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Factors influencing production of lipase under metal supplementation by bacterial strain, *Bacillus subtilis BDG-8*

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Abstract

Lipases are biocatalyst having wide applications in industries due to their versatile properties. In the present study, a lipolytic bacterial strain, *Bacillus subtilis* BDG-8 was isolated from an oil based industrial soil. The effect of selenium and nickel as a media supplement on enhancement of lipase production, was studied individually with the isolated strain by varying the concentration of selected metal. 60µg l^{-1} selenium enhanced lipase production to an enzyme activity measuring 7.8 U ml^{-1} while 40 µg l^{-1} nickel gave the maximum enzyme activity equivalent to 7.5 U ml^{-1}. However, nickel and selenium together at a range of concentration with an equal w/v ratio, at 60 µg l^{-1} each, showed the maximum lipase activity of 8.5 U ml^{-1}. The effect of pH and temperature on lipase production showed maximum enzyme activity in the presence of each of the metals at pH 7 and 35°C among the other tested ranges. After optimisation of the parameters such as metal concentration, pH and temperature lipase production by *Bacillus subtilis* BDG-8 had increased several folds. This preliminary investigation may consequently lead as to various industrial applications such as treatment of wastewater contaminated with metal or oil with simultaneous lipase production.

Key words

Bacillus subtilis, Enzyme activity, Lipase, Nickel, Selenium, Temperature

Introduction

Lipases are important group of hydrolases, which act under aqueous conditions on the carboxyl ester bonds present in triacylglycerols to liberate fatty acids and glycerol (Gupta *et al.,* 2004).They also possess esterolytic activity and thus have a very diverse substrate range (Gupta and Soni, 2000; Jaeger and Eggert, 2002). Lipases are capable of regioselective and stereoselective biotransformations and allow resolution of racemic mixtures (Sharma *et al.*, 2001). They catalyze variety of reactions, such as partial or complete hydrolysis of triacylglycerols and reactions of esterification, transesterification and interesterification of lipids (Colla *et al.,* 2010). Due to these versatile properties, they find a wide variety of applications in industries such as food dressing, paper manufacture, detergent, cosmetics, pharmaceutical, leather *etc* (Park *et al.,* 2005; Gupta *et al.,* 2007; Grbavcic *et al.,* 2007; Franken *et al.,* 2009).

Although lipase can be derived from several sources, such as plants, animals and microorganisms, enzymes from microbial sources generally meet industrial demands due to easey production. The past few decades had witnessed numerous reports on the isolation and identification of numerous lipase producing microorganisms due to their increasing commercial demand (Sirisha *et al.,* 2010). Lipase producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, oilseeds, and decaying food, compost heaps, coal tips, and hot springs.

Bacterial lipase production is influenced by the type and concentration of carbon and nitrogen sources, the culture pH, the growth temperature and the dissolved oxygen concentration (Elibol and Ozer, 2001). In addition, the effect of various metal ions on lipase production should also be considered as they

generally form complexes with ionized fatty acids, changing their solubility and behaviour at the interface (Matsumae *et al.,* 1994). Several reports are available on the use of zinc, cobalt, copper, manganese, iron, molybdenum in growth media, as a supplement, that had enhanced bacterial lipase production by several folds (Tembhurkar *et al*., 2012). However, there are no reports on the use of selenium as a media supplement for enhancing lipase production till date and the same has been attempted in the current study. Using metal as a media supplement for enhancing bacterial lipase production shows the versatility of bacterial strain to resist metal and hence it can find a dual application in bioremediation of metals accompanied by lipase production. The substrate for producing bacterial lipase is basically oil and hence the same technology can be used for the biodegradation of oil with consecutive lipase production. It is always necessary to optimise the critical parameters in shake flask experiments to enable its scale-up for a large scale production.

The present study aimed at isolation and identification of lipase producing strain from an oil contaminated site and optimising physico-chemical parameters for enhancing its production.

Materials and Methods

Isolation and Identification of lipase producing bacterial strains: Soil samples were aseptically collected in plastic bags from an oil Industry in Tamilnadu, India. The isolation of bacterial strains were carried out by serially diluting the soils samples in saline water and subsequently plating on a tributyrin agar base medium using pour plate method. The plates were incubated at 37 ºC for 24 hrs and observed for zone formation. A clear zone around the colonies indicates the production of lipase (Cardenas *et al*., 2001). The lipase positive strains were further purified, grown in nutrient broth at 37 ºC for 24 hrs and screened for the ability to produce lipase with olive oil. Bacterial isolate BDG -8 showing highest lipase activity was identified and further studies were carried out.

Morphological, physiological and biochemical characteristics of the potent lipolytic strain, BDG-8 was determined by the method described in "Bergey's Manual of Determinative Bacteriology" (Holt *et al*., 1994).

