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Research Article

ISOLATION, PURIFICATION AND MEDIUM OPTIMIZATION OF LIPASE ENZYME PRODUCING STRAINS OF *ASPERGILLUS NIGER* ISOLATED FROM NATURAL SOURCES

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ABSTRACT

Lipases are widely used industrial enzymes, which have found its applications in various industries. In this work we have isolated lipase producing strains from various natural sources and have confirmed the lipase production capability by visible plate technique. The initial production was carried out in solid state and submerged fermentation to access the maximum enzyme production capacity. The medium optimization was done to find out the optimal production medium. Also the effect of temperature and pH were also studied and the thermo stability if the enzyme was also tested. From our studies we have found that the enzyme produced by the isolates by solid state fermentation produced an activity of 6.83 IU/ml, 10.33 IU/ml, 9.63 IU/ml and 6.85 IU/ml for isolates A, B, C and D respectively. Strain improvement can be done in these organisms to improve the yield of lipase production.

Keywords: Lipase, Solid state fermentation, Submerged fermentation, Organic and inorganic nitrogen, Enantio selective.

INTRODUCTION

The demand for industrial enzymes, particularly of microbial origin, is ever increasing owing to their applications in a wide variety of processes [1]. Enzyme-mediated reactions are attractive alternatives to tedious and expensive chemical methods. Enzymes find great use in a large number of fields such as food, dairy, pharmaceutical, detergent, textile, and cosmetic industries. In the above scenario, enzymes such as proteases and amylases have dominated the world market owing to their hydrolytic reactions for proteins and carbohydrates. It is in the last decade that lipases have gained importance to a certain extent over proteases and amylases, especially in the area of organic synthesis [2].

The enantioselective [3] and regioselective nature of lipases have been utilized for the resolution of chiral drugs, fat modification, synthesis of cocoa butter substituent, biofuel and for synthesis of personal care products and flavor enhancers. Thus, lipases are today the enzymes of choice for organic chemists, pharmacists, biophysicists, biochemical and process engineers, biotechnologists, microbiologists and biochemists. Lipases have been proven to be an antibacterial enzyme in the humans [4]. Lipases (triacylglycerol acylhydrolases) belong to the class of serine hydrolases. The natural substrates of lipases are triacylglycerols, having very low solubility in water. Under natural conditions, they catalyze the hydrolysis of ester bonds at the interface between an insoluble substrate phase and the aqueous phase in which the enzyme is dissolved. Lipases which are stable and work at alkaline pH, say 8 to 11, which are usually the suitable wash conditions for enzymated-detergent powders and liquids, have also been found, and these hold good potential for use in the detergent industry.

Research has been carried out on plant lipases, animal lipases, and microbial lipases, particularly bacterial and fungal [5, 6 &7]. It is now well established that microbial lipases are preferred for commercial applications due to their multifold properties, easy extraction procedures, and unlimited supply. The production of lipase enzyme by solid state fermentation achieves high interest because of it low cost nutrient and substrate source such as agro waste. As an extension of this, the thermo stability may be the next important criteria where the scientists pay their attention. The thermo stable lipase enzyme is very much accepted by the scientist in pharmaceutical, food, leather, and some various industries as seen in the reviews of literature. The next important aspect comes to know, as important is the racemic converting ability of the lipase enzyme for various drugs having racemic enantiomers. The reaction may change the activity of the drug in favor to the society.

Considering these three factors the wide scope of producing the lipase enzyme is understood. The above quality makes the future interest for the scientists to work in the lipase. This work was also an attempt towards the same goal. As strong belief is that, the goal can be achieved by identifying new strains by means of isolation and screening from various oil substrates and performing the optimization of the production parameters.

MATERIALS AND METHODS

Collection of oil substrates and screening for lipase producers

The strains used in this study were isolated from the various oil substrates. Five oil substrates were used such as fungal infected coconut, coconut oil cake, soil from coconut oil mill, spoiled bread and castor oil soaked cotton. The collected oil substrates are kept aside for a week and observed for required growth. Screening of lipase producing strains was done by agar plate technique [8].

