Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 2 (5): 469-474, 2007
ISSN 1816-4935
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Lipase Production by *Yarrowia lipolytica* NCIM 3589 in Solid State Fermentation Using Mixed Substrate

Imandi Sarat Babu and Garapati Hanumantha Rao
Department of Chemical Engineering, Center for Biotechnology, Andhra University, Visakhapatnam-530 003, India

Abstract: A comparative study on the extra cellular lipase production in Solid State Fermentation (SSF) using *Yarrowia lipolytica* NCIM 3589 with various mixed substrates has been made. Different parameters such as moisture content, carbon level and nitrogen level of the medium were optimized. The maximum lipase activity of 9.3 units per gram of dry fermented substrate (U g ds^{-1}) was observed with mixed substrate of sugarcane bagasse and wheat bran in seven days of fermentation.

Key words: Lipase, mixed substrate, solid state fermentation, *Yarrowia lipolytica*

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are one of the most important classes of industrial enzymes. They hydrolyse triglycerides into diglyceriods, monoglycerides, glycerol and fatty acids. In recent years, there has been an increasing interest in the study of lipases mainly due to their potential applications as medicines (digestive enzymes), food additives (flavour modifying enzymes), clinical reagents (glyceride-hydrolysing enzymes) and cleaners (detergent additives) (Sharma *et al*., 2001). Additionally, a promising application field for lipases is in the biodegradation of plastics such as Polyhydroxyalkanoates (PHA) and Polycaprolactone (PCL) (Jaeger and Reetz, 1995; Mochizuki *et al*., 1995).

Solid state fermentation is defined as the fermentation of solids in the absence of free water, however, the substrate must possess enough moisture to support the growth and metabolism of microorganisms. Recently, several reports have been published indicating the application of this culture in upgrading food and industrial wastes and in the production of fine chemicals and enzymes. The utilization of by-products and wastes from food and industrial sources has several advantages over submerged fermentation such as superior productivity, simple techniques, reduced energy requirements, low wastewater output, improved product recovery and the reduction in production costs, since they supply the microorganisms with some nutritive substances (Ashok, 2003). In SSF, any type of substrate could be used to enhance the production of enzymes because many food and industrial wastes are rich in fatty acids, triacylglycerols and/or sugars. The use of cheap raw materials would diminish the operating costs of the process. Moreover, total capital investment for lipase production has been reported to be significantly lower in solid state fermentation than in submerged fermentation (Castilho *et al*., 2000).

Most studies on lipolytic enzymes production by bacteria, fungi and yeasts have been performed in submerged fermentation; however, there are only few reports on lipase synthesis in solid state fermentation. In recent years, increasing attention has been paid to the conversion of industrial wastes to lipase by solid state fermentation. There are several reports dealing with extra cellular lipase

Corresponding Author: Imandi Sarat Babu, Department of Chemical Engineering, Center for Biotechnology, Andhra University, Visakhapatnam-530 003, India
Tel: +91-891-2844878 Fax: +91-891-2747969

production by fungi like *Rhizhopus* sp., *Aspergillus* sp., *Pencillium* sp. on different substrates (Christen *et al*., 1995; Kamini *et al*., 1998; Cordova *et al*., 1998; Gombert *et al*., 1999; Miranda *et al*., 1999).

Sugarcane bagasse, wheat bran and rice bran are produced abundantly in India. The present research was undertaken to optimize process conditions: substrate level, moisture content, carbon level and nitrogen level of the medium, for the production of lipase using agricultural residues (sugarcane bagasse, wheat bran and rice bran) by SSF.

MATERIALS AND METHODS

Substrate

Sugarcane bagasse, wheat bran and rice bran were used as the substrates. They were procured from a local market of Visakhapatnam, India and were dried at 60°C for 72 h to reduce the moisture content and ground to the desired size.

Microorganism

Yarrowia lipolytica NCIM 3589 obtained from National Chemical Laboratory, Pune, India, was used throughout the study.

Growth Conditions

The culture was maintained on MGYP slants having the composition (%): Malt extract 0.3, glucose 1.0, yeast extract 0.3, peptone 0.5 and agar agar 2.0. The pH of the medium was adjusted to 6.4-6.8 and culture was incubated at 30°C for 48 h. Subculturing was carried out once in every 2 weeks and the culture was stored at 4°C.

