Research Article

Studies on Effect of Different Process Parameters on Production of Lipase By *Brevibacillus sp*.

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ABSTRACT

The present work was carried out to find factors affecting production of lipase by *Brevibacillus*. Lipases are enzymes, which catalyze the hydrolysis of triglyceride to give di and mono- glycosides, glycerol and free fatty acids. Lipase breaks down neutral fats (triglycerides) into glycerol (an alcohol) and fatty acids. *Brvibacillus* was screened for production of lipase and duration of production of lipase. Experiments were conducted for the optimization of carbon and substrate source sources by conventional one factor at a time method. Glucose or molasses were carbon sources and olive oil and soybean oil were substrate sources used for optimization. One factor at a time method was used to determine optimum levels of media components, viz., different triglycerides (soybean oil, castor oil and) of different concentrations (1% to 5%), carbohydrate (glucose and molasses) of different concentrations (1% to 5%). Among different triglycerides used olive oils was observed as a strong inducer of lipase at a concentration of 2%, and within 48h production of lipase was found to be maximum. Carbon sources played a crucial role for the enhanced production of lipase by *Brevibacillus*. The medium composition at different concentrations (1% to 5%) of molasses and glucose were taken. Among these two carbohydrate sources molasses at a concentration of 4% was the optimum concentration for lipase production.

Keywords: Brevibacillus, molasses, inducer/substrate, optimization.

INTRODUCTION

The *Brevibacillus* are a genus of Gram positive aerobic bacteria related to Bacillus and Paenibacillaceae[1]. *Brevibacillus brevis* are important for food hygiene because of their hydrolytic activities on food components[2] and the ability of some strains to produce food poisoning toxins or to grow at refrigerated temperatures.Taxonomy: Bacteria; Firmicutes; Bacilli; Bacillales; Paenibacillaceae; Brevibacillus[1]. *Brevibacillus* is a motile, rod shaped, spore forming (ellipsoidal or cylindrical) bacteria. Bacteria of the genus 'Bacillus' are aerobic, endospore –forming, gram-positive rods that includes both mesophiles and thermophiles.This strain can be easily isolated from soil[3,4]. These are robust and facultative anaerobe that grows readily in most media. They are of rod shaped of width 0.6-0.8 µm and length 1.5-3 µm.They grow at 37°C and can survive even at a temperature of 50-55°C and at minimum 15°C.It is found in soil and food. The genus is one of the most diverse and commercially useful groups of microorganisms. Representatives of this genus are widely distributed in soil, air and water where they are involved in a range of chemical transformations that rival those of the Pseudomonad's and Actinomycetes.

Appropriate growth media composition of *Brevibacillus* is tryptone (1%), NaCl (0.5%), yeast extract (0.5%), tributerine (0.5%), peptone 0.3%, fatty acid 1%, NaCl 0.1%, KH_2P0_4 0.608%, K_2HP0_4 0.863%, $MgSO_4$ (1 mL 0.5%), and pH was maintained at 7.4 and growth temperature is 37^OC.

Brevibacillus brevis is industrially being investigated for use as a bio control agent. Bacillus brevis is widely found in dairy products [5,6]. The use of the bacterium has also been classified as GILSP (Good Industrial Large Scale Practice) by the Japanese government. This classification indicates that bacterium is a safe organism. *B. brevis* secrets large amounts of biologically active recombinant proteins into the culture medium [7]. This eliminates the need for cell disruption and protein refolding

allowing us to obtain a high yield of purified product. One of the properties of *bacillus* species that make them so attractive for commercial processes is the ability to secrete high levels of protein in to the growth medium[8]. *Bacillus* species are an important source of industrial enzymes, fine biochemical's, antibiotics and insecticides. Three quarters of the market is for enzymes involved in the hydrolysis of natural polymers, including proteolytic enzymes used in the detergents, dairy, and leather industries, lipases used in food industry, biodiesel production and carbohydrates used in the baking, brewing, distilling starch and textile industries. About 2/3rd of these enzymes are produced by fermentation from Bacillus species[5,6].

