Thermostable Lipase Production By Geobacillus Thermodenitrificans In A 5-L Stirred-Tank Bioreactor

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Citation

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Abstract

Lipases from thermophiles have gained interest in recent years as it has various applications in industries. It plays a significant role in industries as it has high stability and resistant to chemical denaturation. Its extensive application in industries requires its production in a large scale. In this study, thermostable lipase production by Geobacillus thermodenitrificans in a 5-L stirred tank bioreactor was evaluated. The cultivation was carried out for 96 hours at 55 ºC in a cultivation medium containing (%; w/v or v/v): glucose 1.0; yeast extract 1.25; NaCl 0.45 and olive oil 0.1. Optimization of physical parameters in the bioreactor will improve the production of the thermosatble lipase. Different physical parameters affecting enzyme production and biomass concentration such as agitation rate (100, 200, 300, 400 and 500 rpm), aeration rate (1, 2, 3 and 4 lpm) and inoculum concentration [0.5, 1.0, 1.5, 2.0, and 2.5% (v/v; 5 x 10^6 cell/ml)] were evaluated. Thermostable lipase activity was enhanced by 53% in the bioreactor and the biomass concentration increased by 23% after optimizing the physical parameters in the stirred tank bioreactor. Maximum lipase activity of 180 U/ml was obtained at 72 hours of cultivation at 2 lpm, 400 rpm and 2% (v/v; 5 x 10^6 cell/ml) inoculum with a specific activity of 3.62 U/mg. Thermostable lipase production in the bioreactor was about six-fold higher than that attained in a shake flasks culture. From the results obtained in this study, it is possible of producing thermostable lipase in a large-scale which has an extensive application in industry.

INTRODUCTION

Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3), which acts only on an ester-water interface, is capable of catalyzing the hydrolysis of long-chain triglycerides with the formation of diacylglycerol and carboxylate, as well as the reverse reaction with the synthesis of esters formed from fatty acids and glycerols (1). Lipase is present in diverse organisms including animals, plants, fungi and bacteria; however, only microbial thermostable lipases are of commercial importance (2-3). Extracellular microbial lipases can be produced inexpensively in large quantity by fermentation (4).

Thermophilic Bacillus species previously assigned to rRNA group 5 have recently been transferred to a new genus Geobacillus (5 – 7). The Geobacillus species form a phenotypically and phylogenetically coherent group of thermophilic bacilli with high levels of 16S rRNA sequence similarity (98.5-99.2%). This group comprises established species of thermophilic bacilli (Bacillus stearothermophilus, Bacillus thermocatenulatus, Bacillus thermoleovorans, Bacillus kaustophilus, Bacillus thermoglucosidasius and Bacillus thermodenitrificans). Members of the genus Geobacillus may grow at temperatures ranging from 35 to 78°C and contain iso-branched saturated fatty acids (iso-15:0, iso-16:0 and iso-17:0) as the major fatty acids (8). The major requirement for commercial lipases is thermal stability which would allow enzymatic reaction to be performed at higher temperatures and would be helpful to increase conversion rates, substrate solubility, to reduce the contamination of microorganism and the viscosity of the reaction medium (1). Geobacillus sp. has attracted industrial interest for their potential applications in biotechnological processes as sources of thermostable enzymes (9).

The extensive application of thermostable lipases in industries requires its production in a large scale. In this study, production of a thermostable lipase by the thermophilic bacteria G. thermodenitrificans in a 5-L stirred tank bioreactor was investigated. Stirred-tank bioreactor is ideal for industrial applications as this unit is low in both capital and operating costs. The influence of agitation rate, aeration rate and inoculum concentration on biomass and thermostable lipase production were assessed.

MATERIALS AND METHODS

MICROORGANISM

Geobacillus thermodenitrificans was cultured on nutrient agar and maintained at 65ºC. It was previously isolated from a hot spring in Labok, Machang, Kelantan, Malaysia.

CULTIVATION IN ERLENMEYER FLASKS

G. thermodenitrificans was grown in medium containing 1% (w/v) glucose, 0.45% (w/v) NaCl, 1.25% (w/v) yeast extract and 0.1% (v/v) olive oil. Seven percent (v/v, 5 x 10^6 cell/ml) of culture suspension, which was prepared from a 24 hour old culture, was inoculated in 100 ml of medium, pH 6.8. Fermentation was done in a 250 ml Erlenmeyer flask and incubated at 65ºC on an orbital shaker at 200 rpm for 48 hours. Contents of the flasks were collected after 48 hours and centrifuged (6000g, 15 min, room temperature). The supernatant was used for lipase assays and protein content determination.

