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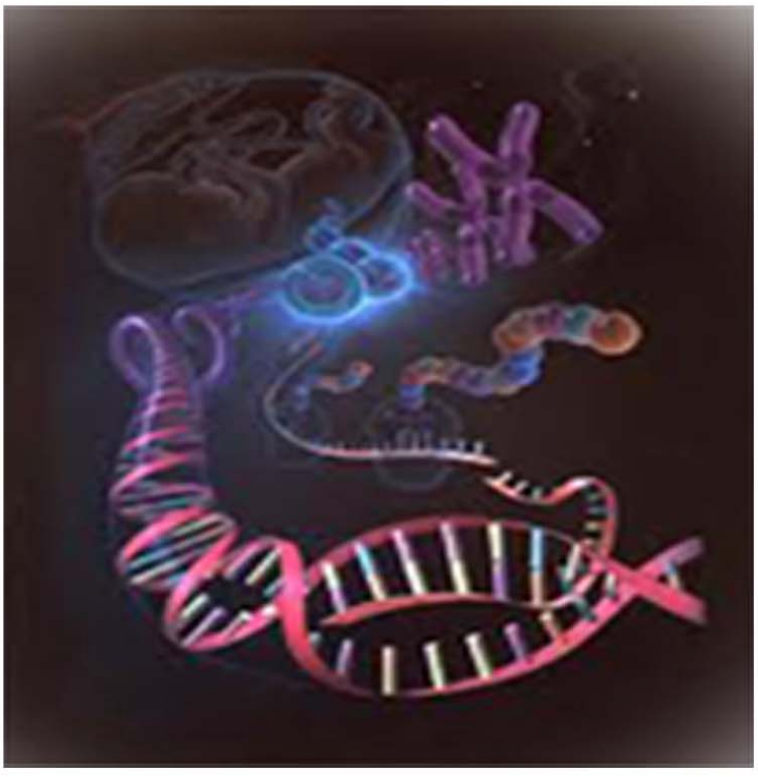

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Research Paper

PRODUCTION, PURIFICATION AND IMMOBILIZATION OF EXTRACELLULAR LIPASES FROM THERMOPHILIC *BACILLUS SUBTILIS* XRF11 AND *BACILLUS LICHENIFORMIS* XRF12 FOR PRODUCTION OF ALKYL ESTERS

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Lipase is known for production of alkyl esters by enzymatic transesterification since long. Thermophilic lipases have great stability at higher temperature with faster rate of reaction and immobilization of these enzymes allows repetitive use of them. In this study, thermophilic lipases were produced, purified and immobilized to be used for transesterification. Purification was carried out by salting out and alcohol precipitation followed by affinity chromatography. Sodium alginate was preferred for immobilization of lipase over varieties of other methods. pNPP assay was used for determination of enzymatic activity. As a result of this study, it was found that 87.0% of peanut oil was converted into ethyl esters after 10 h of reaction at 55ºC. Results of repetitive use of immobilized enzymes showed that after 18 cycles of reaction the production of ethyl esters decreased drastically. Presence of ethyl esters was confirmed by gas chromatographic analysis where it showed presence of C16:0, C18:0, C18:1, C18:3 and C22:0 fatty acids.

Keywords: Thermophilic lipase, Transesterification, Immobilization

INTRODUCTION

Lipase [EC 3.1.1.3] catalyses hydrolysis of triglycerides and produces esters by transesterification reaction. Presently, lipases are considered as choice tools of chemists owing to their ability of catalyzing various types of reactions in non-aqueous environment. These include acidolysis, alcoholysis, aminolysis, esterification and inter-esterfication (Gupta *et al.,* 2004; Hayes, 2004; Saxena *et al.*, 2003). Now a day, it is a choice of biocatalysts as they posses unique chemo, regio and enatio selectivities, which enable the production of drugs, agrochemicals and fine chemicals.(Saxena *et al.*, 2003). Lipases are produced by animals, plants and microorganisms, the majority of lipases used for

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biotechnological purposes have been isolated from bacteria and fungi (Hamid *et al.*, 2003; Sinchaikul *et al.,* 2001). Filamentous fungi, yeasts and *Bacilli* are preferred sources of lipases as they produce extracellular enzymes (Ferrer *et al.*, 2000; Hube *et al.*, 2000; Saxena *et al.*, 2003; Shu *et al.*, 2007; Sinchaikul *et al*., 2001; Vakhlu and Kour, 2006). It was found that thermophilic lipases have higher stability and faster reaction rate as compare to mesophilic. (Hamid *et al.*, 2003; Sinchaikul *et al*., 2001). For immobilization of enzymes several method are in practice. Some of them are entrapments, encapsulation, cross linking, simple adsorption, etc. (Kneževiæ *et al.*, 2004; Minovska *et al.*, 2005). Selection of proper method for immobilization is highly depends on type of enzymes and type of reaction. Entrapment with sodium alginate is one of the easiest and commonly used methods. It also gives good results with most of the lipases. However, environmental parameters need to be standardizing for each reaction system.

