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# Effect of Inoculum Age, Carbon and Nitrogen Sources on the Production of Lipase by Candida Cylindracea 2031 in Batch Fermentation

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**Abstract.** Production of extracellular lipase by Candida cylindracea DSMZ 2031 was studied in a seven liters batch bioreactor, using palm oil (PO), palmitic acid (PA), lauric acid (LA), olive oil (OO) and cooking oil (CO) as carbon source. The effect of carbon and nitrogen sources were studied by measuring the lipase activity. The maximum lipase activity was found to be 12.7 kLU on palm oil as carbon source, urea as nitrogen sources and at 36 h inoculum age. This was achieved at a temperature of 30$^{o}$ C, pH of 6.0, agitation speed of 500 rpm and aeration of 1vvm. Copyright © 2006 Teknik Kimia UNSYIAH

*Keywords*: lipase production, *candida cylindracea*, palm oil, stirred bioreactor

## INTRODUCTION

Interest in lipase enzymes has been greatly developed in the past few years due to their potential application in fat splitting as well as in synthesis of glycerides, the large number of reaction, not necessarily esterification reaction, that they can catalyze. The advantages of the enzymatic hydrolysis over the chemical process are less energy requirements and higher quality of the obtained product. (Posorske, 1984, Noor et al., 2003).

The main uses of industrial lipases are as additives to washing detergents and in food industry, such as cheese ripening, preparation of cocoa butter substitutes and flavour production. Lipases have also been used for tanning, sewage treatment, in cosmetic industries and for transesterification reaction of triglycerides.

Lipase activity has been found in different mold, yeast, and bacteria. Although numerous papers have been published on selection of lipase producers, there is less available information on the fermentation process (Iwai and Tsujisaka, 1984, Suzuki et al., 1988). These information are important in order to identify the optimal operation condition for enzyme production.

The highest activity of lipase obtainable depended on the type cell of used for its production. In fungi, although lipidic substrates and fatty acid generally act as inducers, lipase is produced constitutively. The yeast *Candida cylindracea* is an important lipase producer. Among microbial cells the most commonly used for lipase production is the yeast, *Candida cylindracea*. (Bistline, 1991, Del Rio et al., 1990, Marcrae and Hammond., 1985). *Candida cylindracea* produces extracellular lipases in the presence of lipid material, especially when a fat and a steroid are present simultaneously [Otta et al., 1982). In spite of its wide use, there are not many information in the open literature about the factors and condition that control its

biosynthesis and secretion of lipase production on palm oil by *Candida cylindracea* is available. However, a common characteristic in lipase production is the use of a lipid such as triglycerides or fatty acid, solely or jointly with glucose as carbon source, as inducer of the production. Nevertheless, the role of inducer is not clearly understood (Gordillo et al., 1998). Valero et al. (1991, 1998) showed that lipase production was sensitive to glucose repression. This paper discusses of the effect of carbon and nitrogen sources on the production lipase by *Candida cylindracea.*

## MATERIALS AND METHODS

### *Materials*

Materials used in this research were microorganisms, chemicals for preservation of the microbe in slant, culture medium and for analytical procedures.

*Microorganism*

*Candida cylindracia* DSM 2031 obtained from German Collection of Microorganisms and Cell Cultures, Mascheroder Weg 1 b, Braunschweig, Germany, was maintained on universal yeast-agar (UY) medium at $4^o$ C as described by Benjamin and Pandey (1997).

*Chemicals*

All chemicals and medium components used for the experiments were of analytical grade. There were: peptone, yeast extract, malt extract, $KH_2PO_4$, $MgSO_4.7H_2O$, Urea, $FeCl_3.6H_2O$ were purchased from Sigma Chemical Company, USA. The trace elements are inositol, thiamine hydrochloride and biotin were obtained from Fluka Chemie AG, Switzerland. The carbon substrate used were glucose purchased from Ajax Chemicals, Auburn NSW, Australia, and palm oil (PO) obtained from Palm Oleo Sdn Bhd, Kuala Lumpur.

