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Optimization of Protease Production by Newly Isolated Bacillus Macquariensis from Dairy Industry Waste

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ABSTRACT: Proteolytic aerobic bacteria was isolated from soil sample collected from diary industry waste through a selective enrichment procedure with milk agar medium as the carbon source and identified as *Bacillus macquariensis* by biochemical test with Bergey's manual. We aimed to study the influence of certain nutritional and environmental factors on protease production by this newly isolate. Optimum pH and temperature for the maximum protease production by *Bacillus macquariensis* was found at 9.0(10.12 U/m1/min) and 60^0C (13.11 U/ml/min) respectively. Carbon source starch present media showed the maximum enzyme production (8.52 U/ml/min). Among different nitrogen sources the urea presented media increased the enzyme yield (9.30 U/ml/min). Stimulatory and Inhibitory effects of metal ions such as Se, Ni, Mg, Zn and Cu were tested.Stimulatory effect was observed in Ni and Zn present media.

KEYWORDS: Amylase, *Bacillus macquariensis*, Nitrogen sources, Carbon sources, pH, Temperature, Metal ions.

I. INTRODUCTION

Proteases are among the most important enzymes in present day biotechnology. Proteases refer to a group of enzymes whose catalytic function is to hydrolyze proteins. They are also called proteolytic enzymes or proteinases. Proteases are classified according to their structure or the properties of the active site. There are several kinds of proteases such as serine, metallo, and carboxyl, acidic, neutral, and alkaline proteases. Proteases are the most important class of industrial enzymes and comprise about 25% of commercial enzymes in the world (1). Proteases are classified as acid, neutral and alkaline proteases. These enzymes are widely using in dairy industry as milk clotting agent and meat tenderizing agent in food industry. Reduction of tissue inflammation (clinical and medical) application (2). Another important application of proteases is to break down protein tissue in order to be able to extract a particular substance, such as heparin (3). Alkaline proteases are one of the most widely studied group of enzymes due to their use in many industrial applications such as in food, pharmaceutical, leather, detergent and in recovering silver from used X-ray films (4).

To meet the current largely increased demand, studies on the cost effective production of industrially important enzymes have become the need of today. Microorganisms are the most important sources for enzyme production; they made significant contribution to the production of foods and beverages in the last four decades. Selection of the right

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organism plays a key role in high yield of desirable enzymes (5).The level of enzyme activity produced by an organism from a natural environment is often low and need to be elevated for industrial production. Increase in enzyme levels is often achieved by mutation of organism and media optimization. The microorganisms used for enzyme production are grown in fermenters using as optimized growth medium. Both solid state and submerged fermentation are appliedcommercially. The enzymes produced by the microorganism may be intracellular or secrete into the extracellular medium.

Each application of protease requires unique properties with respect to specificity, stability, temperature and pH dependence. The amylase exhibited activity at a wide range of pH and temperature, desirable characteristics which can lead to its application in detergents as additive and in textile desizing. Among bacteria, *Bacillus sp* is widely used for thermostable α-amylase production to meet industrial needs. They are known to be goodproducers of proteases, and these have been widely used for commercial production of the enzyme for various applications. Therefore, any improvement in the enzyme production, extracellular activity and thermo stability or activity will have a direct impact on the process performance, economics and feasibility. Since the natural isolate produced very low concentration of amylase, in the present study an attempt was made to increase the productivity by optimizing the nature and relative concentration of carbon and nitrogen source with supplemented metal ions.

II. RELATED WORK

The physiochemical properties of *Bacillus subtilis* neutral proteasewas studied and compared theproperties with those of other bacterial alkaline proteases (6).(7) extracted neutral protease from *Bacillus subtilis* and assayed the enzyme activity using the casein digestion method. They also studied the physiochemical properties of the protease enzyme *Bacillus thuringiensis* subsp kurstaki strain grown on gruel based medium, extracted, purified the enzyme metallo protease, separated by ammonium sulphate precipitation and characterized all the protease that exhibited different behaviours towards pH, temperature and thermo stability. (8)studied that proteolytic enzymes of the latex of madar plants (*Colotropis gigantia*) to be rich sources of protease and they are used in the process of unhairing.(9) compared the chemical, physical and microslopial assessment of quality of leathers produced by the traditional liming process and by two enzymic unhearing process such as protease and amylase.

III. MATERIALS AND METHODS

Microorganism

Bacillus macquariensis used in this study which was isolated from sample collected from Dairy industry waste, Mulagumoodu, Tamil Nadu, India andidentified by Goodfellow, (1989) methodology. This strain was routinely maintained on Luria- Bertani agar (LB) plates and stored at 4°C.

