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OPTIMIZATION OF LIPASE PRODUCTION MEDIA PARAMETERS BY A NEWLY ISOLATED *BACILLUS LICHENIFORMS* KDP FROM OIL MILL SOIL

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ABSTRACT

This experimental research is aimed to meet the industrial demand of lipase enzyme using various supplements along with the optimal physical conditions for enhancing the production. *Bacillus licheniforms* KDP was used in this study which has been isolated from the oil mill soil. In the present investigation, selenium is used as a media supplement which has not been previously reported as supplement and thus reported by us for the first time. The maximum lipase production (16.23U/ml) was found to be at 60µm/l. Another metal ion, nickel when separately used as supplement to the medium, the maximum production (17.29U/mL) was found to be 80µm/L. The new isolate produced their maximum extracellular lipase at 7.5 (18.69 U/mL) and 45°C (17.36U/mL). The maximum lipase yield 12.43U/mL and15.38U/mL was found to be in the medium containing 0.1mg/l urea and 1mg/l ammonium nitrate respectively. The correlation analyses of all the experiments proved that the physicochemical parameters and the lipase production are interdependent.

KEYWORDS: **Ammonium nitrate, *Bacillus licheniforms* KDP, Lipase, Nickel, Selenium, Temperature Urea, pH,**

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INTRODUCTION

Lipases (triacylglycerol acylhydrolase, E.C.3.1.1.3) are one of the most important biocatalysts for biotechnological applications. These enzymes are essentially distinguished on the basis of their substrate specificity. In general, lipases have promising applications in organic chemical processing, detergent formulation, synthesis of biosurfactants, the agrochemical industry, waste water treatment, leather (removal of lipids from animal skins), medicine (blood triglyceride assay), paper manufacture, nutrition, cosmetics and pharmaceutical processing [1-4].Microorganisms are known as good source for the production of extracellular lipases than animals and plants [5]. Microbial lipases have gained special industrial attention because of their versatility and availability. The majority of known lipolytic enzymes are from bacterial origin. The availability of lipases with specific characteristics continues to be a limiting factor in widening the scope of industrial applications. The search for novel lipases with different characteristics and for improved lipase production therefore gains significance, justifying the focusing of research interests on new lipolytic microorganisms [6].Lipase production by microorganisms is highly influenced by medium components like nitrogen sources, carbon sources such as fatty acids, triglycerides, and sugars or complex polysaccharides like glycogen and surfactants which can stimulate or repress lipase production. Nitrogen source is usually the most expensive component of microbial growth substrates for production of enzymes. Most bacterial species studied for lipase production are non-pathogenic, mainly because these lipases were aimed to be used for biotechnological applications. It is a fact that extracellular lipases are important microbial virulence factors in addition to their industrial usage. The most useful lipase producer genus used in industry is *Bacillus* among gram-positive bacteria [7]. The bacterium *Bacillus subtilis* considered as a safe organism to produce proteins of commercial interest by US Food and Drug Administration. Because its capability to secrete homologous and heterologous proteins in appreciable quantities into the growth medium [8]. The present study is aimed to enhance the maximal production of lipases by optimization of fermentation parameters.

MATERIALS AND METHODS

Microorganism

Bacillus licheniforms KDP was used in this study which has been isolated from oil mill soil.

Extracellular lipase assay

Samples of the culture medium were withdrawn and centrifuged for 15 min at 10,000 g. The supernatant was used to estimate the extracellular lipase activity by using an olive oil emulsion as a substrate (olive oil 25%, 0.1 M NaOH 7.5%, polyvinyl alcohol (2%) 67.5%). The enzymatic reaction is initiated by adding 1 mL of supernatant to 4 mL of emulsion with 5 mL of 0.1 M of phosphate buffer at pH 7. The enzymatic reaction was maintained for 15 min at 37 ^{0}C on a rotary shaker (150 rpm) and was subsequently stopped by the addition 20 mL of acetone–ethanol mix [1:1(v/v)]. The free fatty acids released during the reaction were then titrated with 0.05 M NaOH [9]. One unit of lipase activity is defined as the amount of lipase inducing the release of 1 mmol of fatty acid per minute at 37 ^{0}C and pH 7.

