INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

www.ijabpt.com Volume-4, Issue-1, Jan-Mar-2013 Coden : IJABPT Copyrights@2013
ISSN : 0976-4550

Received: 30th Oct-2012 *Revised: 05th Nov-2012* *Accepted: 06th Nov-2012*

Research article

ISOLATION AND PURIFICATION OF LIPASE FROM *Bacillus subtilis IN OIL MILL*

Patil M.S. [1*] and Mali P.R. [2]

[1]Department of Microbiology, K.B.P.College, Urun Islampur (MH) India.
[2]Department of Biotechnology, K.B.P.College, Urun Islampur.(MH) India.

*Corresponding Author- Email id patil.madhuri33@gmail.com.

ABSTRACT- In this study, the lipolytic *Bacillus subtilis* was isolated from the oil mill waste by Enrichment technique.The isolated colonies were screened on Tributyrin agar medium, colonies which produce the maximum clear zone of the particular organisms was used for further optimization studies. Among the 3 isolates *Bacillus subtilis* a single isolate was the subjected to solid state fermentation medium and the enzyme characteristics were studied with respect to pH, temperature and incubation period.The production and lipase activity were found maximum 0.45U/mL at pH, 0.45U/mL at temperature 37°C and 0.41U/mL at incubation period 48 hours. The lipase purification steps involved, 60% ammonium sulphate saturation and ion exchange chromatography with DEAE cellulose. To apparent homogeneity as evident by a single band of 62.2kDa on sodium dodecyl sulphate polyacrylamide gel electrophoresis. From the study it was concluded that the commercially important enzyme can be produced by submerged fermentation techniques using frequently available edible oil sources it can be used for the biodegradation of oil effluents.

Keywords-Lipase*, Bacillus subtilis*, Tributyrin agar ,Characterization, SDS-PAGE

INTRODUCTION

An enzyme is a protein produced by a living organism that functions as a biological catalyst.Lipase is one of the important enzyme. Lipase act on lipids. Lipids may be regarded as organic substances relatively insoluble in water, soluble in organic solvents (alcohol, ether etc.), potentially related to fatty acids and utilized by the living cells. Lipase (triacyl glycerol acylhydrolase, E.C.3.1.1.3) catalyzes the hydrolysis of ester bonds at a lipid-water interface and produce glycerol and fatty acids. But these reactions are reversible. Lipase is produced by variety of living organisms, ranging from bacteria to plants and animals. Lipase possesses the unique feature to act at an interface between aqueous and non aqueous (i.e. organic) phase and this feature distinguishes them from esterases. Because of their excellent, capabilities for specific regio-selective reactions in a variety of organic solvents with broad substrate recognition, lipases have emerged as an important biocatalysts in biomedical applications (Pandey et.al.1999, Benjamin and Pandey 1998). They may exhibit specificity for the position of acid in a triglyceride in their natural substrate. Lipase also exhibit stereochemical specificity when reacting with a wide variety of substrate in organic solvents (Baillargeon 1990). It possess characteristic properties like substrate specificity,stereo specificity and the ability to catalyze heterogeneous reactions at the interface of water soluble and water insoluble systems (Shah *et al.,*2007). Lipase enzyme have been found in many species of animals, plants, and microorganisms. The important industrial use for biotechnological applications in dairy industry, oil processing, and production of surfactants (Bapiraju *et al.,* 2004). Microbial lipases are preferred potent sources due to several industrial potential. The world market for lipase has been estimated at approximately US$ 20 million of the industrial enzymes market (Rahman, *et al.*, 2005). Environmental pollution is one of the major problem facing industrialized world today. The need to remediate these sites has led to the development of new technologies that emphasize on the detoxification and destruction of the contaminants rather than the conventional approach of disposal.

Bioremediation, the use of microorganisms or microbial process to detoxify and degrade environmental contaminants is among these new technologies. Different microbes producing lipase are used for the remediation process (Chuks Ugochukw, *et al*.,2008). The exponential increase in the application of lipase in various fields in the last few decades demands extension in both qualitative improvement and quantitative enhancement. Quantitative enhancement requires medium optimization with pH, Temperature and different medium composition. Commercially useful lipases are usually obtained from microorganisms that produce a wide variety of extracellular lipases .The aim of research is to assess the relative abilities of microbes isolated from crude oil polluted soil to degraded crude oil hydrocarbons, to assay and possibly determine some kinetic parameters of crude lipase extract from the microbial isolates.

