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Original Research Article

Optimization of cultural parameters for lipase production by *Bacillus subtilis* Y-IVI

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A B S T R A C T

A bacterial strain isolated from oil contaminated soil and identified as *Bacillus subtilis* Y-IVI based on 16S rDNA analysis was screened for lipase production on tributyrin agar. Maximum lipase production was observed at 48 h of growth. Starch was preferred over other carbon sources for optimum production of lipase at a concentration of 1.5%. Peptone was found to be an ideal nitrogen source for production at a concentration of 1.5%. Among the natural oils, olive oil was able to induce more lipase production. Basal medium supplemented with tween 80 enhanced lipase production to a significant level.

K e y w o r d s

Bacillus subtilis Y-IVI *sp*., Lipase, Tributyrin agar, Starch, Peptone, Tween 80

Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1. 1.3) are members of the family of hydrolases that act on carboxylic ester bonds. Their physiological role is to hydrolyze triglycerides to diglycerides, monoglycerides, fatty acids and glycerol. The interest in lipases arises due to their ability to catalyze the hydrolysis as well as synthesis of fatty acid esters. Lipases act on a variety of substrates including natural oils, synthetic triglycerides and esters of fatty acids. Many lipases are resistant to solvents hence they find use in the synthesis of chiral drugs (Reddy and Pallavi, 2012). They show varied substrate and positional specificities and hence employed in various industries like leather and detergent. Lipases have become an integral part of the modern food industry. The use of enzymes to improve the traditional chemical processes of food manufacture has been developed in the recent past. Yoneda *et al.* (1996) have patented a process on *Pseudomonas* lipase, which was claimed to be useful in food processing and oil manufacture. Alcoholysis of cod liver oil for the production of omega-3 polyunsaturated fatty acids was investigated by using Pseudomonas lipase (Zuyi and Ward, 1993). Lipases have also been used for the degradation of waste water contaminants such as olive oil from oil mills (Vitolo *et al.*, 1998).

Lipases are produced by a wide range of microorganisms namely, bacteria, fungi, archaea and eukaryote, as well as by animals and plants. Nevertheless, for commercial purposes microbial lipases are preferred (Reddy and Pallavi, 2012). Production of lipases by an organism is influenced by several extrinsic and intrinsic factors. During our investigations, a bacterial strain *Bacillus subtilis* Y-IVI isolated from oil contaminated soil was found to be efficient lipase producer. In this regard, an effort was made to optimize the cultural parameters for maximum production of lipase. Results pertaining to the influence of different carbon and nitrogen sources, oils and surfactants and their optimization for maximum production of lipase are presented in this paper.

Materials and Methods

Isolation

A large number of soil samples contaminated with different edible oils were collected aseptically and isolation of lipase producing bacteria was made by spread plate method using serial dilution on nutrient agar medium supplemented with 1% olive oil as substrate. Colonies showing hydrolytic zones in the form of halos around the colonies were picked up and subcultured.

Screening

The primary screening of isolated bacterial strains was made by the method suggested by Limpon *et al*. (2006). The lipolytic activity and lipolytic potential (R/r) of the isolated strains was assessed using tributyrin agar medium and spirit blue agar medium.

Lipase assay

The lipase activity in the culture filterate was assayed by titrimetry (Venkateshwarlu and Reddy, 1993). The reaction mixture containing 2ml of enzyme, 5ml of citrate phosphate buffer (pH 8.0), 2 ml of triacetin was incubated at 37^{o}C for 3 hours. At the end of incubation period the reaction was terminated by adding 10 ml of ethanol and the mixture was titrated against 0.05M NaOH using phenolphthalein indicator. The activity of enzyme was expressed in terms of enzyme units. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1µmol of equivalent fatty acid (ml/min) under the standard assay conditions**.**

Statistical analysis

The data obtained was subjected to relevant statistical analysis using Statistical Package for the Social Sciences (SPSS) 12.0 Software version.

Optimization of cultural parameters for lipase production

The best formulated production medium was selected to determine the ideal parameters for the optimum production of lipase. The optimization of medium components was carried out at the optimum pH 7 and temperature 30^{o}C by substituting the respective components present in the production medium.

Carbon sources

The effect of carbon sources on lipase production was investigated by using different carbon sources namely glucose, sucrose, maltose, lactose. They were tested individually by substituting the starch present in the basal formulated production medium at the concentration 1.5g/l. Later, the maximum enzyme inducing carbon source was further optimized for concentrations (1%, 1.5% and 2%).