In this study, thermophilic extracellular enzymes were produced from two bacteria which were purified and immobilized. Reaction for transesterification was carried out for more than one time after standardization to determine the efficient of immobilized enzyme.

MATERIALS AND METHODS

Isolation and screening of extracellular lipase producing microbes

Thermophiles were isolated from hot water spring of "Lasundra", Gujarat, India. Media containing tributyrin as sole carbon source was used as differential media. Streak plate method was preformed for isolation of microbes.

Plates were incubated at 55ºC until visible colonies appear. Isolated microbes were identified by 16s rRNA sequencing.

PRODUCTION OF EXTRACELLULAR LIPASE

Selected strains were grown in tributyrin broth (Tributyrin-10 ml, Magnesium sulphate-0.2 g, Calcium chloride-0.02 g, Monopotassium phosphate-1.0 g, Dipotassium phosphate-1.0 g, Ammonium nitrate-1.0 g, Ferric chloride-0.05 g, pH-7.0) at 55°C on a rotary shaker at 120 rpm to induce lipase production. Aliquots of the media were taken at regular intervals for determination of lipase activity. Lipase activity was determined by pNPP assay. At a particular time when the activity of the enzyme reached maximum, the broth was collected and processed for enzyme extraction and purification using different methods.

pNPP ASSAY

3 ml of substrate solution (Phosphate buffer (90 ml) + gum arabic (100 mg) + sodium deoxycholate (207 mg) along with 30 mg of pNPP [dissolve in isopropanol]) was pipetted into a cuvette and warmed at 55°C for 10 min. 10 µl of enzymes were added in the cuvette and mixed by inverting. Absorbance was taken at 405 nm at regular intervals of 1 min. Activity of enzyme was calculated by comparison with standard lipase and which was define as the amount of enzyme releasing 1 µmol pNP per minute per mL under assay condition.

PURIFICATION OF LIPASE

Extracellular lipases were first precipitated either by ammonium salt precipitation or ethanol

precipitation which was further purified by affinity chromography using DEAE cellulose.

AMMONIUM SULPHATE/ ALCOHOL PRECIPITATION

At a particular time when pNPP activity of the medium reached maximum; all the cells were removed from the broth by centrifugation. Centrifugation was done at 8000 rpm for 15 min at 4°C. After centrifugation, broth was collected and to the broth benzimidine (2mM) and sodium azide (0.02%) was added to enhance the enzyme stability. After that ammonium sulphate/alcohol was added upto 40% (w/v) and allowed to precipitate for at least 4 h at 4°C. After 4 h of incubation precipitates were collected by centrifugation at 10,000 rpm for 15 min at 4°C. To the remaining supernatant ammonium sulphate/ alcohol was added to 80% (w/v) and allowed to precipitate for 12 h. After 12 h of incubation precipitates were collected by centrifugation at 10,000 rpm for 15 min at 4°C. Precipitates were dissolved in phosphate buffer pH: 7.2 (100mM) containing benzimidine (2mM), EDTA (2mM) and sodium azide (0.02%). Salt particles were removed by filtering through molecular weight cutoff filters (MWCO) of 3kD at 4ºC. As a result of filtration, all the salt particles were removed from the buffer and activity of lipase was determined by pNPP. However, filtration is not required for the enzymes purified with ethanol as it gives lipase in powder form after drying. This can be stored at low temperature for other applications.