Fermentation was carried out in shake flasks using a complex medium as follows: 20 g l^{-1} olive oil (emulsified in 2% gum acacia), 10 g l^{-1} egg yolk, 4.0 g l^{-1} Ammonium chloride, 0.25 g l^{-1} magnesium sulphate heptahydrate, 0.5 g l^{-1} dipotassium phosphate and 5.0 g l^{-1} calcium carbonate. The fermentation media with an initial pH of 7 was then inoculated with 5 ml of cells suspension and incubated at 37°C on incubatory shaker (120 rpm) for 48hrs.

Effect of metal ions: The effect of metal ions such as nickel and selenium individually and in combination on lipase production by bacterial strain BDG-8 was tested by varying the concentration of selected metal, in the range of 10 -100 µg l^{-1} in the fermentation media keeping other factors constant. Enzymatic activities were determined in triplicate and reported as mean ± SD.

Effect of initial pH: The effect of initial pH was accessed in the range of 5.5 to 7.5 for producing lipase using shake flask cultures. The fermentation media amended with the optimum concentration of the metal micronutrient (either selenium or nickel) was adjusted to the required initial pH by addition of either 1N NaOH or 1N HCl while keep other parameters constant.

Effect of temperature: The effect of incubation temperature on bacterial lipase production was tested by incubating the bacterial culture on an incubator at a required temperature (25, 35, 40, 45°C), while keeping the pH of fermentation media constant along with the optimum concentration of either nickel or selenium.

Lipase assay: Lipase activity in the broth was determined titrimetrically on the basis of olive oil hydrolysis (Macedo *et al*., 1997). One ml sample solution was added to the assay substrate containing 10 ml of 10% homogenized olive oil in 10% gum acacia, 2 ml of 0.6% $CaCl_2$ solution and 5 ml of 0.2 mol l^{-1} citrate buffer, pH 7.0. The enzyme substrate mixture was incubated on an orbital shaker at 100 rpm at 37°C for 1 h. To stop the reaction, 20 ml ethanol acetone mixture (1:1) was added to the reaction mixture. Liberated fatty acids were titrated with 0.1 mol l^{-1} NaOH. Extracellular lipase activity was expressed as units per ml of broth. One 'lipase unit' (U) was defined as the amount of enzyme that released one mole fatty acid per minute. Enzymatic activities were determined in triplicate and reported as mean ± SD.

Statistical Analysis: All the experiments were repeated three times and graphically represented as mean±SD. Microsoft Excel 2007 was used to calculate mean and SD.

Results and Discussion

The search is always on with greatest interest for microorganisms from natural sources with desired lipolytic properties due to wide applications of lipase (Gupta *et al.,* 2004). In the current study, soil samples collected from oil industry, when subjected to serial dilution and subsequently plated on a solid enrichment media, yielded nearly 21 distinct bacterial colonies. Among the isolated colonies, all of them could produce clear zones on the solid media containing tributyrine indicating that they were lipolytic strain. Further, their lipase producing capability was quantified using olive oil. BDG-8 was a potent lipolytic strain, among other strains isolated in this study, showing highest lipase production and hence it was selected for further studies.

Morphologically BDG-8 showed an irregular, rough, flat, opaque colony with an entire margin on nutrient agar plates. It was a gram-positive sporulating rod occurring singly or in pairs. It could hydrolyze starch, gelatin, tributyrine, casein, glycerol and could also produce acid in glucose, fructose and sucrose broth.

