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Production and Optimization of Enzymes By Probiotic bacteria isolated from Indian Major Carp *Catla catla*

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ABSTRACT

Microorganisms in particular have been regarded as treasure sources of useful enzymes. They are usually capable of digesting insoluble nutrient materials such as cellulose, protein and starch. Enzymes are among the most important bio products and are being utilized in a large number of processes in the areas of industrial, environmental and food biotechnology. Considerable information are available concerning the intestinal microflora of homeotherms and also the role of those intestinal microflora in digestion; but very little information is available concerning the bacterial population in the gastrointestinal tract. Fresh water dwelling fishes have scanty information on gut microflora associated with them and there is a paucity of knowledge regarding microbial enzyme activity in fish gastrointestinal tracts. Although some of the enzymes like amylase, proteases, Lipase, Cellulase have been reported to be associated in the gut of fish. These enzymes are most important and have the great significance contributes of about 25% of the total enzyme market. It has a number of potential applications in food, pharmaceutical and fine chemical industries. In present study the deals with the Production and Optimization of Enzymes by Probiotic bacteria isolated from Indian Major carp Catla catla

***Key words**: Microflora, Enzymes, Amylase, Proteases, Lipase, Cellulase, Catla catla*

I.INTRODUCTION

Gut microorganisms might have positive effects in the digestive process of the host fish as they are able to breakdown complex molecules, e.g., starch, cellulose, protein and Lipid. It has only been during the last decade that there has been an improved understanding of the importance of commensal intestinal microbiota in fish intestine [1]. The bacterial flora of the gastrointestinal tract in general represents a very important and diversified enzymatic potential, and it seems logical to think that the enzymatic mass lodged in the digestive tract might interfere in a considerable way with a major part of the metabolism of the host animal. Some fish species acquire many of their intestinal enzymes from the microflora inhabiting their guts [2]. Enzyme producing micro organisms were directly indicated by the formation of distinct clearing zones on the substantially darker

background of the medium within two to three days of incubation. The bacterial species isolated from gut for the qualitative detection of different enzymatic activities [3]. Fish receive bacteria in the digestive tract from the aquatic environment through water and food that are populated with bacteria. Being rich in nutrient, the environment of the digestive tract of fish confers a favorable culture environment for the microorganisms [4].

Amylases are among the important industrial enzymes & also have great significance in biotechnological studies. Microbial production of amylase is more effective than that of other sources as the technique is easy, cost effective, fast & can be modified to obtain enzymes of desired characteristics [5].The first enzyme produced industrially was an amylase from a fungal source in 1894, which was used for the treatment of digestive disorders. The microbial amylase could be potentially useful in various pharmaceutical, fine-chemical industries etc., with the event of new frontiers in biotechnology, use of amylase has widened in clinical research, medical chemistry & starch analytical chemistry. Amylases are also used in baking, brewing, textile, detergent, paper & distilling industries [6].

Cellulose is the most abundant and renewable biopolymer on earth. The enormous potential as are renewable source of energy was recognized only after cellulose degrading enzymes or "cellulases" had been identified. Cellulase is implicated in several food processing, textile, paper, pharmaceutical and other related industries [7]. The bacterial flora of the gastro intestinal tract of fishes in general, represents a very important and diversified enzymatic potential (Clements, 1997). Bacteria, which has high growth rate as compared to fungi has good potential to be used in cellulase production. However, the application of bacteria in producing cellulase is not widely used[8].

Lipases are considered to be the largest group of industrial enzyme, subsequent to proteases and amylases. Lipases are glycerol ester hydrolases which hydrolyze ester linkages of glycerides at water–oil interface [9]. During hydrolysis reaction they take acyl group from glycerides and form lipase–acyl composite, and then transfer the acyl group to OH group of water. Lipase occurs widely throughout the world's flora and fauna [10]. In eukaryotes, lipases are involved in various stages of lipid metabolism including fat digestion, absorption, reconstitution, and lipoprotein metabolism. In plants, lipases are found in energy reserve tissues [11].

Proteases are enzymes that are widespread in nature. They breakdown proteins by hydrolysis of the peptide bond that exists between two amino acids of a polypeptide chain. With the modern world focusing on eco-friendly products and product output, more and more chemical processes are being replaced by enzymatic methods [12]. Proteases can be classified according to their active pH range into neutral, acidic and alkaline proteases. Proteolytic enzyme producers are also helpful for the health of the ecosystems of this earth as these microbes decompose the dead and decaying animal or plant tissues in water or land. They can create pollution free environment and they are responsible for the recycling of nutrients [13]. The protease enzyme constitutes two thirds of total enzymes used in various industries and it accounts for about 60% of the total worldwide sale in the market.

Microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications [14]. In order to meet the industrial requirements as well as increasing demand of global enzyme market, isolation and optimization of enzyme production conditions of new promising strains should be a continuous process [15]. Therefore, the present study aims at Production and quantitative assay of enzymes produced by microbes isolated from the intestine of Indian Carp *Catla catla* and Optimizing the production of enzymes such as amylase, cellulase, lipase and protease respectively.

II. MATERIALS AND METHODS

Isolation and Screening of Microbes for Extra-Cellular Enzyme Production

Sample Collection

The fish Indian Carp *Catla catla* were collected by the Fish farm in chittar, Kanyakumari District, Tamil Nadu, India.

Microbial Culture

Microbial culture of the intestinal mucosa collected from the test fish was carried out for bacterial enumeration and isolation. For this purpose, the homogenate of the intestinal mucosa of the test fish was used after five serial 1:10 dilutions (Beveridge *et al*., 1991). Samples (0.1ml) were taken from each dilution and poured aseptically within a laminar flow on sterile tryptone soy agar (TSA) plates, in duplicate. These culture plates were incubated at 34°C for 24 hours. They were then examined for the development of bacterial colonies. The well-separated colonies with apparently different morphological appearance (colony colour, configuration, surface, margin and opacity) were streaked separately on TSA plates to obtain pure cultures. Single, isolated colonies from the streaked plates were transferred to TSA slants and maintained in a refrigerator (4°C) for further study.

To enumerate amylase, cellulase, lipase and protease producing bacteria, diluted gut homogenate was poured on starch-agar, carboxy methyl cellulose (CMC) agar, tributyrin agar and skim milk agar media containing plates respectively. These culture plates were incubated at 34°C for 24 hours. A detailed description of the media compositions is described below in 2.1.4 (Mondal *et al*. 2008). By multiplying the number of colonies formed on each plate by the reciprocal of dilution, colony numbers per unit sample volume of gut homogenate were determined.

Quantitative Extracellular Enzyme production by *Bacillus sp*. TSM1, *Bacillus licheniformis* TSM2, *Bacillus cereus* TSM3 and *Pseudomonas mendocina* TSM4

Selective broth media were used for the production of the respective enzymes. Quantitative estimation of the amylase and cellulase activities were determined following the standard methods using dinitrosalicylic acid and casein digestion and para-Ni-trophenyl palmitate assay methods were used for the quantitative estimation of protease and lipase.

Production and Quantitative Assay of Amylase

Production of Amylase

The culture medium used in this work for amylase production contained (g/L) Starch, 10; peptone, 10; yeast extract, 20; KH_2PO_4, 0.05; $MnCl_2.4H_2O$, 0.015; $MgSO_4.7H_2O$, 0.25; $CaCl_2.2H_2O$, 0.05; and $FeSO_4.7H_2O$, 0.01 and pH maintained to 7.0. 50 ml of production medium in four 250 ml Erlenmeyer flasks were inoculated with 5% inoculum of overnight grown cultures *Bacillus sp*., *Bacillus licheniformis*, *Bacillus cereus* and *Pseudomonas mendocina* and incubated at 30°C on rotary shaker at 100 rpm for 48 hours. 2ml of each broth was harvested aseptically and centrifuged at 10,000 rpm for 20 minutes at 4°C and the supernatant thus obtained was used for amylase assay.

Determination of Amylase Activity

Amylase activity was determined by sepctrophotometric method. According to procedure 1.0 ml of culture filtrate was taken in test tube in duplicate and 1.0 ml of substrate (2% starch) was added. The test tubes were covered and incubated in water bath maintained at 37°C for 15 minutes. The reaction was stopped by addition of 2.0 ml DNS reagent to each tube and kept in boiling water bath for 5 minutes. After cooling at room temperature, the absorbance was read at 540 nm by spectrophotometer. A unit of amylase activity was defined as the amount of amylase required to catalyze the liberation of reducing sugar equivalent to 1 µ mol of maltose per minute under the assay condition.