Fermentation Media

Media for enzyme production with the following composition: (g/L):- Groundnut oil cake 23g, Potassium dihydrogen phosphate - 2g, Magnesium chloride - 1g,Sodium chloride -1g,Ammonium nitrate -1.5g, pH -7.6. Distilled water - 1000ml Sterilized by autoclaving at 121^{o}C for 15 minutes.To optimize thefollowing physical and chemical parameters theexperiments were carried out in 250mlErlenmeyer flasks contained 100ml media and thecultures were incubated with shaking 250rpm for48 h. Cells were harvested from the culture brothby centrifugation at 10000 x g for 25 min and thesupernatants was used as sources of extracellularamylases.

Effect of pH and temperature on α-amylaseproduction

The physical parameters such as pH andtemperature were optimized by varying its rangefrom 4 to 9 and 30 to 60^{0}C respectively. Cultureswere incubated with shaking 250rpm for 48 h.

Effect of different carbon sources on proteaseproduction

The effect of different carbon sources such, glucose, Starch, Sucrose, Barley andArrow root on protease production by *Bacillus macquariensis* was investigated. Eachcarbon source effect was studied separately byadded 1 % (w/v) initial

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concentration to theabove said fermentation media (without milk).The cultures were incubated with shaking 250rpmat the optimized temperature 60^0C and pH 9.0 for48 h.

Effect of different nitrogen sources on protease production

The Nitrogen source taken for the presentinvestigation is Sodium nitrate, Potassium nitrate, casein, Peptone, Yeastextract, Urea respectively to studythe effect on amylase production by *Bacillus macquariensis* .Each nitrogen sourceeffect was studied separately by added 1 % (w/v)initial concentration to the above saidfermentation media .Thecultures were incubated with shaking 250 rpm atthe optimized temperature 60^0C and pH 9.0 for 48 h.

Effect of metal ions on protease production

Effects of different metal ions such as Na2+, Ca2, Ni2+,Zn2+, Mn2+ and Cu2+ on protease productionwas tested by amended with 1 mM/L finalconcentration of each metal ions separately in thestandard culture media. The cultures wereincubated at the optimized temperature 60^0C and pH 9.0 for 48 h.

Enzyme Assay

For protease assay, the method adopted by kunitz (1947) was modified and used.Stock tyrosine was prepared by dissolving 50 mg of tyrosine in 1 N HCI and then made up to 100ml using distilled water. The working standard was prepared by taking 10ml of stock and made to 100ml using distilled water. 0.5ml of 2% casein (Qualigens fine chemicals) was taken in test tubes labelled as test and control. To this 1ml of citrate phosphate buffer was added. The tubes were incubated at 37^0C for 5min. Then 2ml of enzyme solution was added to the tube labeled as test. The tubes were incubated at 37ºC for 30min. After incubation 2ml of 10% TCA was added to both control and test tubes. The tubes were shaken well. Then 2ml of enzyme was added to the control tube. Tubes were centrifuged and supernatants were taken for the assay.

Standard tyrosines of volume 0.2, 0.4, 0.6, 0.8, 1.0 ml were taken in five tubes. Then 0.5ml of supernatant was taken in two test tubes. The volumes in all the tubes were made to 2.4ml using distilled water. Then 2ml of 0.5N of sodium hydroxide was added to all the tubes followed by the addition of 0.6ml of folin's ciocalteau reagent. Tubes were incubated at room temperature for 10 to 20min. Absorbance's was measured at 620nm in an UV-spectrophotometer.A graph was plotted taking concentration along X – axis and optical density along Y – axis, sample values were interpolated to get their concentration. One unit (U) of enzyme is defined as the amount of enzyme required to liberate 1µg of tyrosine under standard assay conditions.

IV. RESULTS AND DISCUSSION

Characteristics and identification of protease producing strain

The cells were yellowish white, mucoid,motile rods occurring insemicurved, thick separate and utilization of carbohydrates such asGlucose, sucrose andDextrose, positive.The organism was oxidase-positive, catalasepositive,Voges Proskaeur (add creatine ascatalyst with reagents) reaction- negative, starchhydrolysis - positive, attacked glucose oxidativewith acid and gas production, showed growth at 55^0C, but not grew in 6.5% NaC1. The organismwas identified as *Bacillus macquariensis*.