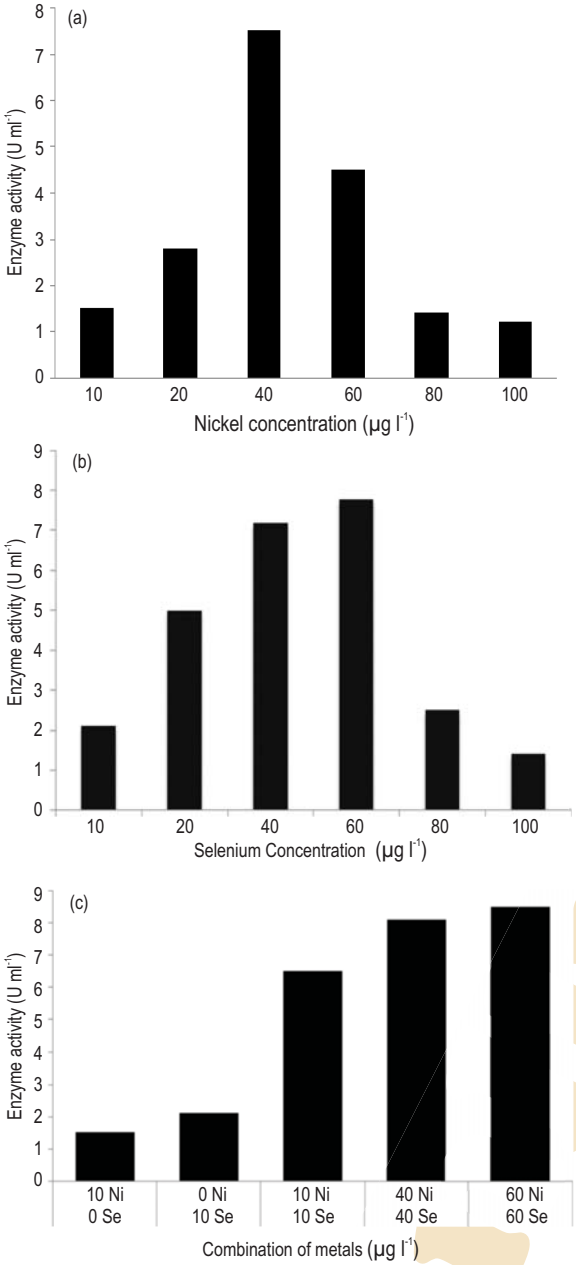

Fig. 1 : Effect of (a) Ni (b) Se and (c)Ni + Se on extracellular lipase production by *Bacillus subtilis* BDG-8

Strain BDG-8 showed positive test for nitrate reduction, citrate utilization and Voges Proskauer test. However, it showed negative results for oxidase activity, urea hydrolysis, methyl red test and hydrogen sulphide gas production (Table 1). Based on the results from morphological, physiological and biochemical characteristics, lipolytic strain BDG-8 was identified as *Bacillus subtilis*.

In the present study (Fig 1(a) it was observed that increasing the concentration of nickel in growth media increased the lipase production by several folds (7.5U ml^{-1} at 40 µg l^{-1}) with the standard deviation value of 2.46. However, nickel at a concentration greater than 40µg l^{-1} decreased the lipase production (1.2 U ml^{-1} at 100µg l^{-1}) that was also accompanied with decrease in biomass. Reduction in biomass and lipase production may be due to metal toxicity posed on the microorganism. Enzyme production by bacteria is biomass dependent. Generally at optimum concentration nickel provides perhaps, the simplest case of trace metal major nutrient interaction with microorganisms (Sujatha and Dhandayuthapani, 2013).

It was observed that increasing selenium concentration increased lipase production upto 60 µg l^{-1} of selenium (7.8 U ml^{-1}) beyond which the activity decreased rapidly to a greater extent (Fig 1b), with a standard deviation value of 2.74. This can be attributed to the fact that higher concentration of selenium reduced *Bacillus subtilis* BDG-8 growth proving lethal to the organism and consequently decreasing lipase production. Selenium, as a media supplement has increased the lipase production over many folds (7.8 U ml^{-1}). Further, improving other parameters may subsequently increase production lipase to several higher folds. Masami *et al*. (2000) reported that selenium is an important trace element in cell division process and in maintaining internal membrane integrity. In the present study we found that 60 µg l^{-1} Se was the optimum concentration because maximum biomass was observed.

Experiments with combination of selenium and nickel in equal ratio revealed that lipase production increased extensively with increasing concentration of combination of metals. Combination of nickel and selenium at 60 µg l^{-1} concentration of each showed highest enzyme activity of 8.5 U ml^{-1}. Although, nickel concentration higher than 40 µg l^{-1} individually reduced the bacterial growth and lipase production, it did not decrease the enzyme activity when combined with selenium at a similar concentration (Fig 1c), with the standard deviation value of 3.27.

It was observed that lipase activity was within detectable range at various pH tested in this study. In the presence of selenium, as media supplement, the optimum initial pH value for maximum lipase production was 7 with an enzyme activity of 7.8 U ml^{-1}. Similarly in the presence of nickel, the maximum lipase activity (7.5 U ml^{-1}) was observed at pH 7 with the standard deviation value of 3.19. It is worth mentioning that the enzyme activity was observed higher in the presence of selenium than with nickel in all the tested values of pH, except at a pH of 6.5.In the present study, the reduction of enzyme activity beyond the optimum value may be due to the suppression of bacterial growth and consequently enzyme inhibition (Fig 2(a).