MATERIALS AND METHODS

Screening of microorganisms

Sample was collected from oil mill and enriched in nutrient agar medium.Enriched sample was used for plating to get only lipolytic isolates.Samples was plated containing (gm/lit): beef extract 3.0, peptone 5.0, sodium chloride 5.0, agar 15.0, calcium chloride 0.05, and glycerol tributyrate 0.2ml. After incubation for 48 hours colonies showing clear zone were picked. The culture was examined for various morphological and biochemical characteristics as per Bergey's Manual of determinative Bacteriology.

Substrate

Groundnut oil cake was used as substrate and their biotechnological applications (Ramachandran et al., 2006) and was dried at room temperature to reduce the moisture content and ground to the desired size.

Inoculum Preparation

In order to prepare the inoculum, a loopfull of cells from a freshly grown slant was transferred into 50 ml of minimal media (without agar) KH_2PO_4 3.0 g, Na_2HPO_4 6.0 g, NaCl 5.0 g, NH_4Cl 2.0 g, $MgSO_4$ 0.1 g in 1 lit. of distilled water and incubated at 30^oC in a shaking incubator at 180 rpm for 24 h (Oswal *et al*., 2002).

Media Preparation

10 gm of desired oil cake was suspended in 90 ml of minimal media. It was then autoclaved at 15 lbs pressure, 120^oC for 20 minutes.

Solid State Fermentation

The above prepared medium was inoculated with 5 ml of inoculum. After through mixing, all the flasks were incubated at desired temperature in a shaking incubator for 48 hours. After a stipulated period samples were drawn. The fermented matter was homogenized and a small amount of sample was taken from each flask for extraction and subsequent analysis.

Enzyme Extraction

The crude enzyme from the fermented material was extracted by simple extraction method. The fermented substrate was mixed thoroughly with 90 ml of 0.05 M of Sorenson phosphate buffer (pH 8.0) and then shaking the mixture in a rotary shaker (180 rpm) at 30^oC for 48 hrs. The crude enzyme obtained from centrifugation and was used to determine enzyme activity.

Lipase assay

The crude enzyme obtained from centrifugation was assayed for lipase activity. It was done by using following steps.

Protocol for lipase assay

27 ml substrate solution was taken in a 100 ml capacity beaker. Add 20 ml enzyme solution to it. The reaction mixture was stirred continuously on magnetic stirred for 30 min. at appropriate temperature. After stirring the reaction mixture, 2-3 drops of phenolphthalein indicator was added to it .It was titrated against 1 N NaOH solution. The end point was colourless to pink. Units of lipases were calculated in terms of fatty acids produced per ml which were calculated in terms of acetic acid released in their reaction mixture under defined set of assay condition. Units of enzyme were calculated by using the following formula given below:

Units of enzyme/ml of enzyme solution = $\dfrac{A \times N \times 60}{V \times T}$

[In terms of acetic acid] Where,

A =Titration reading (Final-Initial), N =Normality of NaOH (1N), 60=Molecular weight of acetic acid, V =Volume of enzyme in ml, T =Time in min.

In this way the lipase production of each isolate was determined.   .

Purification of Extracellular Lipase-
To precipitate lipase by ammonium sulphate experiment was conducted at 50%, 60%, 70%, 80% and 90% saturation of ammonium sulphate salt. The 60% ammonium sulphate saturated and precipitated protein was dialyzed against distilled water for 24 hr. Dialysis removed 10-20 kDa impurities from the sample and it proved very effective. This fraction was loaded to ion exchange chromatography with DEAE cellulose column and a total fractions were collected. SDS-PAGE was done.

RESULTS AND DISCUSSION

1) Enrichment and isolation of Lipase producing bacteria-
After enrichment total three different strains were isolated and Gram stained. They were designated by numbers from L1, L2 and L3.

