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Solid State Fermentation of Agro Waste for Industrial Lipase Production

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Abstract: Enzymes are among the most important products obtained for human needs through microbial sources. A large number of industrial processes in the areas of industrial, environmental and food biotechnology utilize enzymes. Current developments in biotechnology are yielding new applications for enzymes. Solid-state Fermentation holds tremendous potential for the production of enzymes. It can be of special interest in those processes where the crude fermented product may be used directly as the enzyme source. Potentially many high value products as enzymes, metabolites, antibiotics could be produced in SSF, and almost all known microbial enzymes can be produced under SSF systems. In recent years, the SSF processes have been increasingly applied for the production of lipase enzyme. A strain of *Pseudomonas aeruginosa* was used for the production of lipase in solid cultures. The study included collection of agro-wastes including Sugarcane waste, Dal waste, Corn waste, Ground nut, Safflower and Niger seed waste. Lipase are used widely in detergents, food, flavour industry, biocatalyst resolution of pharmaceuticals, esters and amino acid derivatives, making of fine chemicals, agrochemicals, biosensor, a bioremediation cosmetics and perfumery.

The present study was undertaken to examine the effectiveness of agro-wastes as a substrate for lipase production, purification, and characterization of the enzyme.

Keywords: Solid state fermentation, Lipase enzyme, *Pseudomonas aeruginosa*

I. INTRODUCTION

Enzymes are biocatalysts produced by living cells to bring about specific biochemical reactions [1]. Lipases (triacylglycerol acylhydrolases (E.C.3.1.1.3) belongs to the class of hydrolases which catalyze the hydrolysis of triglycerides to glycerol and free fatty acids [2]. Lipases are able to catalyze esterification, interesterification and transesterification reactions in (SmF) in non-aqueous media [3,4]. Solid-state (substrate) fermentation (SSF) is generally defined as the growth of the micro-organisms on (moist) solid material in absence or near absence of free water. The water content of a typical submerged fermentation is more than 95 percent whereas the water content of a solid mash in SSF varies from 40 percent to 80 percent [5]. Lipases are a class of hydrolases that are primarily responsible for the hydrolysis of acylglycerides. They are ubiquitous and indispensable for the bioconversion of lipids (triacylglycerol) in nature. Recent studies on the structure of several lipases have provided some clues for understanding their hydrolytic activity, interfacial activation and stereo selectivity of lipases [6]. Commercially lipase have a wide variety of applications. In the area of detergents, lipase is used widely, it also plays an important role in the production of food ingredients. An example is the lipase-catalyses transesterification reaction replacing palmitic acid by stearic acid to provide the stearic oleic-stearic triglyceride with the desired melting point for use in chocolate (cocoa butter substitute). Other applications of increasing interest include use of lipases in removing the pitch from pulp in the paper industry, in flavor development for dairy products and beverages, and in synthetic organic chemistry [7]. The focus of this study was concentrated on the solid state fermentation of agro-waste substrates for maximum lipase production.

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II. MATERIALS AND METHODS

Materials

All chemical compounds including K_2HPO_4, NaCl, $MgSO_4.7H_2O$, $CaCl_2$, K_2HPO_4 and $CaCl_2$ were analytical grades and were purchased from Merck. Agro wastes were collected from nearer places of Bagalkot, which included wastes of sugar cane, dal, corn, ground nut, niger and safflower.

Microorganism and Inoculum Preparation

Bacterial strain *Pseudomonas aeruginosa* was used for the study was taken from the collection unit of Biogenics, Hubli. The strain was then revived for the further use. The strain was cultivated on agar medium at room temperature for 24 hours and was kept at 4°C. *Pseudomonas aeruginosa* was grown in a medium containing (g/L): K_2HPO_4 (2.0), NaCl (1.0), $MgSO_4.7H_2O$ (0.1), $CaCl_2$ (0.050), K_2HPO_4 and $CaCl_2$, for the incubation period of 24 hours [8-10].

Lipase Production in SSF

Six different agro-industrial wastes (sugar cane, dal, corn, ground nut, niger and safflower) were dried and ground in an electric mill and then sieved. Ten grams of each dried and powdered substrate was transferred in a series of 250 mL Erlenmeyer flasks and moistened with water (50%), and sterilized at 15 lbs/in at 121°C for 15 minutes. Afterwards the flasks were inoculated with 2 mL of spore suspension and incubated in fermenter at room temperature for 24 hours.