Effect of media pH on α - amylase production

Among the physical parameters, the pH of the growth medium plays an important role byinducing morphological change in the organism and in enzyme secretion. The pH change observed during the growth of the organism also affects product stability in the medium. Most of the Bacillus strains used commercially for the production of protease by SmF have an optimum pH between 5.8 -8.0 for growth and enzyme production. In our study *Bacillus macquariensis*was cultivated at different initial pH (range from 4 to 9) with the standard media. The maximum protease10.12 U/mL was found at pH 9 grown cultures (Fig.1). Further increase or decrease in the pH resulted decrease in the activity of amylase.This resultis in good agreement with (10), (11).

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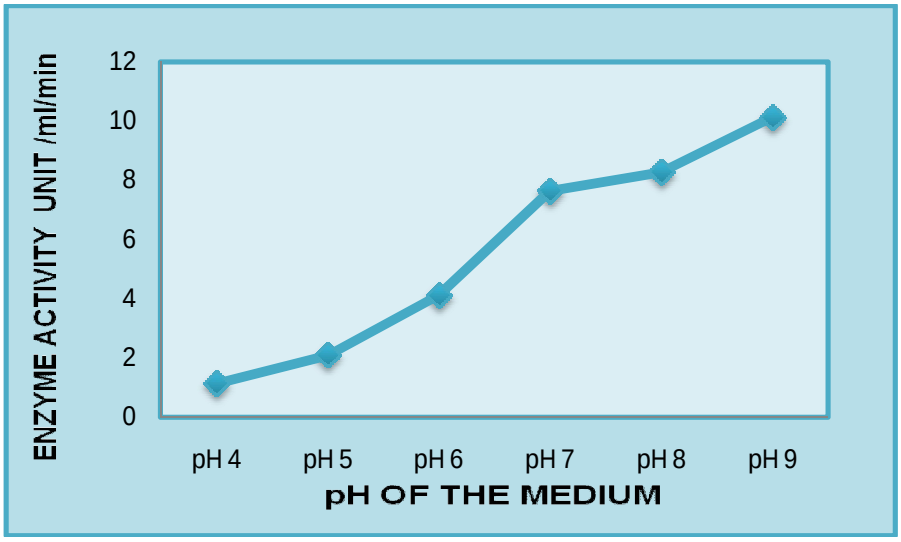

Fig 1: Effect of media pH on protease production

Effect of media temperature on α – amylaseproduction

In the present investigation the effect of different initialtemperature on protease production was studied by grown the strain *Bacillus macquariensis*at temperature range from 30 to 60^{0}C. The bacterium grown satisfactorily and produced the enzyme at temperature range from 45 to 60^{0}C but the maximal protease activity was achieved at 60^{0}C (13.11 U/mL) (Fig.2). Since the maximum protease production was obtained at 60^{0}C this new isolate is thermophilic and possessed the ability to produce thermostable protease. A reduction inenzyme activity was observed at temperatures above 60^{0}C. The mechanism of temperature control in enzyme production is not well understood (12). A link exists between enzyme synthesis which is controlled by temperature and oxygen uptake (13).(14) reported 60^{0}C was the best temperature for protease production in case of *B. SubstilisPE-11*.

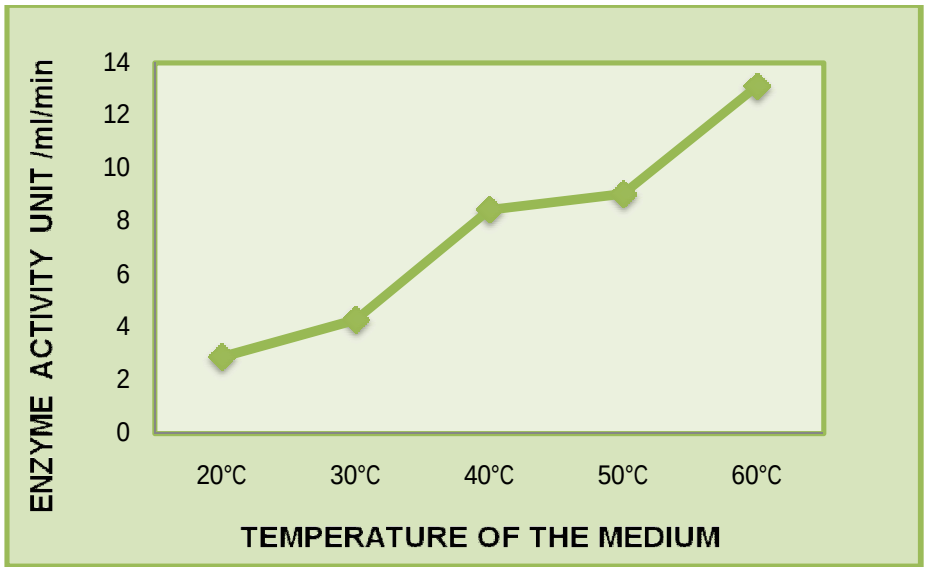

Fig 2: Effect of media temperature on protease production

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Effect of different carbon sources on protease production

