pH on lipase production by *Bacillus* sp and the results are summarized in fig 2. pH 9.0 was found to be the optimum for enzyme production (125 Eu/ml). Most of the published literature responds pH 8.0 as optimum pH for lipase production in thermophilic *Bacillus p* (Hasan *et al*, 2001, Kim *et al.,* 1994, Mohammad *et al,* 2014). Increased cultivation of the organism at an unfavorable pH, reduce the growth rate of organism and also has marked effects on the level of enzyme production (Bhosale, *et al*., 2012) as evidence by drastically reduced enzyme production at all other pH.

Effect of carbon sources:

Lipase production by thermophilic *Bacillus sp* was determined by measuring the enzyme activity when the strain was growing on different carbon sources. The highest lipase production was obtained with 1% ribose and raffinose (165Eu/ml) followed by gum acacia (140Eu/ml) and lactose (115Eu/ml). Dextrin, sucrose, maltose most of the carbon sources except xylose could stimulate lipase activity as they supported the growth of Thermophilic *Bacillus* sp (fig. 3). These results are in agreement with those reported by Sztajer *et al*., (2001). Ability of an organism to drive a metabolic reaction and grow in presence of specific carbon source is dependent on typical enzymatic machinery present in the cell. In the present study *Bacillus* grew well in presence of ribose and raffinose. Gum acacia, lactose and mannitol also supported for maximum enzyme production. However, lowest enzyme units recorded in the presence of xylose indicating that xylose is not suitable for lipase production.

Effect of nitrogen sources:

The addition effect of different nitrogen sources on enzyme production is shown in fig 4. The maximum production of lipase was observed with 1% ammonium sulphate (140Eu/ml) compared to glycerol (35Eu/ml) and pyruvate (50Eu/ml). Lipase production was also noticeable in presence of peptone, arginine and bile salts. Ammonium sulphate is highly soluble in water. Hence, readily available nitrogen source for microbial growth. Further, its

high ionic strength releases ammonium ions in aqueous media that can be easily incorporated as part of different amino acids and hence plays important role in protein synthesis process.

Effect of metal salts:

The effect of different metal salts added to the production medium on lipase production is presented in fig 5. Salts of ferric ions ($FeCl_3$) highly stimulated (300Eu/ml) the lipase production by *Bacillus* sp whereas, enzyme production was considerably high when $ZnCl_2$ and $MgCl_2$ were supplemented in the medium. Presence of KCl did not affected enzyme production and the production increase was marginal in presence of $CaCl_2$, NaCl and $MnCl_2$.

Effect of different substrates:

Supplementation of different lipidic substrate in production medium greatly affected the lipase production capacity of *Bacillus* sp. The enzyme production was unchanged when tributyrin in production media was replaced with castor oil, eucalyptus oil, soya bean oil and coconut oil as indicated in fig 6. However, production was highly stimulated when sesame oil was used as a substrate (165Eu/ml) and was increased considerably in presence of mustard oil (110Eu/ml) and clove oil (77Eu/ml). Lipase is an extra-cellular inducible enzyme whose production is only detectable in presence of suitable substrate. Further, the growth and enzyme production capacity of an organism while growing on different substrate depend on its substrate utilization efficiency that ultimately depends on the presence of specific metabolic route required for metabolism of substrate.

Effect of thiols:

In present study we also examined the effects of different thiol compounds on lipase production by *Bacillus* sp. Interestingly, supplementation of different thiols in production medium either did not affected or stimulated the lipase production (fig. 7). The effect was not detectable in the presence of dithiothreitol, β-mercaptoethanol and thiourea. However, addition of sodium sulphite, cysteine, sodium thiosulphate and methionine gradually increased the lipase production. Methionine and cysteine are sulphur containing amino acids that plays an important role in stabilizing tertiary structure of protein molecules as well as they act as good nitrogen source for microbial growth. Enhanced lipase production in presence of Methionine and cysteine indicated good growth of *Bacillus* sp that might have supported higher production of enzyme.