Lipids are insoluble in water and need to be broken down extra cellularly into their more polar components to facilitate absorption if they are to function as nutrients for the cell.

EXPERIMENTAL

Materials

The bacterial strain used in the present study was isolated in our laboratory (IICT, Hyderabad) and identified as Brevibacillus. The organism was cultured in nutrient agar and slants were preserved in refrigerator for further use. All the media components were procured from SD Fine Chem. Mumbai.

Methods

Growth curve of Brevibacillus

Brevibacillus was inoculated in to nutrient broth (100mL). Then, for every one hour 3ml of sample was collected and the optical density was measured at 600nm using spectrophotometer (Shimadzu 1700, Japan). Growth curve was drawn between optical density and Time to know the duration of different stages of growth.

Screening for Lipase activity

Media was prepared using tryptone (1%), NaCl (0.5%), yeast extract (0.5%), tributerine (0.5%) and agar (1.5%). The bacterium was inoculated at the centre of the petri plate containing sterilized and solidified medium. Incubated it at 37^{0}C & after each 24 h it was observed for production of lipase till 144h. There was formation of clear zone which indicated that the enzyme lipase had been released from *Brevibacillus*, and it degraded the substrate (tributerine) present in the medium which resulted in the formation of clear zones [9,10,11,12]. The increase in the diameter of zone was measured after each 24hrs. A graph was plot taking time (h) on x axis and diameter of clear zone (mm) on y axis.

Microorganism and seed culture

Growth medium composition and conditions for Brevibacillus

Media for *Brevibacillus* was prepared by adding peptone 0.3%, soybean oil 1%, NaCl 0.1%, KH_2P0_4 0.608%, K_2HP0_4 0.863%, $MgSO_4$ (1 mL 0.5%), and pH was maintained at 7.4, volume was made up to 100 mL with double distilled water.

Culture growing on solid media was transferred into liquid broth having composition as defined earlier except agar. Small amount of culture from slant was taken and inoculated in to the liquid media, it was incubated at 37^{0}C, and 150 rpm for 24 hrs. Growth was indicated by turbidity. This culture was utilized for further tests.

Lipase assay

Lipase assay is done to estimate how much of enzyme was being produced. One unit of lipase activity was defined as the amount of enzyme releasing one mole of free fatty acid in one minute under standard assay condition.

$$\text{Lipase activity (mg/mL/min)} = \frac{\text{Volume of alkali consumed x Normality of NaOH}}{\text{Time of incubation x Volume of enzyme solution}}$$

Enzyme Assay

Lipolytic activity was measured according to the method of Sigurgisladottir et al 1993 with slight modification using pNP-laurate as substrate [13]. The enzyme activity was determined at 410 nm. Each reading was taken in triplicate and the activity was calculated as amount of enzyme required liberating one micromole equivalent fatty acid per mL per minute.

The activity was determined according to the method of Sigurgisladottir et al. (1993) with slight modification in brief the method followed is explained here. To 0.8 ml of 0.05 M phosphate buffer (pH 8.0), 0.1 ml enzyme and 0.1 ml 0.01 M pNP-laurate (Sigma, USA) was added. The reaction was carried out at 60ºC for 30 min, after which 0.25 ml 0.1 M Na_2CO_3 was added. The mixture was centrifuged and the activity was determined at 420 nm. One unit of lipase activity is defined as the amount of enzyme, which liberates 1 µg of p-Nitrophenol from pNP laurate as substrate in 30 min under standard assay conditions. (Lowry et al. 1951)

Optimization of carbohydrate

Two different carbohydrates (glucose and Molasses) were selected for two different sets of experiments and their concentrations were varied from 1 % to 5 %.

Glucose as carbohydrate source

All the other media components (as mentioned earlier) were kept constant glucose was taken as carbohydrate source and its concentrations were varied from 1% to 5%. Samples were collected every 24h and were assayed for amount of enzyme produced by enzyme assay procedure.

