ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

# Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 7 (1): 112-117, 2008
ISSN 1682-296X


# Optimisation of *Candida rugosa* Lipase Esterase Activity

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**Abstract:** We aimed to optimize esterase production of *Candida rugosa* lipase (CRL). Active culture of *C. rugosa* (DSM 2031) was revived and the culture medium containing the most frequently used ingredients was optimized using a fraction of factorial design method, Taguchi. Temperature and pH of the culture was also optimized using one factor at a time method. The optimum combination of the major medium ingredients, in order of their magnitude, was (g $L^{-1}$): Corn Steep Liquor (CSL) powder, (40), triolein (glyceril trioleate) (10), glucose (0) and oleic acid (2). The optimum temperature and pH were 30°C and 7, appropriately. Using this combination and conditions, esterase activity of the enzyme preparation was increased up to 9 U $mL^{-1}$, which was equivalent to 20611 U $mL^{-1}$ of Sigma® lipase lipolytic activity.

**Key words:** *Candida rugosa*, esterase activity, lipase production, optimization, Taguchi method

## INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyze both hydrolysis and esterification of esters made up of glycerol and long chain fatty acids (Sharma *et al*., 2001). Lipases can be found in many organisms, but those originated from microorganisms are cheaper and more stable.

The yeast *Candida rugosa* is an important producer of lipase and *C. rugosa* lipase (CRL) has been used in many biotransformations, organic acids and alcohols, detergent making, food and flavour industry, production of ice cream and single cell protein, production of carbohydrate esters, amino acid derivatives, biocide making, biosensor modulations, bioremediation, cosmetics and perfumery (Benjamin and Pandey, 1998; Bornscheuer and Kazalauskas, 1999; Ferrer *et al*., 2001; Dominguez de Maria *et al*., 2006). It has been claimed that CRL poses versatile catalytic reactions, broad specificities and more applications than any other biocatalyst. It has also been introduced as the best lipase for detergent industries (Ratledge and Tan, 1990).

The molecular mass of CRL is 60 kDa, containing 534 amino acids and is moderately glycosylated (Bornscheuer and Kazlauskas, 1999; Lotti and Alberghina, 2000). *C. rugosa* secretes multiple lipase isoenzymes (CRLs), which are strongly related in their amino acid sequence with the same molecular weight, but partially differ in their catalytic properties (Alberghina and Lotti, 1997; Lotti and Monticelli *et al*., 1998; Ferrer *et al*., 2001; Dominguez de Maria *et al*., 2006). Heterologous expression of CRL isoenzymes has been reported as well (Brocca *et al*., 1998). Commercial CRLs contain 2-11 percent proteins and the remaining compositions are sugars or inert vehicles.

Different carbon sources (Valero *et al*., 1991; Obradors *et al*., 1993; Benjamin and Pandey, 1996; Benjamin and Pandey, 1997; Lakshmi *et al*., 1999; Sanchez *et al*., 1999; Dalmau *et al*., 2000; Song *et al*., 2001; Tan *et al*., 2003; Wei *et al*., 2004; Dominguez de Maria *et al*., 2006), nitrogen sources (Benjamin and Pandey, 1996; Dalmau *et al*., 1998; Lakshmi *et al*., 1999; Sanchez *et al*., 1999; Dalmau *et al*., 2000; Tan *et al*., 2003; Wei *et al*., 2004), culture temperatures (Benjamin and Pandey, 1997; Dalmau *et al*., 1998; Sanchez *et al*., 1999; Dalmau *et al*., 2000; Song *et al*., 2001; Wei *et al*., 2004) and pH values (Dalmau *et al*., 1998; Sanchez *et al*., 1999; Dalmau *et al*., 2000; Song *et al*., 2001; Tan *et al*., 2003; Wei *et al*., 2004) have been reported for CRL production. The composition of culture medium influences on the ratio of CRL isoforms and therefore on the catalytic activity (Ferrer *et al*., 2001; Dominguez de Maria *et al*., 2006). However, there is a lack of comprehensive optimization procedure for CRL esterase activity. There is also an inconsistency on repressive/inductive effect of glucose on CRL production (Obradors *et al*., 1993; Song *et al*., 2001). Nowadays, CRL is frequently used in esterification biotransformations for production of chiral pharmaceuticals, such as *S-ibuprofen* (Contesini and de Oliverira Carvalho, 2006; Won *et al*., 2006). Therefore, we decided to optimize esterase production of *Candida rugosa* lipase (CRL) and clarify the effect of glucose on this issue, to use the enzyme preparation for esterification biotransformations in future study.