Nitrogen sources

To test the effect of nitrogen sources on lipase production different organic nitrogen sources viz., beef extract, yeast extract, proteose peptone, tryptone and two inorganic nitrogen sources namely ammonium sulphate and potassium nitrate were used. They were individually tested by replacing the peptone present in the basal formulated production medium at the concentration of 1.0%. Later, the maximum enzyme inducing nitrogen source was further optimized by varying its concentrations (1%, 1.5% and 2%).

Oil source and surfactants

The lipase production was enhanced by incorporation of different lipid sources namely olive oil, coconut oil, groundnut oil, triacetin, tributyrin and surfactants (Tween 20, Tween 40, Triton –X) in the optimized medium. They were tested individually at the fraction of 2% in the medium.

Results and Discussion

The native bacterial strain (LP5) under investigation was isolated from oil contaminated soil and identified on basis of 16S rDNA sequence as *Bacillus subtilis* Y-IVI (Accession number: KJ872591).

Production of lipase on ideal medium

Studies were conducted to determine the trends of growth and lipase production. Table 1 shows result of the bacterial growth (OD at 540nm) and lipase production at regular time intervals of 24, 48 and 72 h on the ideal medium. It is evident from the table that lipase production began in late logarithmic phase (after 24h) and reached maximum production after 48 h of incubation. However, the enzyme production decreased after 72 h.

Optimization of cultural conditions

Optimization of various parameters is one of the most important approaches used for achieving the over production of enzymes in large quantities to meet industrial demands (Tanyildizi *et al.*, 2005). A variety of factors such as pH, temperature, duration of incubation, carbon sources and nitrogen sources, oil sources acting as inducers, surfactants and agitation are known to affect the production of lipases. Apart from individual factors, interactions of determinative factors will also have a significant influence on the production of the enzyme (Rahman *et al.*, 2006). In an effort to optimize the conditions for maximum production of enzyme, the effect of cultural conditions on lipase production by the test isolate was studied and the results are presented in Tables 2–7.

Carbon sources

Results pertaining to studies on the influence of different carbon sources are presented in Table 2. A critical analysis of Table 2 reveals that different carbon sources employed varied for the production of lipase. Among the different carbon sources tested, maximum lipase production was observed in presence of starch, and minimum with maltose. The potential of carbon sources on lipase production are in the following order: starch > sucrose > dextrose > lactose > maltose. The influence of different carbon sources on production of lipase was found to be significant ($p<0.001$, $p<0.0001$) by two-way ANOVA at 5% levels.

Similar to present investigations, Immanuel *et al.* (2008) reported that the medium containing starch was more favourable for lipase production than other carbon sources by *Serratia rubidaea*. Studies of Nishio *et al.* (1987) supported the earlier observations

of lipase production by *Pseudomonas fragi* 2239B. Enhancement of lipase production by *Bacillus circulans* with starch was reported by Sztajer and Malszewska (1988). Similarly Rahman *et al.* (2006) demonstrated that lipase production in *P. aeruginosa* YS-7 was quite low when glucose and glycerol were employed as the sole carbon sources.

In view of the demonstrated fact that starch supports the high levels of lipase production, an attempt was made to optimize the starch concentration in the medium for the maximum production of lipase and the results are presented in Table 3. It is evident from the table that increases in the concentration of starch till 1.5% had increased the lipase production, however, on further increase production decreased. The maximum production was observed at 1.5% of starch concentration with the growth OD of 1.10.

Nitrogen sources

Many researchers studied the influence of various nitrogen sources on the production of lipases (Immanuel *et al.,* 2008). Nitrogen requirement for optimal lipase production is species dependent. Results with regard to influence of nitrogen sources on lipase production are presented in Table 4. A critical analysis of the results reveals that a significant amount of lipase production (9.50 EU/ml) was observed in presence of peptone. Ammonium sulphate was found to be a poor source for lipase production. Influence of nitrogen sources on lipase production was in the order of peptone > tryptone > proteose peptone > beef extract > yeast extract > KNO_3 > $(NH_4)_2SO_4$. Thus, it is evident that organic or complex nitrogen sources are preferred for the production of lipase. The statistical analysis by two-way ANOVA showed that the influence of nitrogen sources on growth and lipase production is statistically significant ($p<0.0001$) at 5% levels. Similar to present investigations, Chander *et al.* (1980) reported that the optimal nitrogen source for *Aspergillus wentii* extracellular lipase production was 5% peptone, followed by tryptone.