The enzyme activity determined in tributyrin beef extract-nitrate broth before optimization was 55Eu/ml and the production found in optimized medium (peptone 3%, beef extract 5% , KNO_3 0.1%, ribose 1%, ammonium sulphate 1%, sesame oil 1%, $FeCl_3.7H_2O$ 1% and methionine 50mM) was 350Eu/ml indicating 6.36 fold increase in lipase production by thermoalkalophilic *Bacillus* sp 8C.

Conclusion

In the present study, a thermoalkalophilic lipase producer strain of *Bacillus* sp 8C has been optimized for improved lipase production. The parameters and conditions used for lipase

optimization studies are simple and easy to manage. This study first time demonstrated the role of raffinose and ribose sugars and thiols like methionine in improvement of thermoalkalophilic *Bacillus* produced lipase yield.

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Figure legends:

Fig.1 Effect of incubation period on lipase production by *Bacillus* sp 8C. Each value represents mean of ± percentage error.

Fig. 1 Effect of pH on lipase production by *Bacillus* sp 8C. Each value represents mean of ± percentage error.

Fig. 2 Effect of different carbon sources on lipase production by *Bacillus* sp 8C. Each value represents mean of ± percentage error.

Fig. 3 Effect of different nitrogen sources on lipase production by *Bacillus* sp 8C. Each value represents mean of ± percentage error.

Fig. 4 Effect of metal salts on lipase production by *Bacillus* sp 8C. Each value represents mean of ± percentage error.

Fig. 5 Effect of different substrates on lipase production by *Bacillus* sp 8C. Each value represents mean of ± percentage error.

Fig. 7 Effect of different thiols on lipase production by *Bacillus* sp 8C. Each value represents mean of ± percentage error.

Figure:

Fig. 1

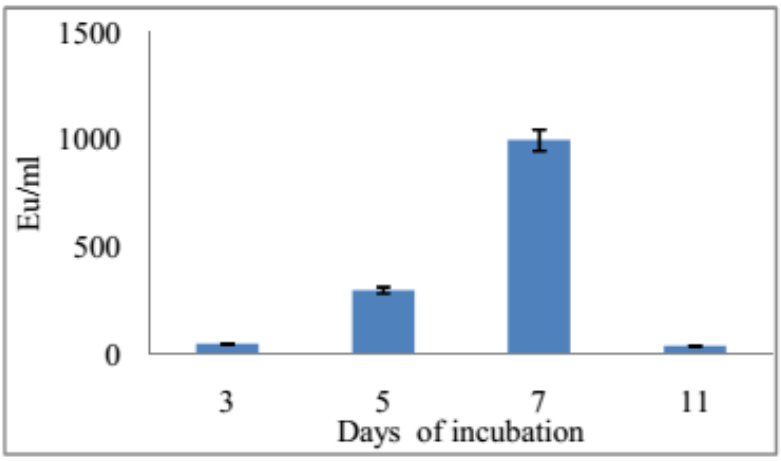


Fig.2

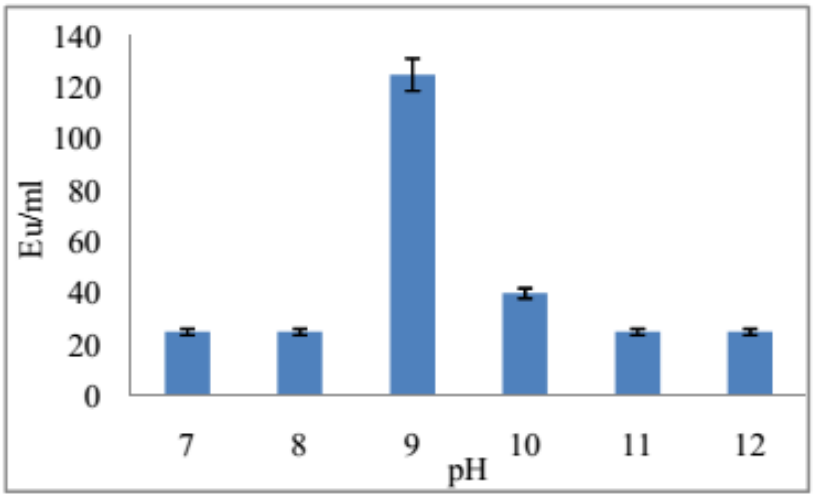


Fig. 3

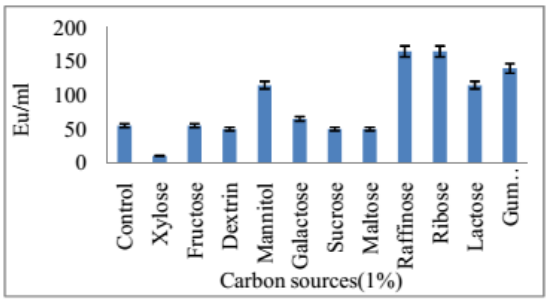


Fig. 4

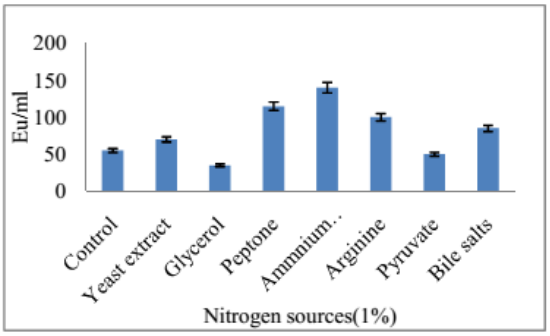


Fig.5

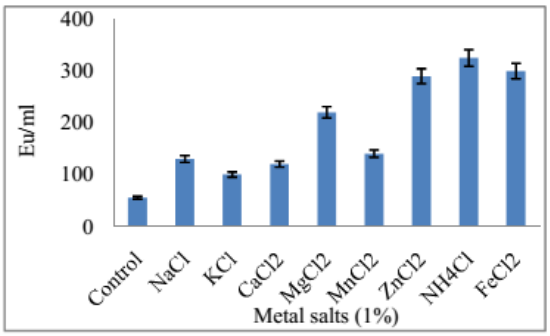


Fig. 6

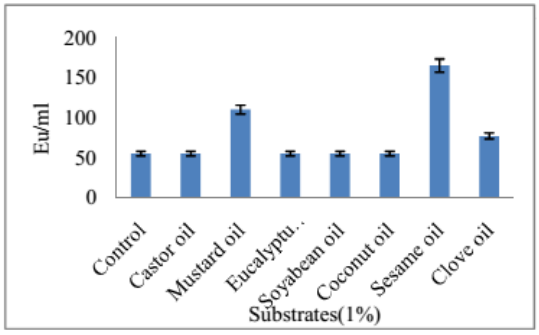
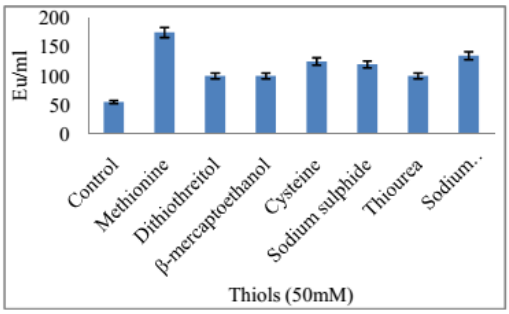


Fig. 7



Tables:

Isolates	Lipase activity (Eu/ml)	Zone of lipolysis (mm)
1C	11.80	28
2C	7.80	15
3C	6.49	12
8C	4.13	10
15C	2.95	11

Table 1: Qualitative and quantitative screening of isolates showing lipolytic activity