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## MATERIALS AND METHODS

This study was conducted in 2006-2007 at Isfahan University of Medical Sciences, Iran.

**Microorganism, medium and culture conditions:** Active culture of *C. rugosa* (DSM 2031) was revived (30°C) on liquid YM medium and then maintained at 4°C (up to maximum 1 month) on YM-agar containing (g $L^{-1}$): peptone from soybeans, 5; malt extract, 3; yeast extract, 3; glucose, 10; agar, 15. Long-term stocks were preserved in 20% (v/v) glycerol at -80°C. The medium was sterilized using autoclave (121°C, 15 PSI, 15 min), but glucose (stock sol.) was autoclaved separately and added to the other ingredients aseptically, considering the final desired concentration. Basal main medium contained (g $L^{-1}$): $KH_2PO_4$, 12.3; $K_2HPO_4$, 5.25; $MgSO_4$, 1; $FeCl_3$, 0.01; urea, 1; $CaCl_2$, 0.0001; yeast extract, 3. The other ingredients were added in different compositions based on Table 1. The yeast was grown in 1 L flasks containing 200 mL of medium. Forty eight hours old cultures of *C. rugosa* on YM-agar were resuspended in 0.9% NaCl solution and each flask was inoculated by 5 mL of this suspension. The flasks were incubated in a shaker-incubator at 30°C, pH 6.3 and 180 rpm.

**Design of experiments (DOE):** The culture medium was optimized using a fraction of factorial design method, Taguchi. Considering the best previous results of the researchers studied lipase production, 9 combinations (L9 orthogonal array) of the major medium ingredients were studied. The factors to be optimized were the following 4 ones in 3 levels: oils, fatty acids, nitrogen sources and glucose concentration (Table 1).

Maximum total (internal + external) esterase activity was considered as the response factor. Using Minitab® 14 (PA, USA) software, the levels of each factor posing the most influence on the esterase activity and also the rank of each factor were identified. Temperature and pH of the culture were also optimized by one factor at a time method, using the best combination of Taguchi's method (Shojaosadati and Asadollahi, 2002).

**Viable count:** Dilutions of the samples were counted by spread plate method after incubation.

**Esterase activity assay:** Esterase activity was determined by the enzymatic method described by Dalmau *et al.* (2000) except that culture samples were centrifuged (5840 x g, 15 min, 4°C), the pellets were collected for internal activity assay and the supernatants were used for extracellular activity assay.

**Extracellular esterase activity assay:** The method of Dalmau *et al.* (2000) was used, except that the formation of *p*-nitrophenol after 1 min was measured at 348 nm.

**Internal esterase activity assay:** The method of Dalmau *et al.* (2000) was used, except that the disrupted cells were passed through the filter (0.2 μm) and after this stage, the procedure was the same as earlier.

**Lipase lipolytic assay:** A convenient method (Baillargoeon *et al.*, 1989) was used to measure lipolytic activity, except that the lipolytic activity unit was defined as the amount of enzyme preparation, which hydrolyzed 1 μeq $h^{-1}$ of the free fatty acid (Sigma® definition).

**Protein assay:** Protein concentration was determined by a well known method (Bradford, 1976) and bovine serum albumin was used as the standard protein in the calibration curve.

## RESULTS

**Optimization of medium for lipase production using Taguchi design:** Considering the best previous results of the researchers studied lipase production (Table 1), combinations of the major medium ingredients were studied using Taguchi fraction of factorial design method.