### *Methods*

*Preparation of universal yeast (UY) agar*

*Candida cylindracea* (DSM 2031) was grown and maintained using the Universal Yeast (UY) - Agar medium at $4^o$ C. The medium consists of ($g.L^{-1}$): glucose 10, peptone 5, yeast extract 3, malt extract 3 and agar 15 (pH 7.0) in distilled water.

*Inoculum Preparation*

Four 500 mL conical flask containing 125 mL growth medium (in $g.L^{-1}$) contained $KH_2PO_4$ 6, $MgSO_4.7H_2O$ 1 , urea 4 , and micronutrients (in $mg.L^{-1}$): $FeCl_3.6H_2O$ 10, inositol 0.4, thiamine hydrochloride 0.2 and biotin 0.8 with glucose 10 $g.L^{-1}$ as carbon sources were sterilized in an autoclave at $121^o$ C for 20 minutes. For preparing the inoculum, a loop full of cells from a freshly grown culture (agar slant) of *Candida cylindracea* was transferred to the flask. The flask was incubated at $30^o \pm 1^o$ C on a rotary shaker at 200 rpm for 36 hrs. The medium used for preparing the inoculum is the same as the production medium but with only glucose as the carbon source. Palm oil (PO) was not used in the inoculum preparation medium,

*Batch fermentation*

Fifty gram of sterile palm oil (PO) in a storage bottle was transferred aseptically using a peristaltic pump to the sterilised medium in the fermentor. The inoculum used 10% of the working volume. This was transferred aseptically by bunsen burning around the septum port. The inoculum medium was of similar composition and concentration as the media for production.

Samples of 50 mL were withdrawn aseptecally at regular time interval for analysis. The fermentation was carried out at a temperature of $30.0^o$ C, pH of 6.0, aeration of 1.0 vvm and stirrer speed of 500 rpm. The pH of the fermentation was maintained constant by addition of sterile 3N NaOH and 3N $H_2SO_4$ solution. Exhaust gases were removed through on the exit gas filter stream at the top of the fermentor.

The exhaust gas line was connected to an off line oxygen / carbon dioxide monitoring unit to measure its composition (Noor et al., 2000).

### *Analytical methods*

*Dry weight biomass*

At regular intervals, a 10 $cm^3$ sample was collected from the bioreactor and filtered through a Whatman filter paper (glass microfibre filters (gf/c), pore diameter 0.45 μm), washed with 10 $cm^3$ of ethanol (70 %), followed by 10 $cm^3$ of n-Hexane to remove traces of crude palm oil, and finally with 40 $cm^3$ distilled water. The filter paper was dried at 85$^o$ C for 10 hours to constant weight (Noor et al., 2000). Sample was measured on either direct or diluted at 640 nm absorbance against water as blank in a spectrophotometer. A calibration curve of asorbance against dry weight was prepared.

*Estimation of glucose*

Glucose was estimated by an enzymatic method using hexokinase and glucose-6-phosphate dehydrogenase (Sigma dianosticsa glucose HK Procedure No. 16-UV). The absorbance of NADPH was measured at 340 nm.

*Estimation of lipase activity*

Lipase activity was determined using tributyrin as the substrate (NOVO Nordisk A/S., 1991). The activity was expressed as unit per mL. One unit of lipase activity was defined as the amount necessary to hydrolyze 1 μmol of ester bond per minute under the assay conditions.

## RESULTS AND DISCUSSIONS

### *Effect of different carbon sources*

Carbon is the main component of cells and some natural fats or oils have been used as carbon sources and inducers for lipase fermentation. The effect of carbon source is shown in Table 1. Plant oils containing palmitic acid, lauric acid such as palm oil and olive oil are suitable for biosynthesis of lipase from *Candida cylindracea* DSM 2031.

To assess the effect of different substrates as carbon sources for lipase production, experiments were carried out using palm oil (PO), olive oil (OO), glucose, palmitic acid (PA), lauric acid (LA) and palm cooking oil (PCO) as carbon sources. PO, OO and glucose, PA, LA and CO were incorporated in the medium to promote lipase induction.