AFFINITY CHROMATOGRAPHY

For affinity chromatography DEAE cellulose was used as a stationary material. The column was prepared in a fiber glass column available in the market. DEAE cellulose was first soaked in phosphate buffer [pH: 7.2 (100mM) containing benzimidine (2mM), EDTA(2mM) and sodium azide (0.02%)] for 2 h to prepare slurry for filling up in the column. The Column was filled slowly with slurry with regular taping of the column from the outside to remove air bubbles produced during pouring of the column. It is very essential to remove air bubbles as it affects the separation process. After packing of the column, it was washed with the same buffer for atleast 1 h at a flow rate of 10ml/min. Crude samples precipitated by ammonium salt were loaded on the top of the column after removal of salt and elution was carried out by salt gradient prepared by mixing different concentration of NaCl in phosphate buffer (0-0.5M). Similarly samples precipitated by alcohol were loaded on the top of the column and elution was carried out by salt gradient prepared by mixing different concentration of NaCl in phosphate buffer (0-0.5M). Fractions of 5 ml were collected in different tubes and lipase activity was determined for each tube. Tubes which gave good activity were pooled. Samples were preserved at low temperature for further analysis or applications

IMMOBILIZATION OF ENZYMES

The sodium alginate entrapment method for immobilization was performed according to the standard method. Alginate solution with a concentration of 2.0% was used for the cell immobilization and was prepared by dissolving sodium alginate in warm water. The solution was sterilized by using an autoclave at 121ºC for 15 min at 15 lbs pressure. Preparation of specific quantity of enzyme was mixed with alginate slurry and stirred for 10 min to get a uniform mixture of

the alginate - enzyme combination. This mixture was extruded drop by drop into a cold sterile 0.2 M $CaCl_2$ solution through a sterile 5 ml syringe from height of 5 cm and kept for curing at 4ºC for 1 h. The beads were hardened by resuspending into a fresh 0.2 M $CaCl_2$ solution for 24 h at 4ºC with gentle agitation. Finally these beads were washed with distilled water to separate excess calcium ions, unbound enzymes and unentraped cells. When the beads were not being used, they were preserved in 0.9% sodium chloride solutions in the refrigerator. (Tan *et al.*, 2004)

TRANSESTERIFICATION

Mixture of ethyl acetate : oil (11:1) was taken in a dry glass bottle (with tight cap) and 10% (w/v) immobilized enzymes was added to the bottle. The mixture was incubated at 55°C with constant stirring at 200 rpm for 12 h. Mixture was allowed to cool. To the reaction mixture, hexane was added and two phases were allowed to separate. Upper hexane phase containing esters was collected for further analysis. For repetitive use of immobilized lipase, the mixture was filtered and beads were washed with sterile distilled water and stored in buffer at low temperature.

GAS CHROMATOGRAPHY

GC instrument were put on and all the parameters were set for the analysis. [Restek 1 capillary column 15 m, Column gradient 150ºC - 270°C FID Detector, detection temp. 30°C, injection volume-1 µL with spilt ratio 1:10]. Instrument was allowed to stabilize for 30 min before injection of samples/standards. 1 µl of sample was injected for the analysis and instrument was allowed to analyze the sample. Obtained data were compared with external standard for determination of composition and concentration of produced biodiesel.

RESULTS AND DISCUSSION

As a result of 16s rRNA sequencing, the two thermophiles were identified as *Bacillus subtilis and* by *Bacillus licheniformis* which were designated as *Bacillus subtilis* xrf11 *and* by *Bacillus licheniformis* xrf12.

PRODUCTION AND PURIFICATION OF LIPASE

A total of 11 of broth containing thermophilic lipase produced by *Bacillus subtilis*xrf11 was collected and purified with different techniques. As a result, an increase in enzyme activity was obtained at each purification step with reduction in product quantity. At the end of the complete purification process, only 10mL of pure enzyme was obtained with maximum specific activity of 506 units/mg of protein. The enzyme was successfully purified upto 28.40 fold (salt precipitated) and 33.26 fold (alcohol precipitated) by affinity chromatography (Table 1). Similarly purification of lipase produced by *Bacillus licheniformis*xrf12 gave highest specific activity of 690 units/mg of protein after purification by DEAE cellulose column previously precipitated with alcohol. (Table 2). Here maximum of 37.70 purification fold was achieved. An important observation was enzyme purified by alcohol precipitation gave higher activity than salt precipitation which implies that alcohol precipitation is a better method for purification of enzymes rather than salt precipitation.