Carbon source in the form of either monosaccharide or polysaccharides may influencethe production of protease enzyme. Many researchers have shown that different carbonsources have varied influence on the production of extracellular enzymes especially among protease producing strains. In our present investigation different carbon sources were used to study their influence on protease production (Fig.3). The influence of starch (8.52 U/mL) was more than the other carbon sources tested. Similarly (15)studied the effect of different carbon sources such as starch, arrow root, lactose, starch, soymeal and sucrose on protease activity.Among the five carbon sources studied, starch, arrow root proved appreciable good for the protease production lactose, starch, soymeal and sucrose are considered good for industrial protease production. (16) ReportedStarch, sucrose and lactose proved appreciably good for the protease production by *Bacillus Sp.* K-30.

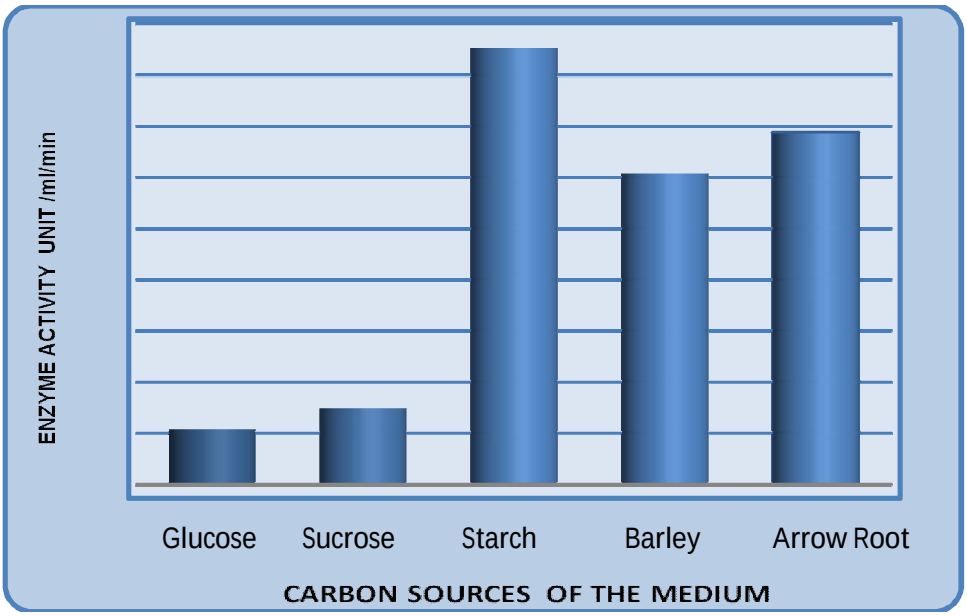

Fig 3: Effect of different carbon sources on protease production

Effect of different nitrogen sources on protease production

Nitrogen sources are the most important secondary energy compounds for microorganism's growth and their production. The nature of these compounds and the concentration used may stimulate or down regulate the production of enzymes. The protease synthesis by several microorganisms has been correlated to the presence or absence of different nitrogen sources and various amino acids in the growth medium.Among the nitrogen sources tested urea amended media showed the maximum productionof protease was 9.3 U/mL. The effects of organic & inorganic nitrogen sources on protease production by *Bacillus Sp* have been reported in the literature. It has been reported some inorganic nitrogen sources gave better enzyme production. The best nitrogen source for protease production was $(NH_4)_2 So_4$(17).