Enzyme Assay

Lipase activity was estimated using olive oil substrate. The assay mixture consisted of 1ml of the substrate emulsion (70 ml emulsifying reagent with 30ml olive oil homogenized for 5 min). The emulsification reagent. (Nacl 17.9 g, KH_2PO_4 0.41 g, Glycerol 540 ml, Gum Arabic 10 g and distilled water to total volume 1.0 L), 0.8 ml of 0.2 M potassium phosphate buffer (pH 7.0) and 0.2 ml of the enzymes were incubated at 550°C for 30 min. The reaction was terminated by adding 2 ml acetone ethanol mixture (1:1 v/v). The amount of fatty acid liberated was determined by titration with 0.01 N NaOH.

$$\text{Enzyme activity (µgm/ml/min)} = \frac{\text{(Volume of alkali consumed)} \times \text{(Normality of alkali)}}{\text{(Time of incubation)} \times \text{(Volume of enzyme solution)}}$$

Selection of Suitable Substrate

Among the various agro wastes and oil seeds, the one which gave maximum activity was selected. This agro waste was then used for the further study. Optimization studies: The optimization factors influencing the lipase production studies were moisture content (%), pH, incubation time (h), incubation temperature (°C) and Carbon sources. At the end of fermentation, crude enzyme was extracted by mixing the fermented substrate with 10 mL of Tris buffer (25 mM; pH 8.0) and then shaking the mixture in an orbital shaker at 10,000 rpm for 10 mins. The obtained extract was filtered and the supernatants were used for lipase assay. Among all the above performed parameters which gave maximum enzyme production were maintained and the production of the enzyme was carried out. This was followed by the purification steps.

Purification

Ammonium sulphate precipitation:

This process is achieved with the help of a salt of high solubility. Salting out is dependent on the hydrophobic nature of the surface of the protein.

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Procedure:

The 85 ml of enzyme extract obtained was saturated to 80% (w/v) with ammonium sulphate. The saturation was carried by adding ammonium sulphate slowly into the crude extract with constant stirring. The stirring was continued till ammonium sulphate was completely dissolved to aid the precipitation. Whole process was carried out in cold condition. The precipitated protein was separated by centrifuging at 10,000 rpm for 10 mins. The protein pellet was dissolved in minimal volume of 25 mM Tris buffer (pH 8.0) (Fig. 1).

Fig. 1. Ammonium sulphate precipitation.

Dialysis:

Dialysis is the technique used to remove the salts from the protein sample. The dialysis membrane acts as a semi-permeable membrane which allows the salts to move out of the membrane into a buffer with lesser ionic strength. Procedure: The protein samples were dialyzed using the low cut off membrane against 500 ml 2.5 mM Tris buffer (pH 8.0) for 24 hrs (Fig. 2).

Fig. 2. Dialysis.

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Column purification:

For the purification of Lipase enzyme two column matrices were used. The first approach was carried with gel filtration column followed by ion-exchange column.

Gel filtration chromatography: Gel filtration chromatography is used for separating molecules of different sizes and is also known as gel exclusion and molecular weight sieve. The basic principle is that molecules are partitioned between the solvent and a stationary phase of defined porosity.

Procedure: The Sephadex G-100 column material was activated by heating the matrix at 500˚C for 30 minutes with 25 mM Tris buffer. After cooling the floating fines were removed and loaded into a column without forming any air bubble the column was allowed to settle and was washed with 100 ml of 25 mM extraction buffer (pH 8.0). The dialyzed enzyme (4 ml) was loaded on to a Sephadex column pre-equilibrated with extraction buffer with simultaneous fraction collection. 25 fractions of 2 ml each were collected and analyzed for the eluted protein by measuring the absorbance at 595 nm followed by screening for the active fractions by finding the activities of the Lipase. The specific activity of the enzyme eluted at every step of purification and the total fold purification was calculated (Fig. 3).

Fig. 3. Gel filtration chromatography.

Ion exchange chromatography: Ion exchange is the separation of proteins on the basis of net charge which depends on the relative numbers of positive and negative charged groups on the surface of the molecule. This net charge will vary with the pH. There are two types of ion-exchangers used groups. The choice of the ion-exchangers is made according to the pH stability of the desired protein:

- Di-Ethyl Amino Ethyl (DEAE) group is an example used in anion exchange to purify negatively charged proteins.
- Carboxy Methyl (CM) group is used in cation exchange for the recovery of positively charged groups.

Procedure:

Pre swollen anion exchanger matrix, DEAE Cellulose was used for the matrix preparation. This matrix was prepared in 25 mM Tris buffer. The prepared ion exchange slurry was packed in 35 ml column without forming any air bubble. The column was allowed to settle and washed with 25 mM of Tris buffer (pH 8.0). The pooled active fraction of the gel filtration column (4 ml) was loaded onto a DEAE Cellulose column pre equilibrated with extraction buffer. The bound enzyme was eluted with linear gradient of 25-500 mM extraction buffer. The 25 fractions of 2 ml were collected. The fractions were analyzed for the eluted protein by reading the absorbance at 595 nm. The fractions were screened for lipase activity and the active fractions were pooled together (Fig. 4).