**Table 1.** Effect of different carbon sources

| Parameter | Range of variable (o C) | Lag period (h) | □ max ($h^{-1}$) | Max. Cell Conc. (g $L^{-1}$) | Max. lipase activity (kLU $L^{-1}$) | Productivity (kLU $L^{-1}$ $h^{-1}$) | $Y_{X/S}$ of lipase production | $Y_{P/S}$ of lipase production |
|---|---|---|---|---|---|---|---|---|
| Different carbon sources | Glucose | 4.5 | 0.137 | 7.54 | 0.22 | 0.006 | 0.71 | 0.06 |
| | Palm oil | 10.5 | 0.101 | 5.8 | 12.7 | 0.368 | 2.36 | 5.75 |
| | Palmitic acid | 10.5 | 0.053 | 4.89 | 1.52 | 0.038 | 5.3 | 1.9 |
| | Lauric acid | 13 | 0.645 | 3.6 | 1.55 | 0.043 | 7.575 | 3.8 |
| | Olive oil | 16 | 0.082 | 5.17 | 2.26 | 0.074 | 2.86 | 1.413 |
| | Palm Cooking oil | 12 | 0.81 | 6.9 | 1.68 | 0.047 | 5.24 | 1.4 |

Operation condition: pH of 6.0, Agitation speed of 500 rpm, temperature 30$^o$ C and aeration 1 vvm, concentration of carbon sources of 10 g.$L^{-1}$.

The fermentation profiles in these six substrates are shown in Figures 1 to 4. The final cell concentration and lipase activity obtained, lag period, the estimated maximum specific growth rate, average enzyme productivity, fatty acid produced, the yield of product – substrate ($Y_{P/S}$), the yield of biomass – substrate ($Y_{X/S}$) and fatty acid produced are shown in Table 1. It can be seen from the Figures that the yeast grew in all the substrates but it exhibited a longer lag phase in olive oil (16 h) compared to the lag in crude palm oil (10.5 h) and in glucose (6.0 h).

Comparing the maximum specific growth of the cells in the six substrates, the yeast grew more rapidly in glucose (0.137 $h^{-1}$) than in other sources (about 0.10 to 0.65 $h^{-1}$). Also the final activity of lipase obtained was higher in PO (12.70 $kLU.L^{-1}$ at 34.5 h) compared to in OO (2.26 $kLU.L^{-1}$ at 36 h) and glucose (0.22 $kLU.L^{-1}$ at 36 h). Also in LA, PA and PCO about (1.52 – 1.62 $kLU.L^{-1}$ at 36 h). However, the final biomass concentration obtained was higher in glucose (7.54 $g.L^{-1}$) and increased with an increase in glucose concentration when compared to other substrates. The enzyme productivity in PO was 0.368 $kLU.L^{-1}.h^{-1}$ and is higher compared to in other oils.

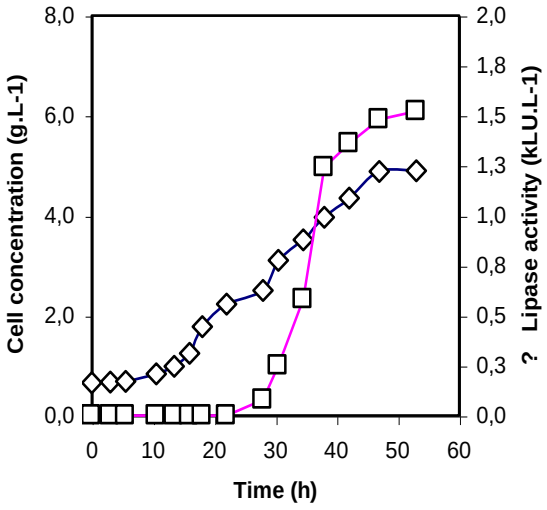


**Figure 1**. Profile of lipase production in 10 g $L^{-1}$ palmitic acid ( pH=6.0, T= 30° C, Aeration = 1 vvm, Agitation = 500 rpm )