Molasses as carbohydrate source

All the other media components (as mentioned earlier) were kept constant molasses was taken as carbohydrate source instead of glucose and concentrations were varied from 1% to 5%. Samples were collected every 24h and were assayed for amount of enzyme produced by enzyme assay procedure.

Optimization of substrate/inducer source

Two different substrates/inducers (olive oil and soybean oil) were selected for two different sets of experiments and their concentrations were varied from 1 % to 5 %.

Olive oil as substrate source

All the other media components (as mentioned earlier)were kept constant and olive oil was taken as substrate (inducer) source and concentrations were varied from 1% to 5%. Samples were collected every 24h and were assayed for amount of enzyme produced by enzyme assay procedure.

Soybean oil as substrate source

All the other media components (as mentioned earlier) were kept constant and soybean oil was taken as substrate (inducer) source and concentration was varied from 1% to 5%. Samples were collected every 24h and were assayed for amount of enzyme produced by enzyme assay procedure.

RESULTS AND DISCUSSION

Growth curve of *Brevibacillus*

Samples were collected optical density was measured and growth curve was plot which indicated that cell entered in to the log phase after 13th h and reached stationary phase after120 th h. The 100mL media prepared was sufficient to maintain growth without being reached to the stationary phase for 120h. So optimization of media components could be carried out till 120th h.

Screening for Lipase activity

From the figure 1 it was clearly identified that the lipase production was more till 96h and fall in the production of enzyme starts after 120h. All the experiments were planned to conduct and analyze enzyme activity before 120h.

Enzyme Assay

Lipase acts on pNP-laurate in the presence ph phosphate buffer pH 8.0 and activity is measured at 420 nm by UV visible spectrophotometry. The enzyme activity graph was plot taking concentration of enzyme (µg/mL) on x axis and enzyme activity (IU) on y axis, which showed a linear relation with amount of enzyme used (R^2 = 0.998). This standard graph further used to estimate amount of enzyme produced from the culture of *brevibacillus*.

Optimization of carbohydrate

All other media components and their concentrations were kept constant except carbohydrate source and its concentration. Two carbohydrates were selected, glucose and molasses and their concentrations were varied from 1% to 5%.

Glucose as carbohydrate source

In five sets of experiments five different concentrations of glucose were used and samples were collected every 24h. Out of all the samples assayed for amount of enzyme produced, it was found that at any concentration of glucose the production of lipase starts after 24h and increased to 96h after which there is a decrease in the production of lipase. Among all the concentrations 4% glucose was found to be the best concentration of glucose for maximum lipase production which is shown in the figure 2.

Molasses as carbohydrate source

In five sets of experiments five different concentrations of molasses were used and samples were collected every 24h. Out of all the samples assayed for amount of enzyme produced, it was found that at any concentration of molasses the production of lipase starts after 24h and increased to 96h after which there is a decrease in the production of lipase. Among all the concentrations 4% molasses was found to be the best concentration for maximum lipase production. It is shown in the figure 3.

Optimization of substrate source

Olive oil as substrate source

Five sets of experiments with five different concentrations of olive oil (1% -5%) it was found that production of lipase reached maximum within 24h with any concentration of olive oil but, 2% olive oil was found to be sufficient (optimum) for maximum production of lipase. This is evident from the figure 4.

Soybean oil as substrate source

Five sets of experiments with five different concentrations of soybean oil (1% -5%) it was found that production of lipase reached maximum within 48h with any concentration of soybean oil and after 48 h slowly the production of lipase decreased. Here 1% soybean oil was found to be sufficient (optimum) for maximum production of lipase. This is evident from the figure 5.