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Fig. 4. Ion exchange chromatography.

Enzyme Characterization

The Enzyme characterization influencing the lipase production studies were pH enzyme assay in different buffers (sodium Acetate buffer: pH 6; Phosphate buffer: pH 7; Tris buffer: pH 8; triss buffer pH 9), Incubation temperature (°C) (RT, 45˚C and 55˚C), Stability of lipase (incubation period 15 min, 30 min, 60 min), Effect of metal ion and inhibitor (incubated at 24 hrs).

The blank sample containing the reaction mixture of respective buffer, 0.1 ml tween80, 0.1 ml $CaCl_2$, additives and the test sample containing 1.7 ml of respective buffer, 0.1 ml tween80, 0.1 ml $CaCl_2$, 0.1 ml enzyme extract and additives was incubated and the OD were measured at 400 nm.

III. RESULTS AND DISCUSSION

Screening and Selection of Substrate

Solid substrate in SSF has a key role in the enzyme production. Most of the lipases of microbial origin are extracellular and excreted through the cell membrane into the culture medium consisting of a suitable solid substrate. Among all the agro wastes tested for lipase production, Niger waste (0.00208 µg/ml/min) holds the best capacity for lipase production by *P. aeruginosa*, which indicates that it contains all the basic nutrients for the bacterial growth and lipase production. Initial studies were carried out on Incubation period for 24 hrs and 48 hrs. Results are revealed in Figs. 5 and 6.

Effect of Incubation Period

Fig. 7 shows that high level of lipase activity was detected in early stages of incubation, i.e., at 24 hrs (0.0022 µg/ml/min) and the enzyme activity steadily decreased beyond 24 hrs which could be due to loss of moisture after prolonged incubation.

Effect of Moisture Content

The substrate Niger waste used in SSF should have enough moisture content to meet the microorganism's requirements for the liberation of value added products such as enzymes. The moisture content beyond the optimum level inhibits the enzyme production because the higher moisture level decreases the porosity due to gummy texture of the substrate that leads to poor oxygen transfer and decreases the diffusion. The lower moisture level, then optimum leads to the poor solubility of the nutrient of solid substrate, improper swelling and higher water tension. Solid substrate-to-moisturizing agent tested for lipase production that the maximum lipase activity was obtained with Niger seeds to mineral salt solution at a ratio of 1:2 (0.00196 µg/ml/min) as shown in Fig. 8.

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Effect of Temperature
Fig.9 shows that Pseudomonas sp produced maximum level of lipase at 45°C where no other temperature was found to be suitable for growth and enzyme secretion.

Effect of pH
Fig. 10 shows that optimum lipase activity at pH 8.0 (0.0019 µgm/ml/min). The activity was found to be decreasing by further increasing the pH values. The pH in and around 8.0 was found to be preferable for the better growth of organism and the lipase production.

Effect of Carbon Source
Fig. 11 shows that among the various carbon sources tested dextrose was found to give maximum result, whereas fructose, casein, sucrose gave relatively good results.

Purification of the Enzyme
Crude enzyme obtained was subjected to Ammonium Sulphate Precipitation and then Dialysis was carried out. After Dialysis the enzyme had a specific activity 559.52 U/µg. Upon further purification of enzyme by Gel filtration chromatography the enzyme had a specific activity of 34.94 U/µg with the fold purity of 16.61%. The enzyme purification was carried out with DEAE- Cellulose column using ion exchange chromatography to obtain pure and homogenous enzyme fold purity of 129.50%. The lipase activity was characterized for the eluted enzyme sample and the molecular weight was found under both fermentation conditions to be 60 kDa by SDS-PAGE (Figs. 12 and 13).

Enzyme Characterization

Effect of temperature:
Effect of temperature on enzyme activity was assayed for different temperature. Fig.14 presents that enzyme activity is optimum at temperature 400c (2318U).

Effect of pH on enzyme activity:
For optimum pH, enzyme assay was carried out in which enzyme activity is maximum at pH 8 (1303.8U). Relatively good activity at pH 6.0 as shown in Fig.15.

Stability of enzyme:
Fig.16 shows that the enzyme activity decreases as time duration increases. It gave relatively good enzyme activity after 30 mins (1932U) of incubation.