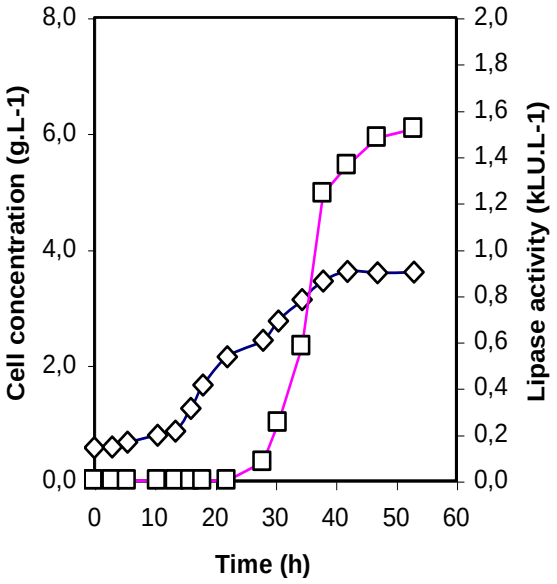


**Figure 2**. Profile of lipase production in 10 g $L^{-1}$ lauric acid ( pH=6.0, T= 30° C, Aeration = 1 vvm, Agitation = 500 rpm )

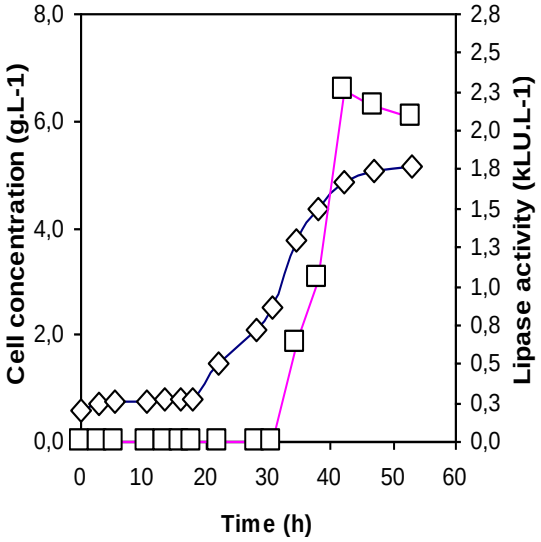


**Figure 3**. Profile of lipase production in 10 g $L^{-1}$ olive oil ( pH=6.0, T= 30° C, Aeration = 1 vvm, Agitation = 500 rpm )

The maximum cell concentration in PO was observed to be lower than when grown in palm cooking oil and glucose, but lipase activity was higher in the former. With OO, oleic acid (composition: 18:2) maybe produced and then consumed by the cells. However with PO, palmitic acid (composition: 16:0) would be the acid

produced and also then consumed by the cells.

In the absence of glucose in the OO and PO and other oil media, the cells were induced to produce lipase to break down the lipid to derive energy and carbon for growth. Initially the growth in PO was exponential (10 to 18 h). After 18 h, the growth is linear as the availability of lipid due to its low solubility in the aqueous medium may be limiting the growth.

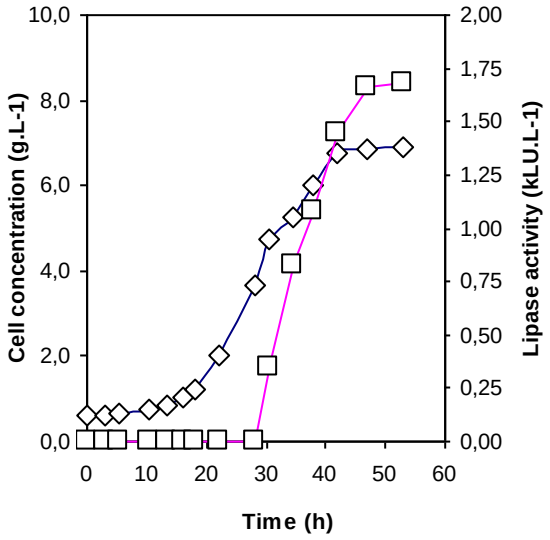


**Figure 4**. Profile of lipase production in 10 g $L^{-1}$ palm cooking oil ( pH=6.0, T= 30° C, Aeration = 1 vvm, Agitation = 500 rpm )