Table 1: Taguchi's L9 orthogonal array for optimization of medium composition

| Parameters experiments | Fatty acids (%) | Oils (%) | Nitrogen sources (%) | Glucose concentration (g $L^{-1}$) |
|---|---|---|---|---|
| 1 | - | - | CSL* (4) | 0 |
| 2 | - | Sesame oil (1) | Soybean flour (3) | 5 |
| 3 | - | Triolein (glyceril trioleate) (1) | Ammonium sulfate (0.9) | 10 |
| 4 | Oleic acid (0.2) | - | Soybean flour (3) | 10 |
| 5 | Oleic acid (0.2) | Sesame oil (1) | Ammonium sulfate (0.9) | 0 |
| 6 | Oleic acid (0.2) | Triolein (1) | CSL (4) | 5 |
| 7 | Palmitic acid (0.2) | - | Ammonium sulfate (0.9) | 5 |
| 8 | Palmitic acid (0.2) | Sesame oil (1) | CSL (4) | 10 |
| 9 | Palmitic acid (0.2) | Triolein (1) | Soybean flour (3) | 0 |

*- CSL: Corn Steep Liquor

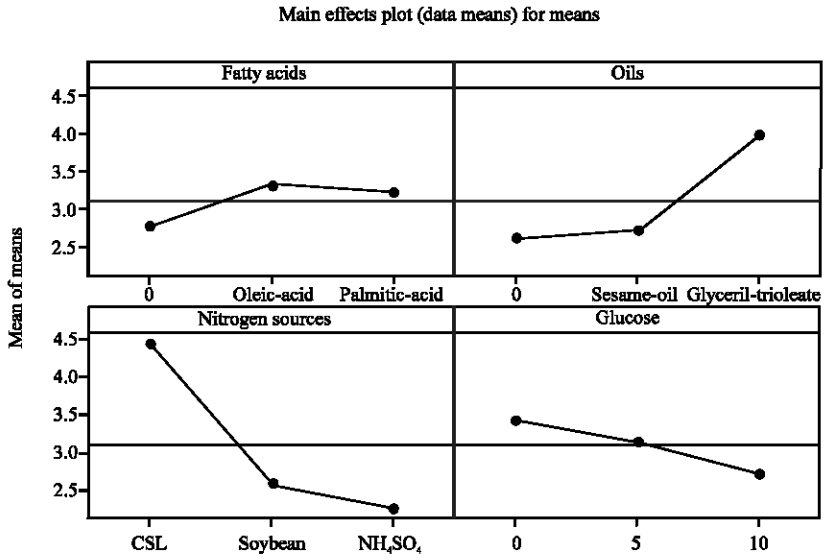


Fig. 1: The mean effects of each factor on total esterase activity. The mean effects of each factor on total esterase activity (the response factor) was plotted by Minitab®14 software

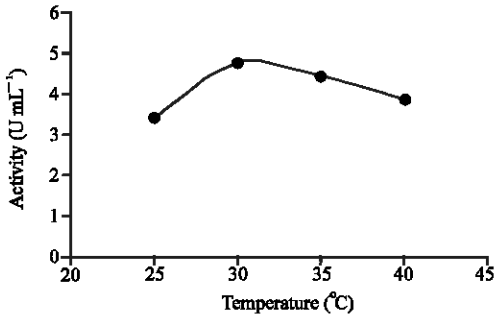


Fig. 2: Effect of different temperatures on esterase activity. Total esterase activity was determined in frequently used temperatures, grown on the optimum combination of the major medium ingredients determined by Taguchi method at pH 6.3, 25 h after the start of culture growth