CULTIVATION IN A 5-L STIRRED-TANK BIOREACTOR

Lipase production by G. thermodenitrificans was carried out in a 5-L stirred-tank bioreactor (INFORS AG, Switzerland) in 2L of medium. The medium (pH 6.8) was sterilized at 121ºC for 20 minutes in an autoclave. The medium was inoculated with 2.0% of (v/v; 5 x 10^6 cell/ml) inoculum and the fermentation was carried out at 55ºC. SPAN 20 10% was used as the antifoam. The impeller speed was initially adjusted to 100 rpm and compressed sterile air was sparged into the medium at the rate of 1 L min^{-1}. Samples (15 ml) were withdrawn every 6 hours and analyzed for lipase activity, protein concentration, pH and cell growth.

CELL GROWTH DETERMINATION

Cell growth was determined based on the method of (10). Biomass concentration was measured as OD_{560} with a Spectronic Unicam Genesys10UV. The obtained values were converted to g cell dry wt/L by using a calibration curve.

LIPASE ACTIVITY DETERMINATION

Lipase activity was determined by using polyvinyl alcohol and olive oil in a 3:1 ratio as the reaction substrate (11). Five milliliters of reaction substrate and 4.0 ml of phosphate buffer 0.2 M (pH 6.8) were incubated for 10 minutes at 65ºC. Then, 1.0 ml of the crude enzyme supernatant of was added to the substrate. The reaction mixture incubated for 30 minutes at 65ºC with shaking at 150 rpm. The enzyme reaction was stopped by adding 20 ml of ethanol-acetone (1:1). The free fatty acid was titrated with 50mM NaOH with 0.1 ml of phenolphthalein as the titration indicator. One unit of lipase activity was defined as the amount of enzyme releasing 1 mole of fatty acid per minute.

PROTEIN CONTENT DETERMINATION

Protein content in the sample was determined using a modified method of (12).

Optimization for maximum thermostable lipase production by G. thermodenitrificans

Lipase production was measured at different aeration rates - 1, 2, 3 and 4 L min^{-1} and agitation speeds -100, 200, 300, 400 and 500 rpm. Inoculum sizes evaluated were 0.5%, 1%, 1.5%, 2% and 2.5% (v/v; 5 x 10^6 cell/ml) inoculum. Samples were collected every 48 hours, centrifuged (6000g, 15 min, room temperature) and lipase activity, protein content, pH and the amount of cell growth determined.

RESULTS AND DISCUSSION

Geobacillus thermodenitrificans as indicated in the name itself, is a heat-loving bacterium which is capable of reducing nitrate to nitrogen. It is Gram-positive rods which forms flat, lobate and off-white colonies. It can grow at 45-70ºC at pH 6-8 in 0.30 % NaCl. The present strain of G. thermodenitrifican was previously isolated from a hot spring and produces thermostable lipase with optimal activity between 55-65°C and pH 6-7. The bacterium produced ~ 29 U/ml of thermostable lipase in 48 hour shake flask fermentation. The protein content in the fermentation flask was ~15 mg/ml and the specific activity was ~ 2 U/mg protein.

Fermentation in a 5-L stirred tank bioreactor increased the production of thermostable lipase. After four days of cultivation in the bioreactor, lipase production was highest at 48 hours of cultivation with 84 U/ml activity (Figure 1A). Enzyme activity then dropped until 96 hours of cultivation with 31.5 U/ml activity. Protein content followed the same pattern, with the highest value, 35 mg/ml, at 48 hours and then dropping afterwards. Bacterial growth also was highest at 48 hours with 0.5 mg/ml cell dry weight.

Cultivation in a stirred tank bioreactor increased thermostable lipase production 3-fold compared to the shake flask. This increase could be due to the higher dissolved oxygen concentration in the bioreactor.

Total lipase activity in a stirred tank bioreactor by Thermus thermophilus increased 5- fold over that obtained in flask culture (13). Our results are consistent with the hypothesis

that fermentation in a bioreactor could improve enzyme production. However, the choice of appropriate temperature, aeration and inoculum concentration are crucial to maximize the culture yield (13).