Shimada et al. (1992) found out lipase activity increased with the chain length of the fatty acid. It was suggested that long-chain fatty acids participated in the expression of lipase genes and induction is controlled at the level of transcription.

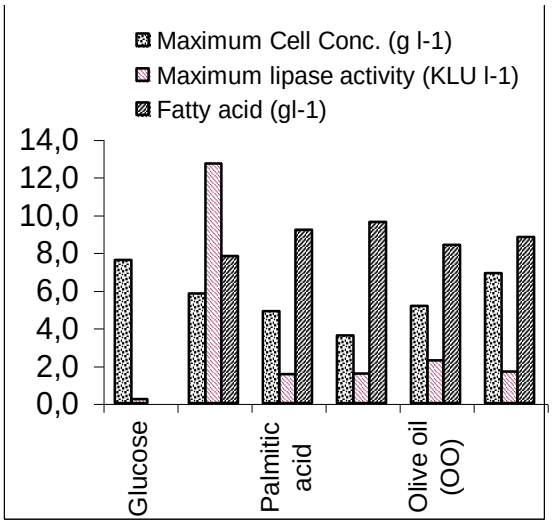


**Figure 5**. Effect of lipase production on different substrates ( pH=6.0, T= 30° C, Aeration = 1 vvm, Agitation = 500 rpm and cooking oil = 10

### *Effect of nitrogen sources on lipase production*

The effect of nitrogen sources in the media on the lipase production was studied. The concentration of each of the nitrogen sources was 1% w/v. The results are given in Table 2.

**Table 2**. Effect of nitrogen sources

| Parameter | Range of variable (o C) | Lag period (h) | □ max ($h^{-1}$) | Max. Cell Conc. ($gL^{-1}$) | Max. lipase activity ($kLU.L^{-1}$) | Produc-tivity ($kLU.L^{-1}.h^{-1}$) | $Y_{X/S}$ of lipase produc-tion | $Y_{P/S}$ of lipase produc-tion |
|---|---|---|---|---|---|---|---|---|
| Different nitrogen sources | Urea | 10.5 | 0.101 | 5.8 | 12.7 | 0.368 | 2.36 | 5.75 |
| | NH4Cl | 10.5 | 0.084 | 5.73 | 10.11 | 0.215 | 1.931 | 1.023 |
| | KNO3 | 6 | 0.107 | 4.92 | 8.88 | 0.222 | 1.313 | 0.87 |
| | NH4NO3 | 5.5 | 0.622 | 5.12 | 9.5 | 0.211 | 1.57 | 3.393 |

Operation condition: pH of 6.0, Agitation speed of 500 rpm and temperature 30° C and aeration 1 vvm.

**Table 3.** Effect of inoculum age

| Parameter | Range of variable (h) | Lag period (h) | □ max ($h^{-1}$) | Max. Cell Conc. ($g.L^{-1}$) | Max. lipase activity ($kLU.L^{-1}$) | Productivity ($kLU.L^{-1}\ h^{-1}$) | $Y_{X/S}$ of lipase production | $Y_{P/S}$ of lipase production |
|---|---|---|---|---|---|---|---|---|
| Inoculum age | 36 h | 10.5 | 0.101 | 5.8 | 12.7 | 0.368 | 2.36 | 5.75 |
| | 48 h | 13 | 0.071 | 5.5 | 12.3 | 0.255 | 1.728 | 4.68 |
| | 60 h | 16.6 | 0.061 | 5.24 | 12.1 | 0.228 | 1.662 | 4.638 |

Operation condition: pH of 6.0, Agitation speed of 500 rpm and temperature 30° C and aeration 1 vvm.