Production and Quantitative Assay of Cellulase

Production of Cellulase

The strains *Bacillus sp*. TSM1, *Bacillus licheniformis* TSM2, *Bacillus cereus* strain TSM3 and *Pseudomonas mendocina* strain TSM4 were individually incubated in the production medium (Carboxymethylcellulose agar (CMC agar) medium ($g \cdot L^{-1}$): L-glutamic acid – 0.3g, NH_4NO_3 -1.4g, KH_2PO_4 – 2g, $CaCl_2$ -0.3g, $MgSO_4$ - 0.3g, Proteose peptone -7.5, $FeSO_4$ – 5g, $MnSO_4$ – 1.6g, $ZnSO_4$ – 1.4g, CMC – 30g, Tween80 – 20ml, pH – 5.6) in a shaker at 30°C for 48 hours.

Determination of Cellulase Activity

The production of reducing sugar (glucose) from CMC substrate through cellulolytic activity was measured at 540 nm by the dinitrosalicylic acid method using glucose as the standard. One cellulase unit (U) was defined as the amount of enzyme per millilitre culture filtrate that released 1 µ mol of glucose per minute.

Production and Quantitative Assay of Lipase

Production of Lipase

Each bacterium was initially cultured using medium containing (w/v) yeast extract (0.15%), peptone (0.5%), sodium chloride (1.0%) and olive oil (0.5%), at pH 7, and 30°C for 24 hours. Then, 5% of enriched seed culture was inoculated into a 50 ml medium (w/v) containing potassium peptone 0.5%, dihydrogen orthophosphate 0.1%; sodium chloride 1% and magnesium sulphate 0.01%. Then it was incubated at 30°C at 100 rpm. After incubation it was centrifuged at 10000 rpm and the supernatant was used for lipase activity determination.

Determination of Lipase Assay

Lipase activity was assayed through spectrophotometric method by using p-nitrophenol palmitate as substrate. The reaction mixture containing 100 l of 50 mM Tris buffer (pH-7.0), 50 l of substrate solution (1mM p-NPP containing 1% Triton X-100), 350 ml of H_2O and the reaction was initiated by adding 100 µl of enzyme solution. After incubation of 10 minutes, the reaction was stopped by adding 1 ml of 2% sodium dodecyl sulphate (SDS) solution. The absorbance was read at 420 nm using UV–Vis–spectorophotometer. One unit of lipase activity was defined as the amount of enzyme releasing 1 µ mol of p-nitrophenol per minute.

Production and Quantitative Assay of Protease

Production of Protease

The culture medium used in this work for protease production contained (g/L) Glucose, 10; Casein, 5.0; Yeast extract, 5.0; K2HPO4, 1.0; MgSO4, 0.2 and pH maintained to 7.2. 50 ml each production medium in 250 ml Erlenmeyer flask was inoculated with 5% inoculum of overnight grown culture of *Bacillus sp*. *Bacillus licheniformis*, *Bacillus cereus* and *Pseudomonas mendocina* strain and incubated at 30°C on rotary shaker at 100 rpm. 2ml of each broth was harvested aseptically and centrifuged at 10,000 rpm for 20 minutes at 4°C and the supernatant thus obtained was used for protease assay.

Determination of Protease Assay

Activity of protease was determined by Anson method with some modifications Enzyme solution (0.5ml) was mixed with 2.0 ml substrate (0.65% casein in 25 mM Tris-HCl buffer) at 37ºC for 30 minutes and after incubation TCA was added to attenuate the reaction. This mixture was centrifuged at 10,000 rpm for 5 minutes and the released amino acids were measured as tyrosine by the method of Folin and Ciocalteu.

Optimization of Various Parameters for Enzyme Production

The physical and nutritional parameters are extremely important in the yield and characteristics of enzymes production. Here in the present study, *Bacillus sp*., *Bacillus licheniformis*, *Bacillus cereus* and *Pseudomonas mendocina* were used to optimize the production of enzymes such as amylase, cellulase, lipase and protease. Different temperature, pH, carbon source and nitrogen source were tested to achieve maximum yield of respective enzymes. The experiments were conducted in 250 ml Erlenmeyer flasks containing the respective media for the production of amylase, cellulase, lipase and protease (as given in the section 2.2.1.1.1, 2.2.1.2.1, 2.2.1.3.1 and 2.2.1.4.1respectively) and were sterilized at 15 lbs pressure for 15 minutes. After sterilization of the broth by autoclaving, the flask were cooled and the strains were inoculated and incubated differently for different parameters as described in the following paragraphs by taking one parameter at one time. Appropriate controls were maintained in all the experiments.