Several previous studies have shown that microbial lipases could be purified upto 80 folds with combination of more than one purification techniques (Huang *et al.*, 2004; Prescott, 2002; Zheng-yu *et al.*, 2007). In all these techniques certain common steps are involved like primary purification by either ammonium sulphate or alcohol to make the filtrate more dense in terms of protein, followed by different kinds of chromatography which includes affinity

Table 1: Purification of Lipase Produced by *Bacillus subtilis* xrf11

	Volume	Total Enzyme Activity/unit	Total Protein (in mg)	Specific Activity (units/mg of protein)	% Yield	Purification fold
Salt Precipitation						
Crude	1000	5600	368.17	15.21	100	1
Ammo. Sulfate	50	4925	75.53	65.2	87.94	4.28
DEAE cellulose	10	1872	4.3	432	33.42	28.40
Alcohol Precipitation						
Crude	1000	5600	368.17	15.21	100	1
Alcohol	50	5103	76.05	67.1	91.12	4.41
DEAE cellulose	10	1892	3.73	506	33.78	33.26

Note: Table shows the activity of crude lipase obtained from xrf11 strain. It also shows the activity of lipase after different purification steps. It was found that lipase precipitated with alcohol was able to give higher activity compared the lipase precipitated by ammonium sulfate. Maximum purification of 33.26 fold was achieved by using affinity chromatography.

Table 2: Purification of Lipase Produced by *Bacillus licheniformis* xrf12

	Volume	Total Enzyme Activity/unit	Total Protein (in mg)	Specific Activity (units/mg of protein)	% Yield	Purification fold
Salt Precipitation						
Crude	1000	6235	340.71	18.3	100	1
Ammo. Sulfate	50	5423	73.18	74.1	86.97	4.04
DEAE cellulose	10	2032	3.2	635	32.59	34.69
Alcohol Precipitation						
Crude	1000	6235	340.71	18.3	100	1
Alcohol	50	5514	70.51	78.2	88.43	4.27
DEAE cellulose	10	2106	3.05	690	33.77	37.70

Note: Table shows the activity of crude lipase obtained from xrf12 strain. It also shows the activity of lipase after different purification steps. It was found that lipase precipitated with alcohol was able to give higher activity compared the lipase precipitated by ammonium sulfate. Maximum purification of 37.70 fold was achieved by using affinity chromatography.

chromatography, ion exchange chromatography, high pressure liquid chromatography for product purification and product collection.(Gupta *et al.,* 2004; Huang *et al.*, 2004; Saxena, Sheoran *et al.*, 2003; Zheng-yu *et al.*, 2007). Many processes involve more than one chromatography in the purification of lipase (Hossack *et al.*, 1979; Mago and Khuller, 1990; Mahmoudabadi and Drucker, 2006; Watanabe and Takakuwa, 1984; Zhao *et al.,* 2008). Among all these methods, a common

Table 3: Activity of Immobilized Lipase

Conditions	Specific Activity (Free Enzyme)	Specific Activity % (Immobbilized Enzyme)	Activity of immobilized enzyme compared to free enzyme
Bacillus subtilis xrf11			
Immobilized cells	-	20.1	4.65
Enzyme purified by ammo. Sulphate + DEAE cellulose	432	375.6	86.94
Enzyme purified by alcohol + DEAE cellulose	506	428.2	84.62
Bacillus licheniformis xrf12			
Immobilized cells	-	37.2	5.85
Enzyme purified by ammo. Sulphate + DEAE cellulose	635	567.3	89.33
Enzyme purified by alcohol + DEAE cellulose	690	578.2	83.79

Note: Table shows the activity of free and immobilized lipase obtained from xrf11 and xrf12 strain. Comparison of between free and immobilized enzyme also shown here which shows that immobilized form of enzymes are also able 80.0% of activity as compared to free enzymes.

observation made that the higher numbers of purification steps give better quality of enzymes but lesser quantity enzyme. So it is very essential to choose a proper method for purification which can give highly purified product in minimum number of purification steps.

IMMOBILIZATION OF ENZYMES AND TRANSESTERIFICATION

It was found that immobilized enzymes of *Bacillus subtilis* xrf11 gave specific activity of 428.2 units/mg of protein and *Bacillus licheniformis* xrf12 gave 578.2 units/mg of proteins, which is around 80.0%

Figure 1: Product Obtained at Each Reaction Cycle : *Figure Shows The % Of Product Produced By The Immobilized Beads. From The Figure It Was Noted That as Upto 18-21 Cycle Of Reaction More Than 80% Of Product is Obtained Which Reduces Drastically And Reached Below 20% After 30th Cycles*