CONCLUSIONS

Carbohydrate source (glucose and molasses), and inducer/substrate source (soybean and olive oil) were optimized by one factor at a time method. Among the two different carbohydrate sources used it was found that when molasses at a concentration of 4% was used, the production of lipase was more when compared to production of lipase when glucose was used and when molasses was used, even after 96h production of lipase was not decreased. Even in economic point of view molasses is the best option as carbohydrate source for increased production of lipase. And when we observed the two substrate/inducers for lipase production it was found that when we use olive oil production of lipase starts immediately within 24h and kept on decreasing after 24h. So maximum production of lipase was seen in a short time period of 24 h with olive oil as substrate. 2% olive oil is the optimum concentration to be used for maximum production of lipase though it is time saving olive oil is not cost effective. With soybean oil production of lipase reaches maximum near 48h and then starts decreasing. Optimum concentration of soybean oil to be used for maximum lipase production is 2%. For commercialization 2% molasses and 1% soybean oil were found to be optimum or the production of lipase.

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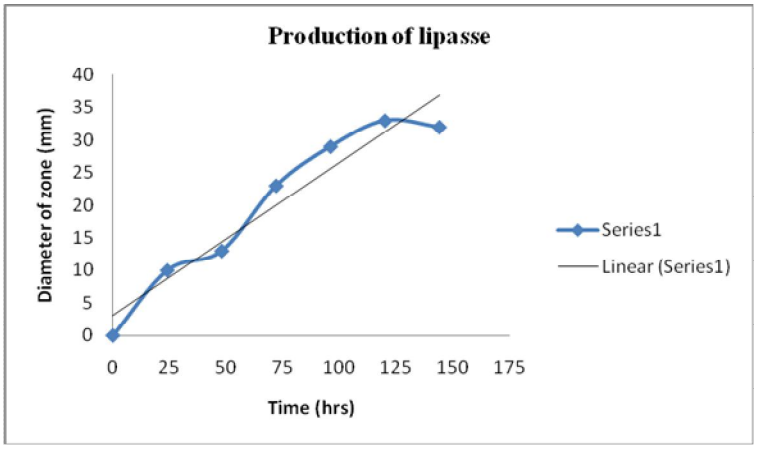