Effect of Different Temperature on Enzyme Production

To determine the optimum temperature for maximum enzyme production, the test strains were inoculated in respective production media at different temperatures. 50 ml each of sterilized media were inoculated with 0.5ml each of inoculum in individual conical flask (250 ml) and incubated over a period of 2 days at different temperatures (25, 30, 35, 40 and 45ºC). After incubation, the amylase, cellulase, lipase and protease production were estimated from the culture supernatant by spectrophotometric assay method.

Effect of different pH on Enzyme Production

The effect of different pH on enzyme production were determined by preparing respective production medium with different pH ranges of 5,6,7,8 and 9 in individual conical flasks. Then these flasks were sterilized at 121ºC for 15 minutes. After sterilization the flasks were allowed to cool (37ºC). After that, 0.5 ml each of inoculum were added in each flask and incubated at 30ºC for 2 days in a shaker at 150 rpm. After incubation, the production of respective enzymes was determined in the culture supernatant by spectrophotometric assay method.

Effect of Different Carbon Sources on Enzyme Production

To test the effect of different carbon sources on enzymes production by *Bacillus sp*. *Bacillus licheniformis*, *Bacillus cereus* and *Pseudomonas mendocina*, 5 different carbon sources were screened. They were glucose, fructose, maltose, starch and cellulose. They were supplied individually in addition to the respective production media at a concentration of 1% taken in conical flasks. Then the flasks were sterilized at 121ºC for 15 minutes and allowed to cool (37ºC). Then 0.5 ml each of inoculum were added in each flask and incubated at 30ºC for 2 days in a shaker at 150 rpm. Then the enzyme productions were estimated in the culture supernatant by spectrometric assay method.

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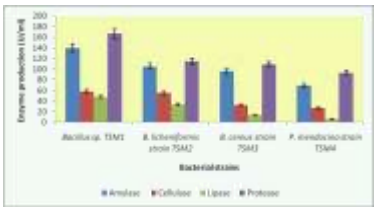
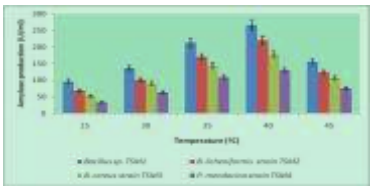
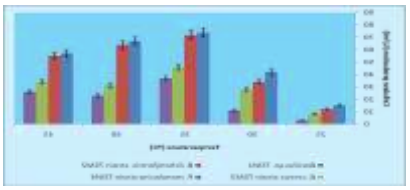
Effect of Different Nitrogen Sources on Enzyme Production

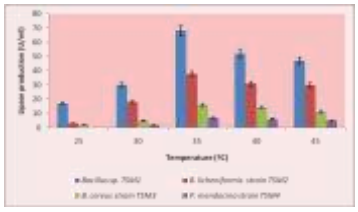
The effect of different nitrogen sources on enzyme production by *Bacillus sp*, *Bacillus licheniformis*,, *Bacillus cereus* and *Pseudomonas mendocina* were determined. 5 different nitrogen sources such as yeast extract, peptone, glycine, ammonium sulphate and ammonium nitrate were individually tested. They were added individually in addition to the respective production media for amylase, cellulase, lipase and protease at a

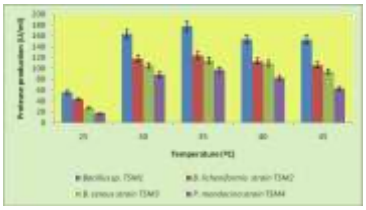


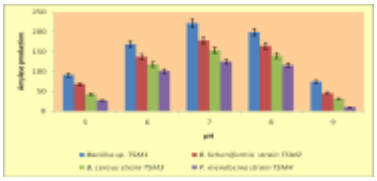




concentration of 1% taken in conical flasks. The flasks were sterilized at 121ºC for 15minutes and allowed to cool at 37ºC. Then, 0.5 ml each of inoculum were added in each flask and incubated at 30ºC for 7 days in a shaker at 150 rpm. Finally the enzyme production was determined in the culture supernatant by spectrophotometric assay method.