Figure 6 shows that the higher lipase activity was produced with urea compared to the other sources. It was reported that secretion of lipase was maximum on addition of ammonium phosphate, by organism *Rhodotorula glutinis* (Shimada et al., 1992).

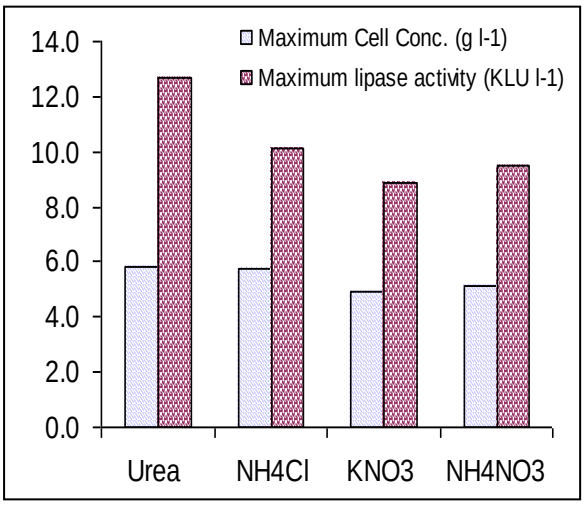


**Figure 6**. Effect of nitrogen sources on lipase production ( pH=6.0, T= 30° C, Aeration = 1 vvm, Agitation = 500 rpm and palm oil = 10 g $L^{-1}$)

### *Effect of inoculum age on lipase productio*n

The selection of inoculum age was at the optimum operation condition at pH 6, temperature of 30° C, agitation speed of 500 rpm, aeration 1 vvm and palm oil concentration 10 $g.L^{-1}$.

The fermentation profiles to show the effect of inoculum age are shown in Figures 7. and Table 3. The effect of inoculum age was done with 36 h to 60 h incubation time at 30° C, 200 rpm shaker speed. The results showed that the lipase activity was maximum in the 36 h old culture.

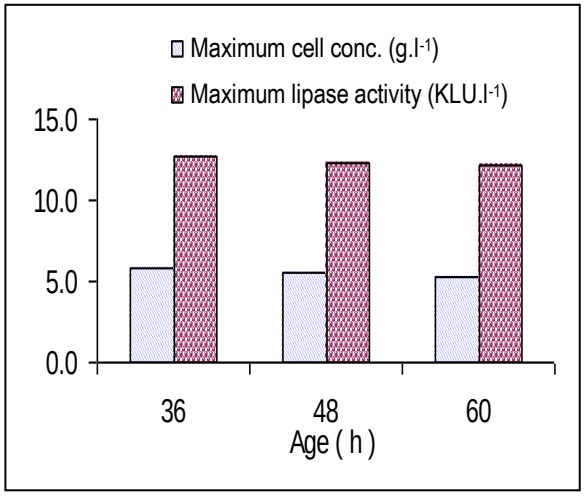


**Figure 7**. Effect of inoculum age on lipase production ( pH=6.0, T= 30° C, Aeration = 1 vvm, Agitation = 500 rpm and glucose = 10 g $L^{-1}$)

## CONCLUSION

Increasing palm oil concentration improved the synthesis of lipase but did not enhance growth of *Candida cylindricae.* Experiments carried out using palm oil (PO), olive oil (OO), glucose, palmitic acid (PA), lauric acid (LA) and palm cooking oil (PCO) as carbon sources, showed that the highest lipase activity was recorded with PO.

Different sources of nitrogen were tested in order to determine their influence on the synthesis of lipase. Results showed

that maximum lipase activity was recorded when the media utilized urea.

The effect of inoculum age was done which varied from 36 h to 60 h at the incubation temperature of 30° C and shaker speed of 200 rpm. The results showed that lipase activity was maximum with a 36 h old culture as inoculum.

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