2) Biochemical characterization and tentative identification of isolates- (Table no.-1 & 2)
From table no. 1 and 2 and with reference to Bergey's Manual of Systemic Bacteriology Vol. I & II all the three isolates were tentatively identified i.e. as *Staphylococcus,Bacillus subtilis,Bacillus megaterium.*

3) Determination of lipase production by SSF-
Amongst three isolates only isolate L2 showed better zone of clearance i.e. indication of lipase activity. Thus, for fermentation L2 isolate was used. Then lipase assay was done. So the enzyme activity was 0.43 Units/ml.

4) **Factors affecting enzyme activity**

a) Effect of pH –(Table no.-3.1)

Maximum lipase activity was obtained 0.45 Units/ml at pH 8.

b) Effect of temperature – (Table no.-3.2)

Maximum lipase activity was obtained 0.45 Units/ml at temp.37

c) Effect of incubation time –(Table no.-3.4)

Maximum lipase activity was obtained 0.45 Units/ml at incubation time period 48 hr.

5) Purification of lipase-

Purification of lipase was carried by using ammonium sulphate precipitation**-** Results revealed that 60% saturation was proved to be effective. The 60% ammonium sulphate saturated and precipitated protein was dialyzed against distilled water for 24 h. Dialysis removed 10-20 kDa impurities from the sample and it proved very effective.

This fraction was loaded to ion exchange chromatography with DEAE cellulose column and total fractions were collected .**SDS-PAGE analysis** showed a single band of purified lipase after DEAE Cellulose column chromatography. Comparison with low molecular weight Protein markers, molecular weight of purified lipase was determined to be 62.2kDa (Figure 1).

 Coden : IJABPT Copyrights@2013 ISSN : 0976-4550

Table no.1– Biochemical characteristics of lipase producing isolates.

Sr. No.	Test	Lipase producing bacteria isolates		
1	Catalase	+	+	+
2	Starch	-	+	+
3	Gelatine	-	+	+
4	Casein	-	+	+
5	V.P	-	+	-
6	Nitrate reduction	+	-	-
7	Glucose	+	+	+

Table no. 2– Tentative identified isolates

Isolate No.	Tentative identified isolates
L1	*Staphylococcus*
L2	*Bacillus subtilis*
L3	*Bacillus Megateruim*

Table no.3.1– Lipase activity at different pH of isolate L2

pH of medium	Lipase activity (Unit per ml)
7	0.25
8	0.45
9	0.36
10	0.25

Table no.3.2- Lipase activity at different temperature of isolate L2

Temperature of medium	Lipase activity (Unit per ml)
20	0.14
30	0.24
37	0.45
40	0.32