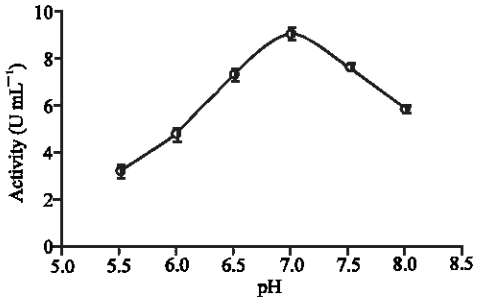


Fig. 3: Effect of different pH values on esterase activity. Total esterase activity was determined in frequently used pH values, grown on the optimum combination of the major medium ingredients determined by Taguchi method, 25 h after the start of culture growth. Data points (duplicate) are averages and error bars are standard deviations

Using the software (Fig. 1), optimum combination of the major medium ingredients, in order of their magnitude, were as follows (g $L^{-1}$): Corn Steep Liquor (CSL) powder (40), triolein (glyceril trioleate) (10), glucose (0) and oleic acid (2). This combination had not been used in the experiments, therefore the result was predicted (5.89 U $mL^{-1}$) by the software and confirmed by the experiment (5.9 U $mL^{-1}$) using the combination of medium ingredients.

**Optimization of conditions for lipase production using one factor at a time method :** Using the best combination of medium ingredients determined above and changing only one condition at a time, such as temperature or pH, these conditions were optimized as well. Total esterase activity was determined in frequently used temperatures. The esterase activities between 25-30°C were increased (Fig. 2) and reached to the maximum at 30°C. After this temperature, the activity declined.

Using the best combination of medium ingredients determined previously and changing only pH values, the optimum pH was determined at 30°C. Total esterase activity was determined in frequently used pH values. The optimum pH was 7 (Fig. 3).

**Time course of the enzyme activity:** In order to investigate the best time for harvesting CRL from the

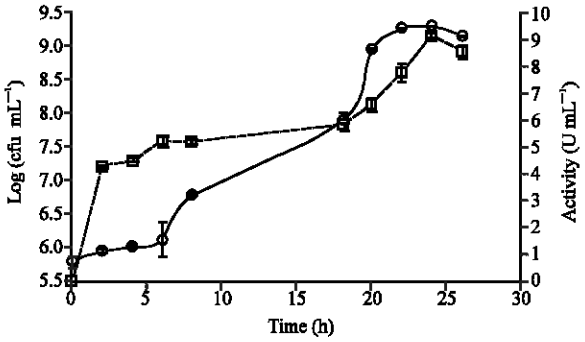


Fig. 4: Time course of growth and esterase activity. Microbial count (○) and total esterase activity (□) of the culture, were determined during the growth. Data points (duplicate) are averages and error bars are standard deviations

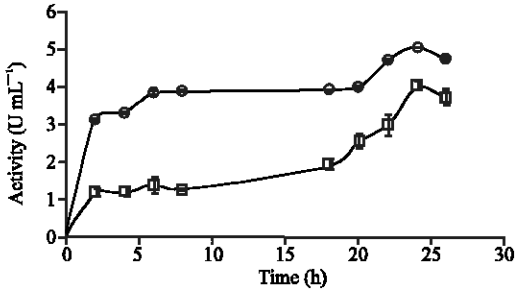


Fig. 5: Comparison of internal and external esterase activity. Internal (○) and external (□) activities were determined during the time. Data points (duplicate) are averages and error bars are standard deviations

culture growing in the optimized medium at optimized conditions, total esterase activity was studied during the growth. Two steps were seen in the growth curve as well as esterase activity and the latter one seemed to be growth dependent (Fig. 4). The maximum growth and activity was observed at 24 h.

To find out the proportion of intracellular and extracellular esterase activity, these activities were determined during the growth. The patterns of these activities seemed to be almost similar (Fig. 5). The ratio of internal to external activity was increased between hours 0-24 from 0.37 to 0.79.