Optimization of Fermentation Parameters

Lipase production by strain *Bacillus licheniforms* KDP was conducted in 250 ml Erlenmeyer flasks with 50 ml of the minimal medium containing Glucose 1%, NaCl 0.1%, $MgSO_4.7H_2O$ 0.05%, KH_2PO_4 0.1%, $CaCl_2.2H_2O$ 0.04%, Olive oil 1.0 (v/v). Extracellular enzyme from the flask was harvested by centrifugation at 10,000 rpm for 15 min and supernatant was used as extracellular enzyme source. The remaining nutrients and physical parameters were optimized as

follows.Optimization of different nutrient and physical parameters for lipase production were studied by maintaining all factors constant except the one being studied. Fermentation was carried out in 250 mL Erlenmeyer flasks containing 50 mL of sterilized minimal media. Effect of pH on lipase production was studied by cultivating the isolate in different initial pH values (pH 4–8) of the minimal medium. Effect of temperature on enzyme production was studied by incubating the organism at various temperatures ranging from 25 to 60°C. Flasks were kept on shaker at 180 rpm for 48 h.The effects of different medium components such as metal ions and nitrogen sources were studied. Different concentration of metal ions such as Selenium and Nickel (10 to 100 µm/L) was used as supplements to study the growth and lipase production. The influence of different concentration of nitrogen sources such as Urea and Ammonium nitrate (0.1 to 1 mg/L) was studied by supplementing in minimal medium along with the 1% olive oil. Minimal medium without selenium, nickel, urea and ammonium nitrate was considered as control and all the experiments were run in triplicates. All the experiments were carried out in the 250 mL Erlenmeyer flasks containing 50ml of minimal medium with optimized temperature and pH at 180 rpm for 48h.

Statistical Analysis

All the data was statistically analyzed to find the coefficient of correlations (Pearson) between the variables by Software - MINITAM Release 12.2. The Pearson product moment correlation coefficient was calculated to measure the degree of linear relationship between the variables. The correlation coefficient assumes a value between -1 and +1. If one variable tends to increase as the other decreases, the correlation coefficient is negative. Conversely, if the two variables tend to increase together the correlation coefficient is positive.

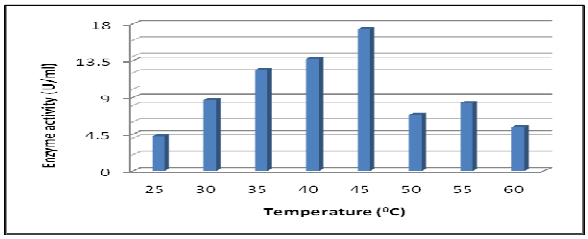
RESULTS AND DISCUSSIONS

Growth conditions affect the synthesis of lipase by microorganisms. Carbon sources, nitrogen sources, the presence of activators and inhibitors, incubation temperature, pH, inoculum amount and oxygen tension can influence lipase production [10].

Effect of temperature on lipase production

Lipase production was studied in the temperature range 25-60°C at an interval of 5°C. Production was carried out in the minimal medium containing 1.0 % v/v olive oil (pH 7.0) and at 250 rpm for 48 hrs. The maximum lipase activity (17.36 U/mL) was obtained at the temperature of 45°C (Fig.1). The production declined at temperatures above 50 °C. Temperature range 35 to 45^0C grown culture showed very high degree of positive correlation (γ= +0.966) whereas 50 °C and above showed the moderate degree of negative correlation (γ= -0.503). The correlation analysis indicates that the temperature and lipase production are interdependent. One of the physical parameter such as incubation temperature can influence on lipase production by microorganisms [10]. Low temperatures decrease lipase export to the supernatant phase and high temperatures result in enzyme denaturation [11]. Optimization of temperature is vital for cell growth and enzyme production. *Bacillus sp* strain L2 produced maximum lipase at the optimum temperature at 37- 40^0C [12].