The concentration of nitrogen source also influences the lipase production. Results pertaining to the concentration of peptone on lipase production are presented in Table 5. It is evident from the table that the present isolate exhibited maximum lipase in 1.5% peptone concentration and further increase in peptone concentration there was a decline in the lipase production. The statistical analysis by two-way ANOVA showed that the peptone levels on growth and lipase production by the selected isolate are statistically significant ($p<0.0001$ and $p<0.05$ respectively) at 5% levels. Similarly, Zhang and Guan (2009) reported that *Enterobacter agglomerans* exhibited maximum production of lipase at 2.5% yeast extract concentration.

Oil sources and surfactants

Lipases are induced mostly in presence of fats or oils in the culture medium. In the present investigations, four different oils were tested for their influence lipase production and the results are presented in Table 6. It is evident from the table that LP5 produced maximum lipase (9.50 Eu/ml) in presence of olive oil with a growth OD of 1.100. It is followed by coconut oil. Groundnut oil and castor oil were found to be poor sources for lipase induction. Gulati *et al.* (2005) reported that neem oil was the best suited oil for lipase production by *Fusarium globulosum*.

Surfactants in the fermentation medium enhance the secretion of proteins by altering the cell membrane permeability. Therefore,

addition of surfactants is suggested for the enhanced production of lipase. The results presented in Table 7 reveal that among the five surfactants employed, Tween-80 was found to enhance the production of lipase. Similarly, the studies conducted on lipase production by *P. aeruginosa* EF2 indicated the positive influence of Tween-80 (Gilbert *et al.* 1991). Tributyrin was the next preferred surfactant for lipase production. However, Triton-X inhibited the lipase production. The statistical analysis by two-way ANOVA showed that the influence of oil substrates, surfactants on lipase production is statistically significant ($p<0.0001$, $p<0.05$) at 5% levels.

Table.1 Production of lipase by *B. subtilis* Y-IVI on ideal medium

24 h		48h		72h	
Growth	**EU/ml**	**Growth**	**EU/ml**	**Growth**	**Eu/ml**
0.375	5.50	0.603	9.0	0.712	0.75

Table.2 Influence of different carbon sources on lipases production by *B. subtilis* Y-IVI

Carbon source	Growth	Eu/ml
With out carbon source	0.144	0.10
Lactose	0.255	0.50
Maltose	0.209	0.25
Starch	1.100	9.5
Dextrose	0.274	1.25
Sucrose	0.206	1.75

*All the mean values are statistically significant ($p<0.0001$, $p<0.05$) at 5% levels.

Table.3 Optimization of starch levels for the maximum production of lipases by *B. subtilis* Y-IVI

Starch (in %)	Growth (O.D)	Eu/ml
1.0	0.296	0.25
1.5	1.100	9.5
2.0	0.339	2.50

*All the mean values are statistically significant ($p<0.0001$, $p<0.05$) at 5% levels.

Table.4 Influence of different nitrogen sources on production of lipase by *B. subtilis* **Y-IVI**

Nitrogen source	Growth	Eu/ml
Peptone	1.100	9.50
Beef extract	0.292	0.75
Yeast extract	0.52	0.50
Proteose peptone	0.091	0.80
Tryptone	0.804	1.25
$(NH_4)_2SO_4$	0.018	0.25
KNO_3	0.005	0.25
Control	0.001	0.01

*All the mean values are statistically significant ($p<0.0001$, $p<0.05$) at 5% levels.

Table.5 Optimization of peptone levels for the maximum production of lipase by *B.subtilis* Y-IVI

%peptone	Growth	Eu/ml
1.0	0.061	7.5
1.5	1.100	9.5
2.0	0.031	3.0

*All the mean values are statistically significant ($p<0.0001$, $p<0.05$) at 5% levels.

Table.6 Influence of different oil substrates on the production of lipase by *B. subtilis* Y-IVI

Oil substrate	Growth	Eu/ml
Groundnut oil	0.332	1.5
Coconut oil	0.200	6.0
Castor oil	0.036	1.0
Olive oil	1.100	9.5

*All the mean values are statistically significant ($p<0.0001$, $p<0.05$) at 5% levels.

Table.7 Influence of different surfactants on the production of lipase by *B. subtilis* Y-IVI

Surfactants	Growth	Eu/ml
Tween20	0.46	1.0
Tween80	0.081	8.5
Tributyrin	0.011	2.5
Triton-x	0.106	Nil
Triacetin	0.027	1.0

*All the mean values are statistically significant ($p<0.0001$, $p<0.05$) at 5% levels.

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