Effect of metal ions and inhibitors:
In proteins there is active site which is responsible for its activity. Amino acids present in its active site. Metal ion binding at active site may increase its activity or may inhibit its activity. It depends on enzyme is metalloid or non metalloid. In present study Fig.17 shows that enzyme activity is inhibited by meal ion.

Kmax and Vmax value:
Kmax establishes the value for the intra cellular level of substrate. Kmax as equal to the substrate at which initial velocity is one half the maximal velocity. The maximum rate observed when the enzyme is in the form of the enzyme substrate complex. When 1/V is plotted against 1/S straight line is obtained this line have a slope of Km/Vmax, and intercept of 1/Vmax on the 1/V axis and an intercept of -1/Kmax on the 1/S axis as in Fig.18. This has the advantage of allowing a much more accurate determination of Vmax value.

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IV. CONCLUSION

The present study explores an agro industrial residue (Niger waste) which has the potential to produce lipase in an economically feasible process by a new promising bacterial strain of *P. aeruginosa*. Such a high level of lipase activity (0.0022 µg/ml/min) was achieved in 24 h of solid state fermentation at 45°C. Lipase activity with optimized growth conditions by *P. aeruginosa* led to 1.83 folds enhancement in lipase biosynthesis. It is concluded from the results that lipase produced by *P. aeruginosa* was stable in acidic pH and even at higher temperatures. In conclusion, this work will be of great value for production of lipase commercially by *P. aeruginosa* with high enzyme activity.

Fig. 5. Agrowastes and *Pseudomonas aeruginosa*.

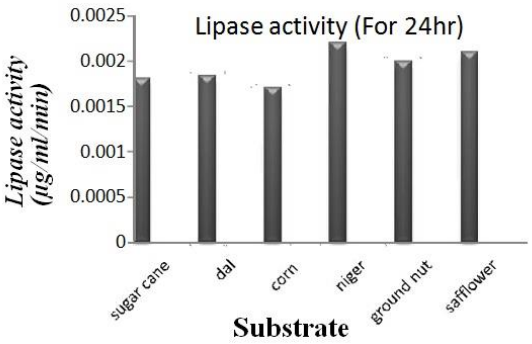

Fig. 6. Lipase activity for 24 hrs.

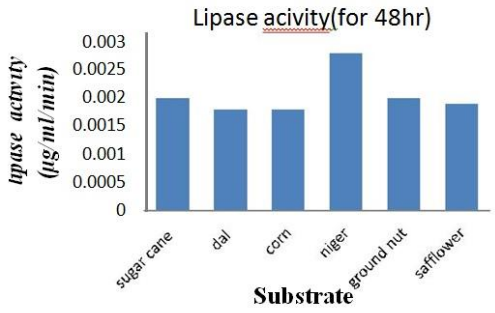

Fig. 7. Lipase activity for 48 hrs.

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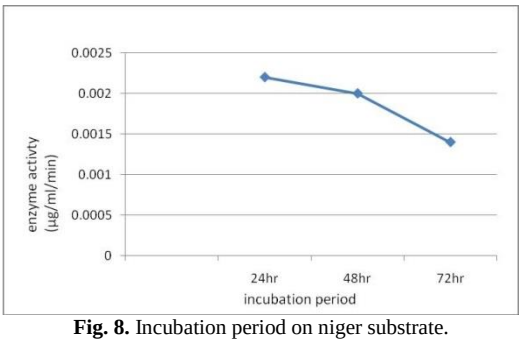

Fig. 8. Incubation period on niger substrate.

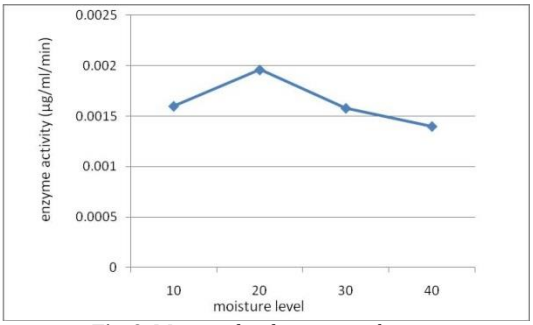

Fig. 9. Moisture level on niger substrate.

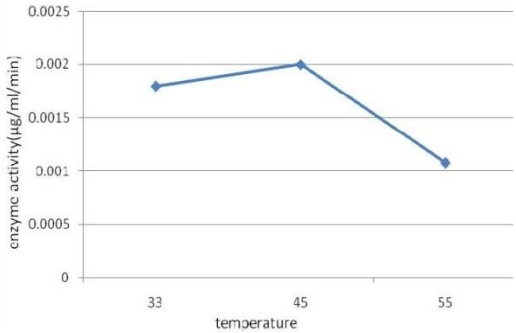

Fig. 10. Effect of temperature on niger substrate.