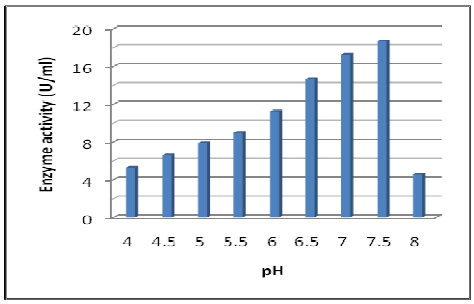
Figure.1
Effect of different initial temperature on lipase production by B.licheniforms KDP



Effect of pH on lipase production

The initial pH of the culture medium was found to be one of the most important critical environmental parameter which affects the growth and enzyme production by *B. licheniforms* KDP. The results are given in Fig.2. Maximum biomass and lipase production was obtained at an initial medium pH of 7.5 (18.69 U/mL). The pH 7.5 was found to be the optimum pH for the lipase production by *B. licheniforms* KDP because in this pH grown culture showed the perfect correlation (γ= +1). Lipase production and biomass decreased significantly at pH of 8.0 and above. The similar optimal pH 7.5 condition was also reported for lipase production by *Bacillus megaterium* AKG-1, *Bacillus stearothermophilus* MTCC 37 and *Bacillus licheniformis* MTCC 10498 [13-15]. Kumar et al., [16] reported that the very few thermostable lipases from *Bacillus sp* are active in alkaline (pH 9.0–10.0).

Figure. 2
Effect of different initial pH on lipase production by B. licheniforms KDP



Effect of different concentration of Urea and Ammonium nitrate on lipase activity

Urea and ammonium nitrate were tested as inorganic nitrogen sources at a range between 0.1 to 1mg/l in media supplemented with 1% v/v olive oil. The maximum lipase yield and biomass were found to be in medium containing 0.1mg/l urea and 1mg/l ammonium nitrate (Fig.3 & 4). Urea (1.5 % w/v) was found to be an effective inorganic source for the maximum lipase (7.4 U/mL) production by *Alcaligenes viscosus* for 36hrs [17]. In the present study the concentration above the 0.5mg/L urea showed very poor enzyme production because they may be toxic to the culture. Different concentration of ammonium nitrate grown culture showed high degree positive correlation (γ= +0.940). When ammonia nitrate concentration was increased upto 1mg/L showed the significant effects on lipase production. Inorganic nitrogen sources such as sodium nitrate have also been reported to be effective in some microbes [18].These experiment data indicates that the ammonia nitrate is the better nitrogen source than urea.

Figure 3
Effect of different concentration of Urea on lipase production by B. licheniforms KDP

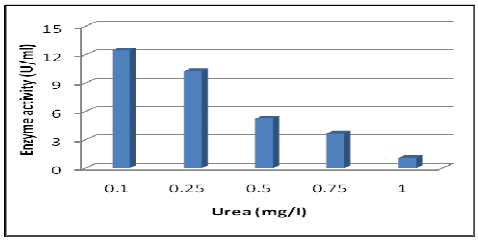
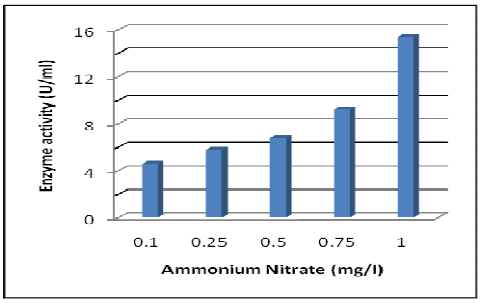