Inoculum Preparation

The yeast *Yarrowia* strain was cultivated in a medium containing peptone 5 g, yeast extract 3 g and sodium chloride 3 g per liter of distilled water. The cells were cultivated in this medium at 30°C on a shaker at 200 rpm for 24 h (Oswal *et al*., 2002).

Media Preparation

Ten grams of substrate was weighed into a 250 mL Erlenmeyer flask and to this a supplementing salt solution was added to the desired moisture level. The composition of the salt solution was as follows (% w w^{-1}): Urea, 0.2; KH_2PO_4, 0.1; $MgSO_4.7H_2O$, 0.05; $CaCl_2$, 0.01; NaCl, 0.01; H_3BO_3, 0.00005; $CuSO_4.5H_2O$, 0.000004; KI, 0.00001; $FeCl_3.4H_2O$, 0.00002; $ZnSO_4.7H_2O$, 0.00004; $MnSO_4.H_2O$, 0.00004; myo-inositol, 0.00000004 and d-biotin, 0.00000008 (Corzo and Revah, 1999). The substrate medium without vitamins was sterilized at 121°C for 15 min. After cooling, the vitamins previously sterilized by filtration were added to the substrate medium.

Solid State Fermentation

The sterilized solid substrate was inoculated with 2 mL of inoculum. The contents were mixed thoroughly and incubated in a slanting position at 30°C.

Enzyme Extraction

The crude enzyme from the fermented material was extracted by simple extraction method. For this, the fermented substrate was mixed thoroughly with 50 mL of 50 mM phosphate buffer (pH 7.0) and then shaking the mixture in a rotary shaker (200 rpm) for 60 min at 37°C, a temperature high enough to increase the extraction efficiency without causing enzyme denaturation (Freire *et al*., 1997). The raw extract was obtained by pressing the mixture and subsequent centrifugation. The supernatant was used to determine enzyme activity (Gombert *et al*., 1999).

Lipase Assay

The activity of lipase was determined as described in the literature (Winkler and Stuckman, 1979) with the following modifications: 1 mL of isopropanal containing 3 mg of p-Nitrophenyl Palmitate (*p*NPP) was mixed with 9 mL of 0.05 M Tris-HCl buffer (pH 8.0), 40 mg of Triton X-100 and 10 mg of gum arabic. Liberation of *p*-nitrophenol at 28°C was detected at 410 nm. One enzyme unit was defined as 1 μmol of *p*-nitrophenol enzymatically released from the substrate per minute (Bruno *et al*., 2004).

Optimization of Medium Parameters

The strategy adopted was to optimize one particular parameter at a time and then include it at its optimum value in the next optimization step, if found beneficial. The parameters optimized were: substrate level, incubation time, moisture content, carbon level and nitrogen level.

RESULTS AND DISCUSSION

Substrate Selection

Among all the substrates, the maximum lipase activity was observed for the combination of sugarcane bagasse with wheat bran (Table 1). These results were in accordance with the observed lipase production by *Pencillium restrictum* (Gombert *et al*., 1999) and *Asprgillus flaavus* USM A 10 on different substrates (Pau and Omar, 2004).

Optimization of Substrate Levels

Surface area occupied by the substrate was an important parameter in the SSF. Figure 1 depicts that an amount of 10 g substrate yields maximum production of lipase. The less yield at higher levels was due to the low mass transfer rate and difficulty in the penetration of the organism (Rao *et al*., 2003).

Table 1: Effect of different substrates on lipase activity

Substrates	Lipase activity (U g ds^{-1})
Rice bran	0.7
Sugarcane bagasse	2.4
Wheat bran	2.1
Rice bran+sugarcane bagasse	1.8
Rice bran+wheat bran	1.2
Sugarcane bagasse+wheat bran	4.7

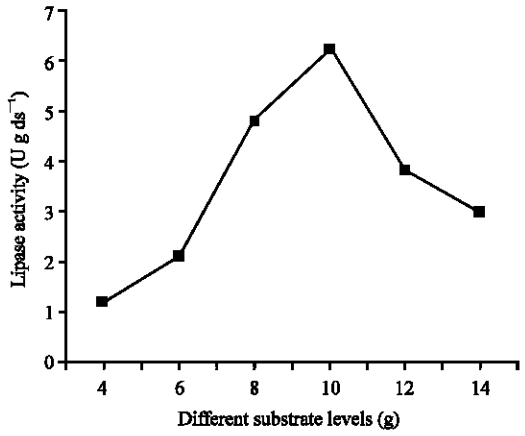

Fig. 1: Effect of different substrate levels on lipase activity

Effect of Incubation Time

The amount of lipase produced was observed daily during a period of nine days. The maximum lipase activity was observed on seventh day as shown in Table 2. After seventh day, it was reduced due to the consumption of nutrient materials.