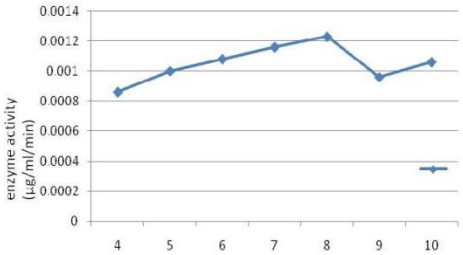

Fig. 11. Effect of pH on niger substrate.

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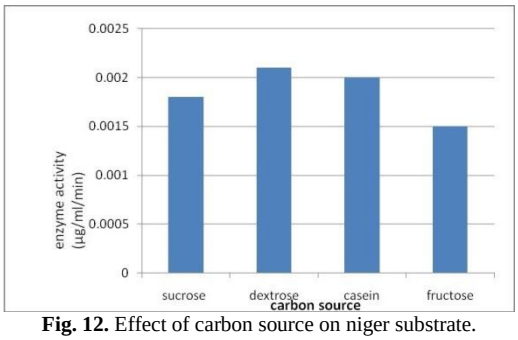

Fig. 12. Effect of carbon source on niger substrate.

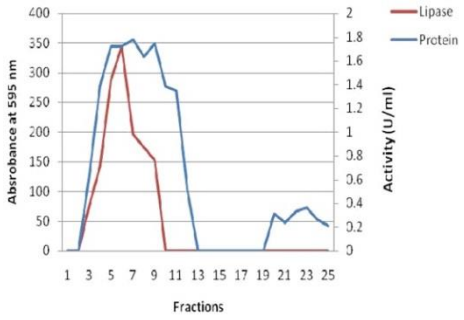

Fig. 13. Gel filtration chromatography.

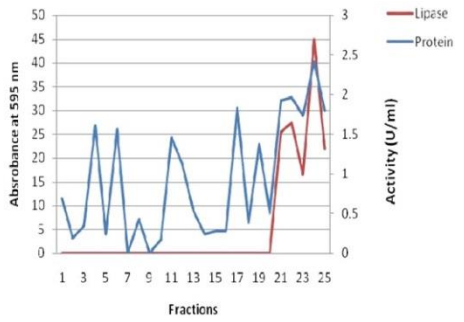

Fig. 14. Ion exchange chromatography.

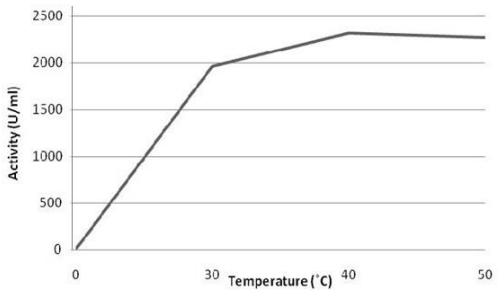

Fig. 15. Effect of temperature on enzyme activity.

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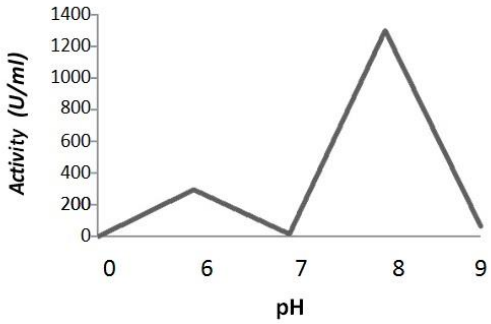

Fig. 16. Effect of pH on enzyme activity.

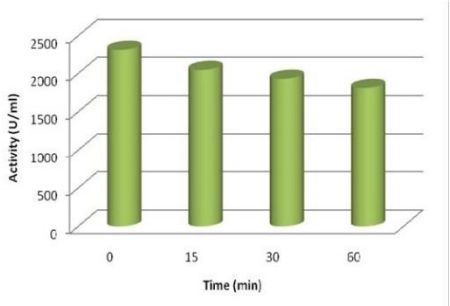

Fig. 17. Stability of enzyme.

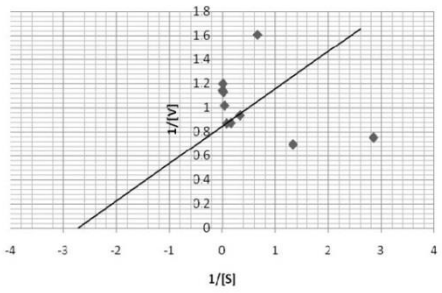

Fig. 18. K_{max} and V_{max} value

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