Each small quantity of oil substrates were transferred into test tubes containing 5ml of yeast extract malt extract broth. The test tubes were suitably labeled as per the collected oil substrates. The test tubes were incubated for 1 week. This incubated broth is subjected to serial dilution. 1ml of serial diluted sample was taken and mixed with 5ml of potato dextrose agar containing 50 μg/ml of rifampcin. The total contents was poured into a petri plate and incubated for 7days at 30˚C. After 7 days the discrete colonies were picked up and transferred to potato dextrose agar slants. The slants were further incubated for 3 days at 30˚C. The slants obtained after 3 days from Potato Dextrose Agar slants may be the desired organisms. The organisms were further confirmed for its lipolytic activity by using the visible plate technique [9]. Emulsified salad oil (mixture of palm oil, coconut oil and olive oil in the even ratio) was prepared and spread at the bottom of the petri dish. 2ml of the broth suspended with the organism was layered on the lipid layer. 6-7ml of molten agar (45 ˚C) was added over the organism layer by tilting and rotating the plates. Total contents were incubated at 30˚C for 4 days.

Production of Lipase by submerged fermentation [10]

The isolates were grown in an inoculation medium containing glucose (10 g/l), peptone (20 g/l), sodium chloride (5 g/l), yeast extract (5 g/l) and the pH was maintained at 6.0. The production medium consists of the inoculation medium supplemented with salt solution. The salt solution was prepared from ammonium sulphite (5 g/l), sodium hydrogen phosphate (6 g/l), potassium hydrogen phosphate (2.0 g/l), magnesium sulphate (3 g/l) and calcium chloride (3 g/l). The organism that exhibited lipolytic activity was subjected to fermentation for the production of lipase enzyme. The

72 hours old culture was prepared as spore suspension by adding 5ml sterile water. This 5ml of spore suspension was added with 45ml of inoculum medium in 250 Erlenmeyer flasks. The total contents were incubated in rotary shaker at 30˚C for 48 hours. 10% inoculum was added with 45ml of production medium. It was incubated at 30˚C for 7 days. At the end of 7 days fermentation, the biomass was treated with 50 ml of distilled water and stirred well for the extracellular Lipase to soluble in aqueous media. After that it was filtered by muslin cloth. Residue was again treated with 50 ml of water and filtered. The filtrate was centrifuged at 15000 rpm for 30 minutes. The clear supernatant was taken as enzyme source.

Production of Lipase by solid state fermentation [11]

The inoculum was prepared as mentioned earlier. 10 gm of solid substrate (wheat bran was taken in Erlenmeyer flask. 1 ml of salt solution was sprinkled over the solid substrate. It was moistened to 70% by adding distilled water. Flask with contents was sterilized by autoclaving. After cooling 10% inoculum was added. It was incubated at 30˚C for 7 days. After 7 days fermentation, the biomass was treated with 50 ml of distilled water and stirred well for the extracellular Lipase to soluble in aqueous media. After that it was filtered by muslin cloth. Residue was again treated with 50ml of water and filtered. The filtrate was centrifuged at maximum speed for 30 minutes. The clear supernatant was taken as enzyme source.

Lipase activity assay [12]

Lipase activity assay was done by titrimetric method using olive oil substrates emulsion. Add 70 ml emulsifying reagent with 30 ml olive oil and homogenized for 5 minutes. 1ml of substrate emulsion was taken in conical flask and 0.8ml of 0.2M potassium phosphate buffer (pH 7.0) was added. After that 0.2ml of enzyme was added. The total contents were incubated at 55 ˚C for 1 minute. Reaction was terminated by adding 2ml of acetone ethanol mixture (1:1 v/v). Total contents were titrated against 0.05N sodium Hydroxide using phenolphthalein as indicator.

Effect of carbon source on the production of Lipase enzyme

1% of various carbon sources such as Glucose, Sucrose, Starch and Tween 80 were added to the substrate wheat bran and it was fermented with the isolated strain for the production of lipase enzyme. It was incubated for 7 days. The enzyme activity was then calculated.

Effect of organic nitrogen source on the production of Lipase enzyme

1% of various organic nitrogen sources such as Urea, Yeast extract, Nutrient broth and Soya Bean Meal were added to the substrate wheat bran and it was fermented with the isolated strain for the production of lipase enzyme. It was incubated for 7 days. The enzyme activity was then calculated.

Effect of inorganic nitrogen source on the production of Lipase enzyme

1% of various inorganic nitrogen sources such as Ammonium sulphate, Ammonium nitrate, Ammonium phosphate and Sodium nitrate were added to the substrate wheat bran and it was fermented with the isolated strain for the production of lipase enzyme. It was incubated for 7 days. The enzyme activity was then calculated.

Effect of temperature on the production of Lipase enzyme

Fermentation for the production of lipase enzyme was carried out under various temperatures such as 25˚C, 30˚C, 35˚C and 40˚C in Erlenmeyer flask. It was incubated for 7 days. The enzyme activity was then calculated.