. Kanimozhi *et al.* (2011) and Sugihara *et al.* (1991) earlier reported that *Bacillus* sp. prefers pH around 7.0 for best growth

Table 1 : Morphological, physiological and biochemical characteristics of *Bacillus subtilis* BDG-8

Characters	Response
Morphological characteristics	
Colony morphology	irregular, rough, flat, opaque, entire margin
Cell morphology	Rod
Gram reaction	+
Motility	-
Physiological characteristics	
Growth under aerobic condition	+
Growth under anaerobic condition	-
Biochemical characteristics	
Production of catalase	+
Oxidase	-
Indole production	-
Methyl red test	-
Voges-Proskauer	+
Starch hydrolysis	+
Hydrogen sulphide gas production	-
Gelatin hydrolysis	+
Casein hydrolysis	+
Nitrate reduction	+
Citrate utilization	+
Urea hydrolysis	-
Glycerol hydrolysis	+
Tributyrine hydrolysis	+
Fermentation of acid	
Glucose	+
Fructose	+
Sucrose	+

(a) +: Positive response; (b) - : negative response

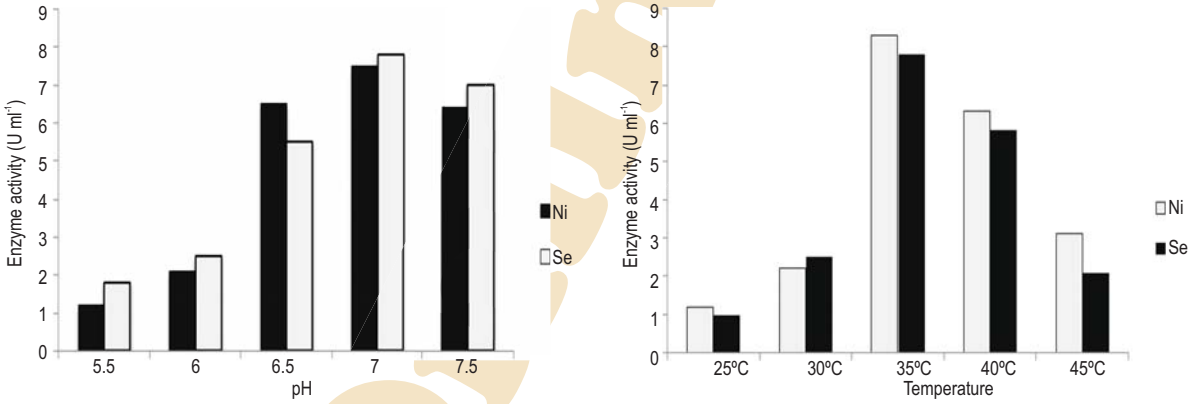

Fig. 2 : (a) pH activity and (b) Incubation temperature-activity profile of lipase enzyme by Bacillus subtilis BDG-8

and lipase production and similar observation were made in this study. However, there are several reports that state that maximum lipase activity was observed at pH values higher than 7 (Gupta *et al.,* 2004 ; Ananthi *et al*., 2013).

Temperature is yet another critical factor for bacterial growth and extracellular lipase production. Lipases are generally produced at a temperature range of 20–45°C (Gupta *et al.,* 2004).

In the current study, the effect of temperature on lipase production was studied independently with optimum pH obtained from previous results using optimum concentration of selenium and nickel as a micronutrient (Fig. 2b). When selenium was used as micronutrient the optimum temperature was 35°C, yielding lipase activity of 8.3 U ml^{-1}. Temperature beyond 35°C showed a steady decrease in enzyme activity (Fig. 2b) that could be due to enzyme inactivation or suppression of cell viability due to reduction in

growth. Nevertheless, the enzyme activity was greatly affected at a temperature less than 35°C. This study reveals that at low temperature the growth of *Bacillus subtilis* BDG-8 was low which reduces the metabolism of the organism to a greater extent resulting in lower production. In the current study, it was observed that the optimum temperature required for lipase production corresponded with the growth temperature (Annamalai *et al.,* 2011).

Similarly, when nickel was used as media supplement, the optimum temperature for maximum lipase activity (7.8 U ml^{-1}) was near 35°C with the standard deviation value of 3.08. Temperature above or lesser than 35°C comparatively showed lesser lipase activity in the current study.At all temperature (25 to 45°C) tested in this study except at 30°C, selenium as a media supplement and micronutrient showed higher lipase activity compared to nickel. This, once again confirmed that selenium can be a better micronutrient compared to nickel for lipase production by *Bacillus subtilis* BDG-8. Optimum temperature for lipase production by *Bacillus subtilis* BDG-8 was found to be 35°C but *Bacillus sp*, *Bacillus sp*. MPTK912 and *Bacillus sp.* PD-12 produced maximum lipase at 30°C , 30°C and 40°C respectively (Etugrul *et al.,* 2007; Mukesh Kumar *et al.,* 2012; Praveen and Sharmishtha, 2011).

Thus, the present study confirms that optimization of critical parameters ultimately results in increase in lipase production by several folds.

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