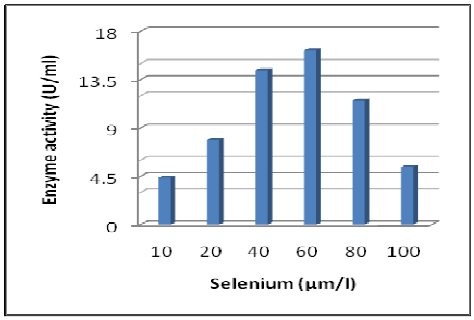
Figure. 4
Effect of different concentration of Ammonium nitrate on lipase production by B. licheniforms KDP



Effect of different concentration of Se on lipase production

Selenium (Se) is a physiologically essential element to organisms and occurs in them as organic forms at low levels. In the present study different concentrations of Se (10 to 100µm/L) were tested for their ability to support biomass and enzyme production by the new isolate. The maximum biomass and enzyme production (16.23 U/mL) were obtained in media supplemented with 60µm/L of Se (Fig. 5). The optimum Se concentration is 60 µm/L because the perfect positive correlation (γ= +1) was found for this concentration. Masami et. al., [19] reported that the bacteria *Bacillus* sp effectively reduced 20mM of selanate (most commonly found form of Se) into 2mM selenite and selenium (non-toxic insoluble elemental) when present the appropriate carbon source and absence of oxygen. This is the first report on the effect of Se on lipase production.

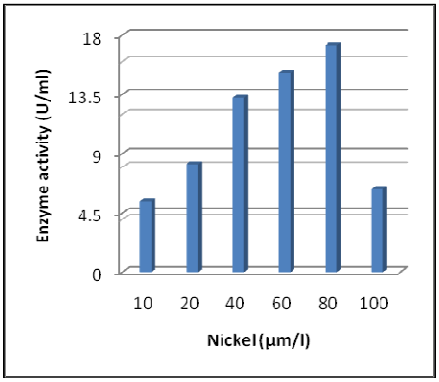
Figure. 5
Effect of different concentration of selenium on lipase production by B. licheniforms KDP



Effect of different concentration of Ni on lipase production

Different concentration of Ni (10 to 100µm/L) was supplemented to the culture and incubated (pH 7.0) at 250 rpm for 48 hrs. The maximum lipase production was found at 80µm/L Ni grown culture. The perfect positive correlation (γ= +1) was found for 60-80µm/L of Ni grown culture. Increasing the concentration of Ni^{2+} from 0.84 to 8.4 µM, increased ethanol production from 35.73 mM to 176.5mM under standard metal concentration by *Clostridium ragsdalei*. Nickel was necessary for growth of *C. ragsdalei*. Growth rate (μ_{max}) of *C. ragsdalei* improved from 0.34 to 0.49 (day^{-1}), and carbon monoxide dehydrogenase (CODH) and hydrogenase (H_2ase)-specific activities improved from 38.45 and 0.35 to 48.5 and 1.66 U/mg protein, respectively, at optimum concentration of Ni^{2+} [20]. Prasad and Manjunath [21] reported that the metal ion Ni^{+} did not support the lipase production by *Bacillus sp.1* (SAN-L1*), Bacillus sp.* 2 (SAN-L15), *Serratia sp*. (SAN-L21), *Pseudomonas sp*. (SAN-L7) and *Staphylococcus sp*. (SAN-L11) which were isolated from industrial effluents. The results are shown in Fig.6.

Figure.6
Effect of different concentration of nickel on lipase production by B. licheniforms KDP



CONCLUSIONS

Urea and Ammonium nitrate were used as inorganic nitrogen sources to enhance the lipase production by newly isolated *B. licheniforms.* Ammonium nitrate was found to be a best nitrogen source than urea. The new isolate produced their maximum extracellular lipase at 7.5 (18.69 U/mL). Maximum extracellular (17.36 U/mL) lipase activity was obtained at 45°C. This indicated that *B. licheniforms* was a thermophilic organism and it could tolerate higher temperature. The metal ions Se and Ni supported maximum production of lipase at 60µm/L and 80µm/L respectively. So in the present investigation the correlation analyses of all the experiments proved that the physicochemical parameters and the lipase production are interdependent.

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