Effect of pH on the production of Lipase enzyme

Various pH such as 6.0, 6.5, 7.0, 7.5 and 8.0 of phosphate buffer of IP standard was prepared and then 1% of the solutions were added to the fermentation substrate for the production of lipase enzyme. Total contents were incubated for 7 days. The enzyme activity was then calculated.

Studies on the thermostability of Lipase produced by the isolated strain by solid state fermentation

The enzyme extracted by the downstream process after fermentation was taken in four test tubes and labelled accordingly. They were subjected to heat treatment for 15 minutes under various temperatures such as 20˚C, 30˚C, 40˚C and 50˚C respectively. Then the lipase activity assay was carried out by titrimetric method using olive oil substrate emulsion method. The enzyme activity was then calculated.

Purification of enzyme produced by the isolated strain by solid state fermentation

The isolated enzymes were purified by the following procedures. Enzymes were subjected to centrifugation at maximum speed for 15 minutes. Then the supernatants were treated with ammonium sulphate to the saturation. Then the precipitate was filtered and the cold acetone was added for the enzyme recovery.

Estimation of total protein by Lowry method

The total protein was calculated by Lowry method which has been described elsewhere.

1 Unit of lipase activity is calculated by the formula

$$\text{Lipase Activity} = \frac{5.61\ \text{Volume of NaOH Normality of NaOH}}{\text{Volume of enzyme taken}}$$

Where 5.61=unit constant for identifying acid value

Unit Definition

1 Unit lipase activity = Amount of enzyme required to release 1 micro mole of fatty acid per ml per minute under above assay conditions.

RESULTS AND DISCUSSION

From the five different source used, four isolates were found to be producing lipase. The visible plate method was observed after the incubation for three days. The plates changes to clear, transparent opaque and white spots of calcium salt from turbid. This shows that the organism breaks the lipids and exhibits lipolytic activity.

Comparison of submerged and solid state fermentation for lipase enzyme production

From the both fermentation procedure done the activity of the lipase was tabulated in table 1 from the observation the maximum production of lipase was found in the solid state fermentation.

Effect of carbon source, nitrogen source and inorganic source on the production of Lipase

Tween 80, soybean meal and ammonium sulphate was found to be the best source for carbon, organic nitrogen and inorganic nitrogen source respectively. The results are given in table 2, 3 and 4.

Effect of Temperature on the production of lipase by solid state fermentation:

The maximum activity for the effect of temperature on lipase production was seen as the 30˚C from the table 5. This was the normal growth temperature for the fungi. There was notable activity found even at 40˚C.

Effect of pH on the production of lipase by solid state fermentation:

The maximum activity for the effect of pH on lipase production was seen as the pH 7.0 from the table 6. This was the neutral pH and the normal growth pH for the fungi. Also in alkaline and acidic pH the enzyme shows some activity.

Thermo stability study of lipase enzyme produced from the isolated strain:

The thermo stability of the lipase enzyme was found to be maximum upto 40˚C. The maximum activity has been found in 30˚C, whereas at 50˚C there is no activity at all due to the denaturation of the enzyme. The results are given in table 7.

Table 1: Comparison of submerged and solid state fermentation for lipase enzyme production

S. No.	Comparative Fermentation Activity		
	Enzyme	Submerged Fermentation	Solid state Fermentation
		Activity in IU/ml	Activity in IU/ml
1	A	3.65±0.12	6.83±0.54
2	B	3.37±0.23	10.33±0.18
3	C	3.65±0.23	9.63±0.09
4	D	3.08±0.20	6.85±0.32

Table 2: Effect of Carbon source on the production of lipase by solid state fermentation

S. No.	Enzyme	Carbon source (Activity in IU/ml)			
		Glucose	Sucrose	Starch	Tween 80
1	A	5.84±0.11	14.73±0.12	7.71±0.11	17.95±0.06
2	B	22.58±0.11	17.67±0.11	10.09±0.08	23.14±0.06
3	C	23.28±0.23	11.50±0.13	4.91±0.00	25.25±0.12
4	D	22.72±0.11	9.40± 0.34	7.4 3±0.17	23.14±0.13

Table 3: Effect of organic nitrogen source on the production of lipase by solid state fermentation

S. No.	Enzyme	Organic nitrogen source (Activity in IU/ml)			
		Nutrient broth	Soyabean meal	YEME	Urea
1	A	5.19±0.24	8.7 ± 0.09	11.92 ±0.11	7.01 ±0.28
2	B	7.43± 0.17	5.33± 0.00	14.31 ±0.11	10.81±0.13
3	C	9.12±0.11	7.43±0.06	19.07±0.06	4.91±0.22
4	D	3.51±0.07	3.51±0.07	12.62±0.22	12.48±0.40