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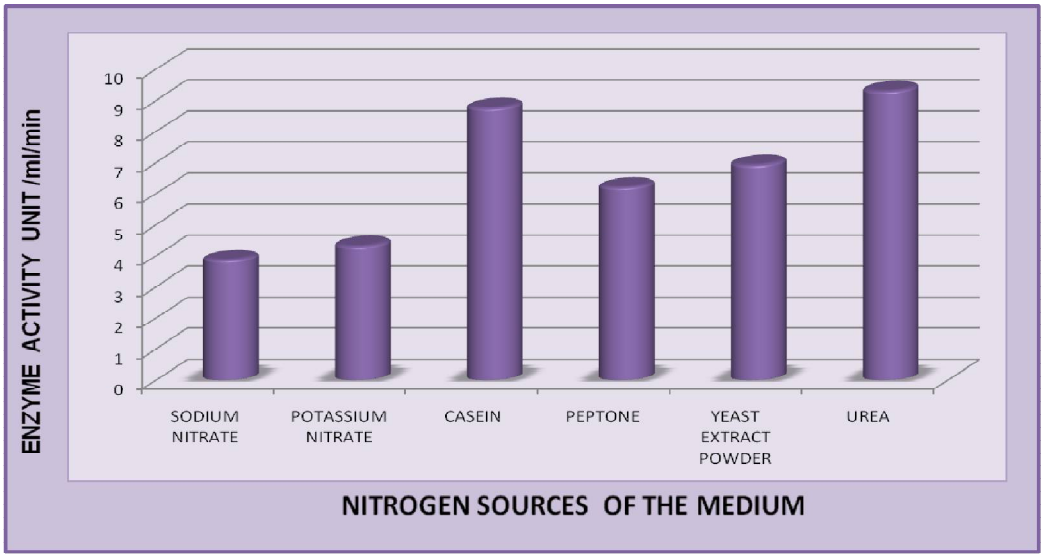

Fig 4: Effect of different nitrogen sources on protease production

Effect of different metal ions on α –amylase production

The influence of 6 kinds of metal ions on the protease activity was studied (Fig.4). Among the metal ions tested nickel and zinc enhanced the activity of protease enzyme produced by *Bacillus macquariensis.* Sodium and potassium was inhibited alkaline protease enzyme activity. (18)reported that mn^{2+}, ca^{2+} and mg^{2+} ions have been described to increase the relative protease activity produced by *Bacillus megatarium* isolated from Thai fish sauce.

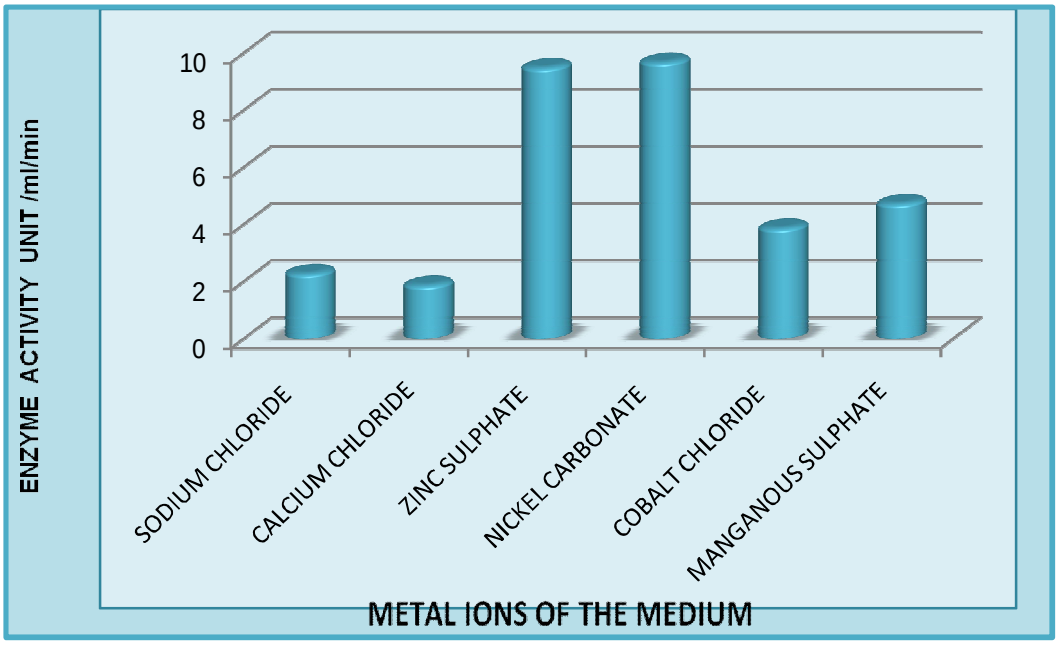

Fig 5: Effect of different metal ions on protease production

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V. CONCLUSION

The enzymes of protease family have great significance due to its wide area of potential application. In the present investigation, B.*Macquariensis*was isolated from diary industry waste. The optimum pH and temperature was found to be 9.0 and 60^0C respectively. Among different carbon and nitrogen sources starch and urea supported to the maximum yield. The metal ions Ni2+ and zinc, enhanced the production at 1mM/L concentration. By this investigation the above said conditions are the optimum for the maximum protease production and which can be used to design new production media.

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