EFFECT OF AGITATION RATE

Agitation rate of the impellers in a stirred tank bioreactor plays an important role in the production of thermostable lipase enzyme. Agitation showed to be an important parameter to ensure nutrient availability in a growth medium having olive oil (14). An agitation rate of 400 rpm produced the highest lipase activity of ~ 125 U/ml with cell growth of 0.55 mg/ml (Figure 2A). Higher agitation may create condition of higher availability of the carbon sources to microorganisms (15).

Agitation helps maintain uniform conditions within the fermenter and promotes effective mass transfer to the liquid medium in the fermenter. Higher agitation also increases gas dispersion, and increaseed gas dispersion allows more mass transfer (15). The 100 - rpm-air-sparged run was oxygen limited due to the low agitation speed, and had a low enzyme activity as the oxygen transfer coefficients are strongly influenced by the amount of agitation Production of lipase by Candida rugosa in a triple impeller system was highest at 600 rpm (16).

EFFECT OF AERATION

Under the conditions studied (400 rpm and 55°C), it was found that cell growth and lipase production occurred only when air was supplied continuously to the bioreactor. The aeration rate in the stirred tank bioreactor is important for growth of the bacteria, and also may increase the oxygen exchange rate and help mix the medium (17).

Aeration at 2 lpm produced the highest lipase enzyme activity level 105.5 U/ml (Figure 2B). Lipase production decreased at other aeration rate. The cell growth also was highest, 0.34 mg/ml at 2 lpm. Aeration rate less than 2 lpm might not provide enough oxygen for cell growth. Whereas, an aeration rate > 2 lpm might exceed the oxygen need and result in lower enzyme activity.

G. thermodentrificans was isolated from high temperature environment where the dissolved oxygen concentration was quite low. Its adaptation to this low oxygen environment could be the reason for lower lipase production and cell growth at higher aeration rates. The intrinsic factor determining cell growth and lipase production was oxygen transfer rate rather than dissolved oxygen concentration. Improvement in oxygen transfer rate by aeration resulted in an increase in lipase yield by Acinetobacter radioresistens and/or a reduction in fermentation time (18).

EFFECT OF INOCULUM SIZE

The larger volume of the stirred tank bioreactor means that a higher inoculum concentration also was needed. An inoculum size of 2% (v/v; $5x10^6$cells/ml) produced the highest lipase activity of 115 U/ml (Figure 2C). Cell growth also was highest at that inoculum size with at 0.60 mg/ml. Inoculum size influences the utilization of glucose in the medium (19). Thus, the higher cell concentration at a time, the greater was the production of enzyme that can be expected (20). However, at a lower level of inoculum, enzyme activity was < 115 U/ml. This could be due to the depletion of oxygen on account of the high cell concentration. Studies on the effect of inoculum size on l-leucine amino peptidase production by Streptomyces gedanensis IFO13427, showed that LAP production progressively increased up to 2% (v/v) (1.2×10^9 CFU/ml) inoculum and decreased thereafter (21).

THERMOPHILIC LIPASE PRODUCTION AFTER PHYSICAL PARAMETERS OPTIMIZATION

All the optimized physical parameters were used and the fermentation was run for four days (Figure 1A). The highest lipase activity was detected at 72 hours of cultivation with ~ 180 U/ml of biomass compared to 84 U/ml prior to optimization. After 72 hours of cultivation, lipase production decreased.

Protein content also was highest 3.62 U/mg protein at 72 hours, an increase ~ 53% over the results obtained prior to optimization. The thermostable lipase activity obtained after physical parameter optimization in the bioreactor was ~ 6-fold higher than that attained in shake flask cultures. The highest cell growth (Figure 1B) also occurred after 72 hours of fermentation with 0.64 mg/ml. This amount was also 23% higher than the growth prior to optimization. Thus, both growth and metabolic activity are higher in cultures grown under optimized parameters in a stirred tank bioreactor. A 4-fold enhancement in lipase production and approximately 3-fold increase in specific activity by B.cepacia was obtained in a 14 l bioreactor within 15 h after statistical optimization following shake flask culture (22).

In conclusion, the results obtained from the present work, indicate that a thermostable lipase can be produced on a bioreactor scale. The enzyme yield from the stirred tank bioreactor was significantly higher than that obtained in

shake flask cultures. Optimization of the physical parameters for the stirred tank bioreactor fermentation also increased production of the thermostable lipase and the biomass concentration. There were no operational problems detected, in spite of the high culture temperature employed. The results obtained in this work suggest the possibility of applying this process to larger-scale systems which will be beneficial for industrial purpose.