Table no.3.3 Lipase activity at different incubation time of isolate L2

Incubation time (hrs.)	Lipase activity (unit per ml)
24	0.29
48	0.41
72	0.23

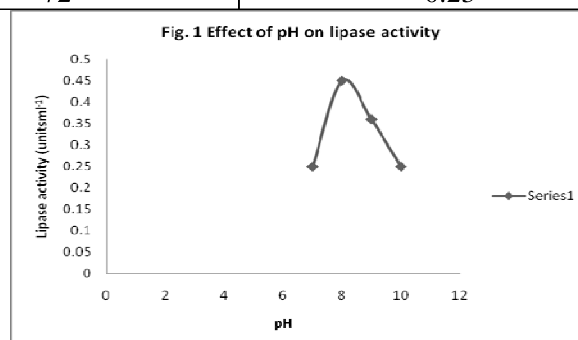

Fig. 1 Effect of pH on lipase activity

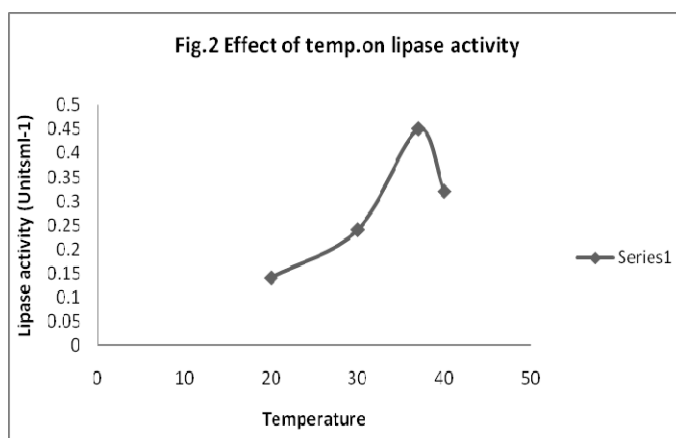

Fig. 2 Effect of Temperature on lipase activity

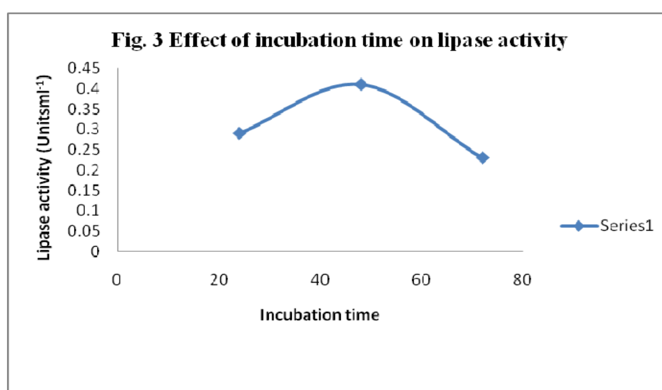

Fig. 3 Effect of Incubation time on lipase activity

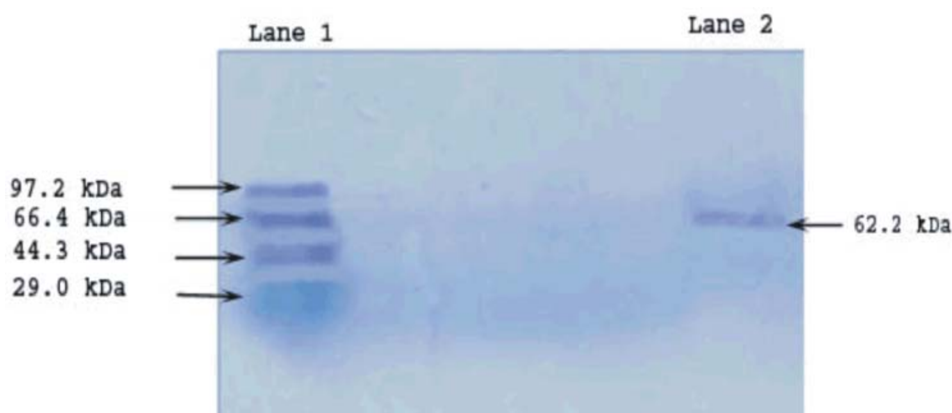

Fig. 4: Photographic representation of SDS-PAGE Gel (Lane 1, Marker Proteins; Lane 2 showing lipase single band)

ACKNOWLEDGEMENTS

The authors are thankful to Mr.P.V.Gaikwad,K.B.P.College,Islampur and Dr.A.K.Salunkhe.

 Coden : IJABPT Copyrights@2013 ISSN : 0976-4550

REFERENCES

Benjamin,S.,Panday,A (1997),"Coconut cake: a potent substrate for production of lipase *Candida rugosa* in solid state fermentation".ActaBiotechnologica 17,241-251.

Benjamin,S.,Panday,A (1998),"Mixed solid substrate fermentation:a novel process for enhanced lipase production by Candida rugosa."ActaBiotechnologica 18,315-324.

Biochemistry,U.Satyanarayana,"Salting out-ammonium sulphate precipitation"65.

Chaturvedhi M,Sing M,Chugh M,Rischi & Kumar Rohul (2010) isolation of lipase producing bacteria from oil contaminated soil for the production of lipase by SSF using ground nut oil cake vol.6,no.4 international journal of biotechnology and biochemistry.

David Plummer,An introduction to Practical Biochemistry,"Ion exchange chromatography" 71-72

Godfrey,T.,West,S (1996)."Introduction to industrial enzymology".In: Godfrey,T.,West,S,(ed).Industrial enzymology,2nd edn.Stockton,NewYork,pp 1-8.

Janarthanan,S Vincent,Practical Biotechnology SDS-PAGE 80-81.