Effect of Moisture Content

Moisture content of the substrate plays a vital role for microbial growth and biochemical activities in SSF. The maximum yield was obtained at 80% moisture content as listed in Table 3. In SSF processes, higher moisture content would lead to decreased porosity, change in wheat bran particle structure, development of stickiness, reduction in gas volume and decreased diffusion. On other hand, the insufficient moisture leads to the reduction of solubility of nutrients present in wheat bran (Babu and Satyanarayana, 1996).

Effect of Carbon Source

The imperative role of different carbon sources on lipase production by this organism was elucidated by incorporating the selected carbon source (1 g) to the mixed substrate. Table 4 presents the results of different carbon sources on lipase activity. Among all the carbon sources, glucose had better impact on productivity.

Effect of Nitrogen Source

Nitrogen source mediated lipase production was well documented in submerged fermentation (Corzo and Revah, 1999) and SSF (Dominguez *et al*., 2003). Table 5 depicts the role of different nitrogen sources on lipase production. Among all the nitrogen sources, urea yields maximum lipase activity. Further studies were conducted by taking urea at different levels. Figure 2 shows that 1 g of the urea yielded maximum lipase activity. However, at higher levels the production was reduced due to the inhibitory effects of urea.

Table 2: Effect of incubation time on lipase activity

Incubation time (days)	Lipase activity (U g ds^{-1})
1	0.8
2	1.4
3	2.5
4	3.2
5	3.9
6	4.5
7	6.8
8	4.8
9	3.7

Table 3: Effect of moisture content on lipase activity

Moisture content % (v/w)	Lipase activity (U g ds^{-1})
30	1.3
40	2.8
50	3.6
60	4.2
70	6.7
80	7.2
90	6.4
100	5.3
110	4.1

Table 4: Effect of different carbon sources on lipase activity

Carbon source	Lipase activity (U g ds^{-1})
Glucose	8.4
Sucrose	7.8
Maltose	7.2
Fructose	7.3

Table 5: Effect of different nitrogen sources on lipase activity

Nitrogen source (g)	Lipase activity (U g ds^{-1})
Yeast extract	7.1
Malt extract	7.9
Urea	8.9
Ammonium sulphate	7.3
Ammonium nitrate	8.0
Sodium nitrate	7.1
Yeast extract+Ammonium sulphate	7.5
Yeast extract+Ammonium nitrate	8.4

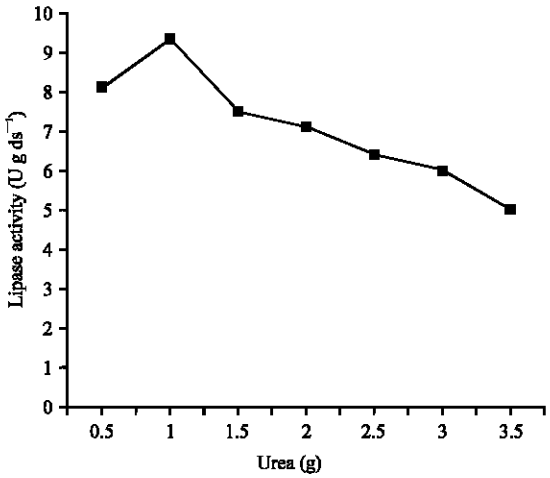

Fig. 2: Effect of urea concentration on lipase activity

CONCLUSIONS

Solid state fermentation of mixed substrate by *Yarrowia lipolytica* NCIM 3589 in the presence of sugarcane bagasse and wheat bran with moisture content of 80% yielded 9.3 U g ds^{-1} of lipase activity in 7 days. The mixed substrate use of sugarcane bagasse and wheat bran for lipase production may have the combined benefit of utilizing a low value waste material while producing a commercially valuable product.

ACKNOWLEDGMENTS

This work was carried out in the Center for Biotechnology, Department of Chemical Engineering Andhra University, Visakhapatnam during 2006 with the financial assistance received from the University Grants Commission (SAP, Phase-III), New Delhi, India.

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