Figure 1

Figure 1: Profile of lipase production and growth of before and after physical parameters optimization, in a 5-L stirred tank bioreactor (A) lipase activity and protein content, (B) growth. All the experiments were carried out independently in triplicates. 1A closed triangles -lipase activity before optimization, closed diamond Â– lipase activity after optimization, closed circles Â– protein content before optimization and closed square Â– protein content after optimization. 1B closed triangle Â– cell dry weight before optimization and closed square Â– cell dry weight after optimization

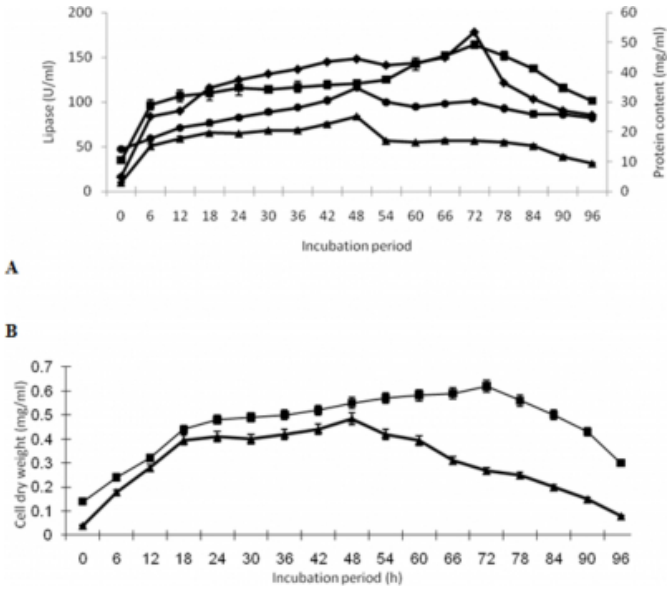
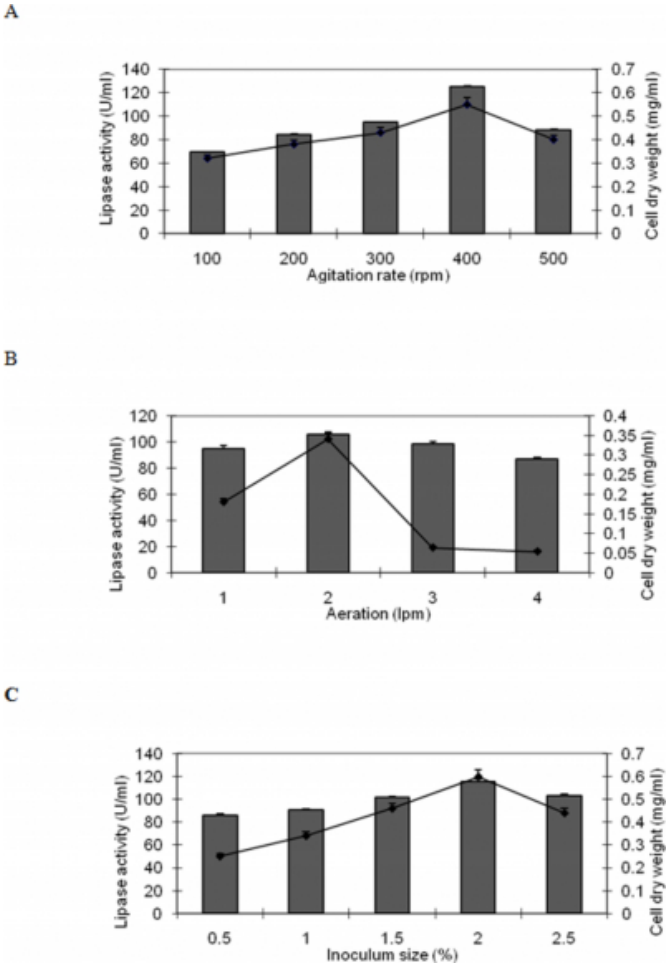


Figure 2

Figure 2: Effects of agitation rate (A), aeration levels (B) and inoculum sizes (C) on lipase production and cell growth. Bar chart – lipase activity and line – cell dry weight.



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