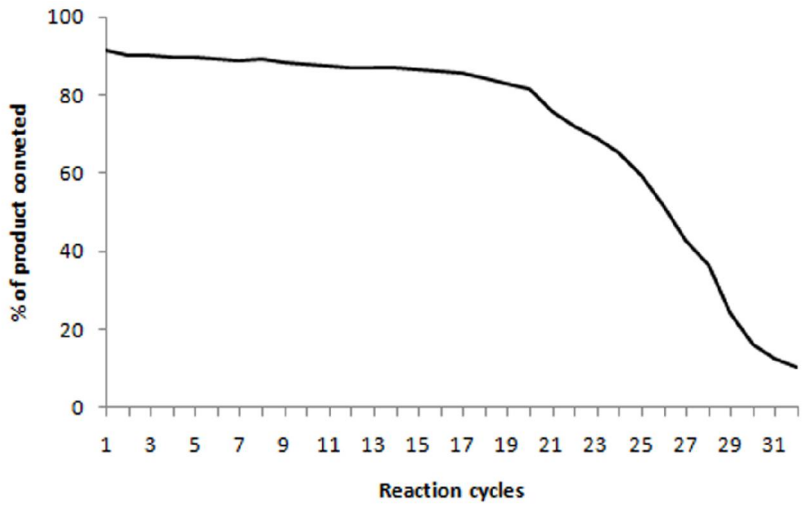
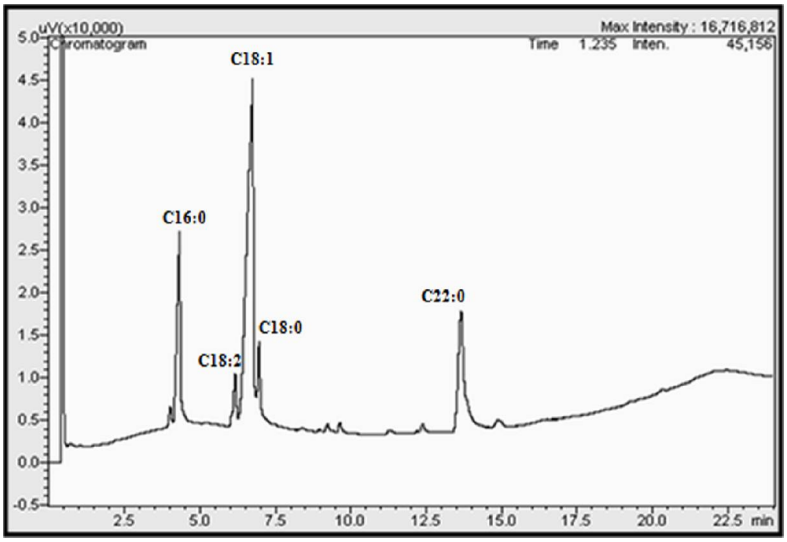


Figure 2: Gas Chromatogram Of Faees Produced By Immobilized Lipase Enzyme (*Bacillus Licheniformis* Xrf12) By Reaction With Peanut Oil - *Five Prominent Peaks Of C18:0. C18:0. C18:1, C18:2 And C22:0 Were Observed In The Product Obtained From Transesterification Of Peanut Oil By Immobilized Enzymes*



of activity as compare to free enzymes (Table 3). In the study immobilized purified enzymes were selected for enzymatic transesterification rather than immobilized cells as the later are not able to give good enzymatic activity. Immobilized enzymes were repeatedly used to determine the reduction of their activity with the reaction cycles. As a result of this study, it was found that immobilized enzymes of *Bacillus subtilis*xrf11 and *Bacillus licheniformis*xrf12 were able to give higher activity (which is about 50% of initial activity) upto 18 to 21 reaction cycles, which later decreased drastically and reached below 20 units/mg of activity at 30th cycle. (Figure 1) This is because after certain reaction cycles beads started to degrade in small pieces. (Sung-in *et al.*, 2003)

RESULTS OF GAS CHROMATOGRAPHY

As a result of transesterification reaction by immobilized lipase xrf12, five prominent peaks of C16:0 (16.55%), C18:0 (5.43%), C18:1(53.83%), C18:2(3.93%) and C22:0(17.96%) were found (Figure 2). These fatty acids make 97.27% of the total fatty acid present in the peanut oil. It was found that almost 87.0% of peanut oil was converted into fatty acid ethyl esters. In case of immobilized enzyme from xrf11 similar kinds of results were observed where a total 81.27% of fatty acids present in the peanut oil were converted into FAEEs.

CONCLUSION

Thermophilic lipases have good stability and activity in their immobilized form which makes them a potential tool for transesterification at higher temperature.

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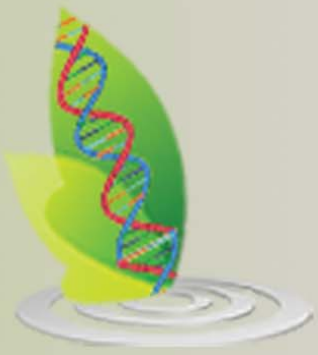

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