Fig. 1: Production of lipase by B*revibacillus* with respect to time

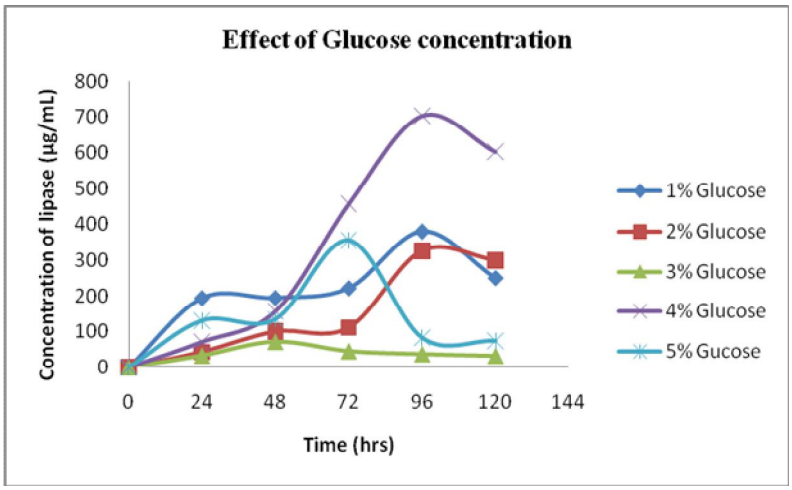

Fig. 2: Effect of glucose concentration on lipase production by *Brevibacillus*

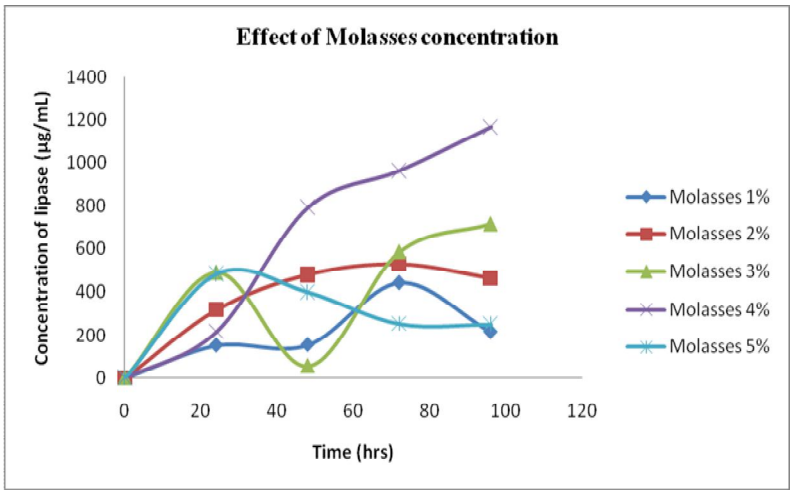

Fig. 3: Effect of molasses concentration on lipase production by *Brevibacillus*

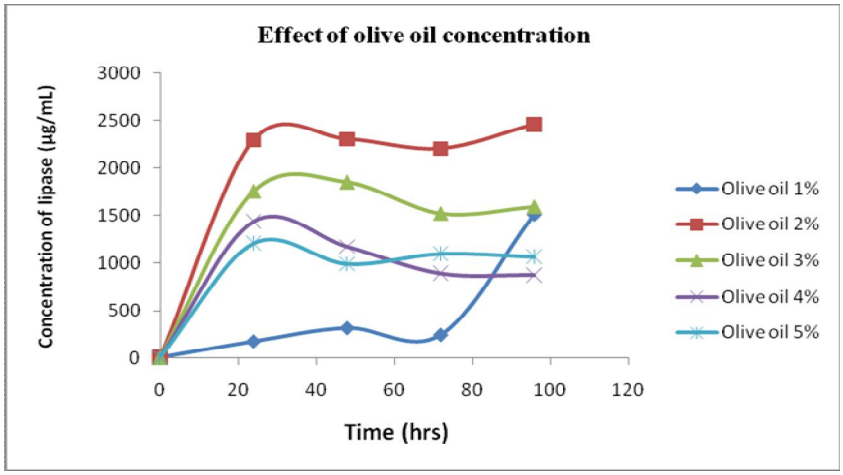

Fig. 4: Effect of olive oil concentration on production of lipase by *Brevibacillus*

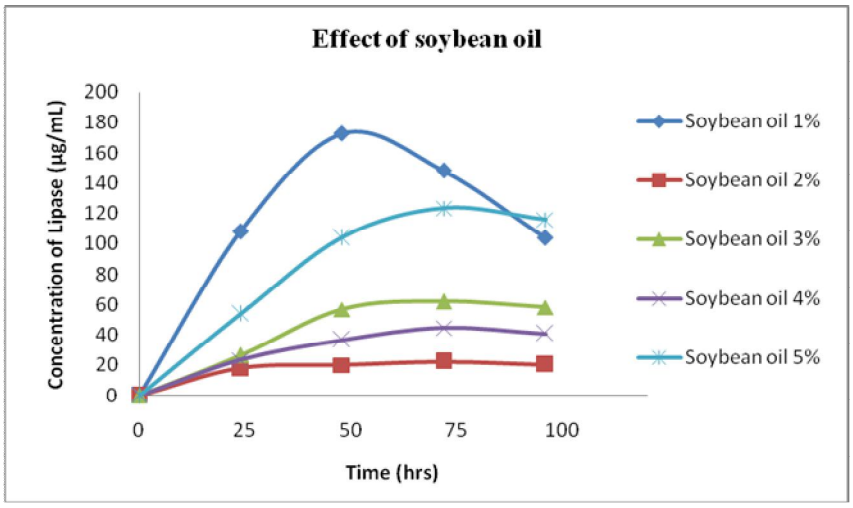

Fig. 5: Effect of soybean oil concentration on production of lipase by *Brevibacillus*

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