Table 4: Effect of In organic nitrogen source on the production of lipase by solid state fermentation

S. No.	Enzyme	In Organic nitrogen source (Activity in IU/ml)			
		NH_4SO_4	NH_4NO_3	NH_4PO_4	$NaNO_3$
1	A	7.79±0.138	7.01± 0.21	7.71 ±0.00	4.91 ±0.13
2	B	14.73±0.05	10.81±0.17	11.32±0.00	9.81±0.06
3	C	11.64±0.30	11.64±0.17	10.38±0.068	14.45± 0.07
4	D	9.81±0.00	3.51± 0.10	7.71±0.118	7.01 ±0.05

Table 5: Effect of Temperature on the production of lipase by solid state fermentation

S. No.	Enzyme	Temperature (Activity in IU/ml)			
		25°C	30°C	35°C	40°C
1	A	4.91±0.06	14.87± 0.82	7.29±0.11	7.29±3.45
2	B	3.79±0.03	22.81±0.06	3.79±0.06	5.75±2.74
3	C	4.34±0.06	17.11±0.11	7.43±0.06	3.08±1.56
4	D	4.91±0.06	18.37±0.0.06	5.38±0.37	4.63±7.07

Table 6: Effect of pH on the production of lipase by solid state fermentation

S. No.	Enzyme	pH (Activity in IU/ml)				
		6.0	6.5	7.0	7.5	8.0
1	A	14.31±0.13	23.48±0.33	24.12±0.03	20.06±0.105	7.43±0.19
2	B	21.04±0.06	23.14±0.1	14.45±0.11	12.62±0.046	7.71±0.00
3	C	20.2 ±0.15	22.04±0.38	21.74±0.10	14.31±0.064	4.91±0.03
4	D	19.64±0.06	21.45±0.05	16.97±0.02	11.78±0.12	5.61±0.004

Table 7: Thermo stability study of lipase enzyme produced from the isolated strain

S. No.	Enzyme	Thermo stability of enzyme (Activity in IU/ml)			
		20°C	30°C	40°C	50°C
1	A	4.2±0.138	16.22±0.11	8.09±3.48	-
2	B	3.5±0.01	12.01±0.03	6.25±2.74	-
3	C	8.7±0.03	14.37±0.01	4.98±1.56	-
4	D	2.8±0.14	15.17±0.11	5.63±7.07	-

Estimation of protein by Lowry method

The total protein content was determined by Lowry method and it was found that the lipase from organism A yield 1.75 mg/ml, lipase from organism B yield 1.11 mg/ml, lipase from organism C yield 1.30 mg/ml and lipase from organism D yield 1.70 mg/ml.

CONCLUSION

The lipase enzyme is one of the major industrially used enzyme and the eye of the researchers, the work was carried out on the topic Lipase Enzyme. The lipase enzyme was produced in the work from the fungal sources, which were taken from the various oil substrates. The oil substrates were collected from various places and then it was allowed to get infected; by keeping aside. Fungi were transferred for the screening purpose by agar plate technique. The screened fungi were tested for its lipolytic activity by visible lipolytic activity method. Then the fungi were subjected to both the submerged and solid state fermentation for the lipase enzyme production and the results were calculated by its lipase activity using titrimetric method (Olive oil substrate emulsion). The results obtained shows that the solid state fermentation is much better process than the submerged fermentation by means of economy, activity and yield. So the further works were carried out by the solid state fermentation process. After this the optimization of the process parameters in the lipase production was done by using various parameters such us the effect of nutrients (carbon, organic nitrogen and inorganic nitrogen sources) effect of pH and the effect of temperature. The results were tabulated and seen that the Tween 80 were found to be the best carbon source, soyabean meal for organic nitrogen and ammoniam sulphate shows the best results than the other nitrogen sources. The neutral pH (7.0) shows the best activity rather than the alkaline and acidic. At a temperature 30˚C, the normal fungal growth temperature, it was seen to be ideal for the organism isolated to produce the lipase enzyme as it shows maximum activity. Furthermore the thermo stability studies were carried out for the produced enzyme in that too the enzyme shows maximum activity at 30˚C. So the enzyme may be useful in the detergent industry where the commercially available enzyme has maximum activity at 40-50˚C. Further more the study extends to the estimation of total protein content in the produced enzyme and was found to be approximately 1.75 mg/ml, 1.1mg/ml 1.30mg/ml and 1.7mg/ml for enzyme for A,B,C,D respectively.

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