## DISCUSSION

CSL as the nitrogen source had the most impact between the factors tested by Taguchi method and showed much better results than ammonium sulfate and soybean flour. Ammonium sulfate (Dalmau *et al*., 1998; Sanchez *et al*., 1999; Dalmau *et al*., 2000; Tan *et al*., 2003; Wei *et al*., 2004) urea (Triantafyllou *et al*., 1993; Benjamin and Pandey, 1996; Lakshmi *et al*., 1999; Sanchez *et al*., 2000) and soybean meal (Tan *et al*., 2003) have been used as nitrogen sources. Previously, Dalmau *et al*. (1998, 2000) and Sanchez *et al*. (1999) had suggested $(NH_4)_2SO_4$ and Benjamin and Pandey (1996) and Lakshmi *et al*. (1999) had announced urea as nitrogen sources for lipase production. This is the first time, which CSL is introduced as N source for this purpose and due to its low price, this finding seems to be interesting for industries, financially.

It was shown that the combination of glyceryl trioleate (an ester) and oleic acid (a fatty acid) increased CRL activity better than each of them solely, or better than the other studied growth substrates as carbon sources and lipase inducers. Researchers have reported various major carbon sources and inducers for production of CRL, including oleic acid (Lakshmi *et al*., 1999; Sanchez *et al*., 1999; Tan *et al*., 2003; Wei *et al*., 2004), palmittic acid (Dalmau *et al*., 2000), olive oil (Valero *et al*., 1991; Benjamin and Pandey, 1996; Song *et al*., 2001), sesame oil (Lakshmi *et al*., 1999) and glycerol trioleate (Wei *et al*., 2004). This is the first report, which a combination of an ester and a fatty acid is introduced as the carbon sources and inducers for lipase production.

The negative effect of glucose on lipase production mentioned by Obradors *et al*. (1993) was also verified in the current study, but was in contrast with the results of Song *et al*. (2001).

It was determined that the optimum temperature for lipase activity was 30°C, which was consistent with some results (Benjamin and Pandey, 1997; Dalmau *et al*., 1998; Sanchez *et al*., 1999; Dalmau *et al*., 2000; Song *et al*., 2001; Wei *et al*., 2004). The optimum pH was 7, but Wei *et al*. (2004), Song *et al*. (2001), Dalmau *et al*. (2000), Sanchez *et al*. (1999) and Lakshmi *et al*. (1999) have not used this pH. Tan *et al*. (2003) was the only researcher who previously optimized pH for lipase activity and reported pH 7 as the optimum value, which is consistent with the current study. Tan *et al*. (2003) only studied pH values with 1 unit difference, but in this work pH was optimized changing the values by 0.5 unit steps, which seems to be more accurate.

Two steps which was seen in the growth curve as well as esterase activity, might be due to switch from low amounts of the carbon sources in yeast extract at early stages to oleic acid and glyceril trioleate, which are metabolized more slowly.

It was shown that 24 h after the start of *C. rugosa* growth is the optimum time to extract esterase from the culture. Lakshmi *et al*. (1999) had reported that the maximum productivity of lipase production was

obtained within 48 h. But, the results of current study and Lotti *et al.* (1998) showed that the maximum yield was obtained earlier due to difference in the media composition. It might be concluded that the latter media are better considering productivity.

The ratio of internal to external activity was increased between hours 0-24 about 2 times, which supports hypothesis of Lotti *et al.* (1998), saying during batch growth in media producing high amounts of lipase, the synthesis of lipase becomes faster than its transport, causing intracellular accumulation.

## CONCLUSION

The culture medium was optimized using a fraction of factorial design method, Taguchi. Temperature and pH of the culture was also optimized using one factor at a time method. Using this combination and conditions, the activity of enzyme preparation was increased to 9 U $mL^{-1}$, which was equivalent to 20611 U $mL^{-1}$ of Sigma® lipase lipolytic activity, with a productivity of 0.362 U $mL^{-1}$ $h^{-1}$. After a semi-purification, in case of using appropriate substrates, this enzyme preparation can be considered as a potent biocatalyst for production of enantiopure pharmaceutical products. It might be concluded that 24 h after growth in the optimized medium at the optimized conditions, will be the optimum time to extract and purify the enzyme as a biocatalyst for esterase activity.

## ACKNOWLEDGMENT

We wish to express our gratitude to Isfahan University of Medical Sciences for financial support of this study.

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