Result

Quantitative extracellular enzyme production by *Bacillus sp*. TSM1, *Bacillus licheniformis* TSM2, *Bacillus cereus*TSM3 and *Pseudomonas mendocina* TSM4

Effect of different temperature on amylase production by *Bacillus sp*. TSM1,*Bacillus licheniformis* TSM2, *Bacillus cereus* TSM3 and *Pseudomonas mendocina* TSM4

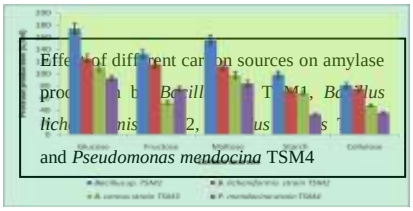
Effect of different temperature on cellulase production by *Bacillus sp*. TSM1, *Bacillus licheniformis* TSM2, *Bacillus cereus* TSM3 and *Pseudomonas mendocina* TSM4

Effect of different temperature on protease production by *Bacillus sp*. TSM1, *Bacillus licheniformis* TSM2, *Bacillus cereus* TSM3 and *Pseudomonas mendocina* TSM4

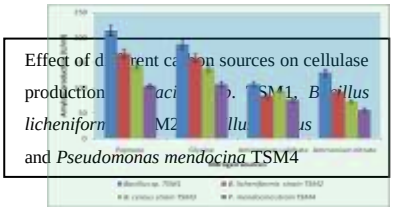
Effect of different pH on amylase production by *Bacillus sp*. TSM1, *Bacillus licheniformis* TSM2, *Bacillus cereus* TSM3 and *Pseudomonas mendocina* TSM

Effect of different pH on lipase production by *Bacillus sp*. TSM1, *Bacillus licheniformis* strain TSM2, *Bacillus cereus* strain TSM3 and *Pseudomonas mendocina* strain TSM4

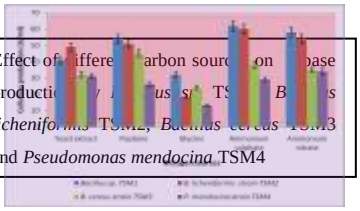
Effect of different pH on protease production by *Bacillus sp*. TSM1, *Bacillus licheniformis* TSM2, *Bacillus cereus* TSM3 and *Pseudomonas mendocina* TSM4



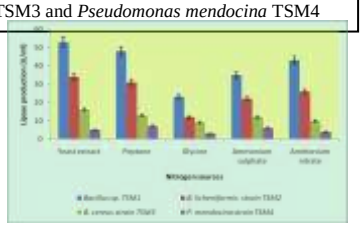
Effect of different carbon sources on amylase production by *Bacillus sp.* TSM1, *Bacillus licheniformis* TSM2, *Bacillus cereus* TSM3 and *Pseudomonas mendocina* TSM4



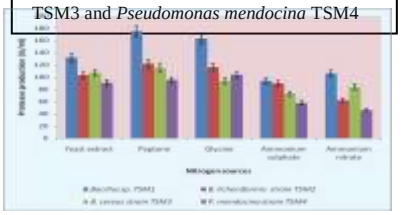
Effect of different carbon sources on cellulase production by *Bacillus sp.* TSM1, *Bacillus licheniformis* TSM2, *Bacillus cereus* TSM3 and *Pseudomonas mendocina* TSM4



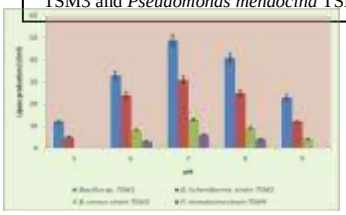
Effect of different carbon sources on protease production by *Bacillus sp.* TSM1, *Bacillus licheniformis* TSM2, *Bacillus cereus* TSM3 and *Pseudomonas mendocina* TSM4



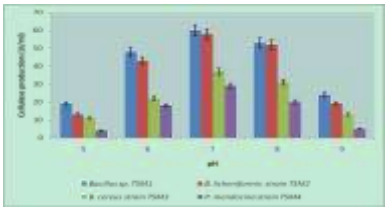
Effect of different carbon sources on Protease production by *Bacillus sp.* TSM1, *Bacillus licheniformis* TSM2, *Bacillus cereus* TSM3 and *Pseudomonas mendocina* TSM4

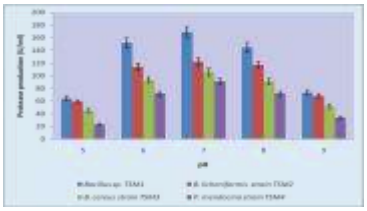


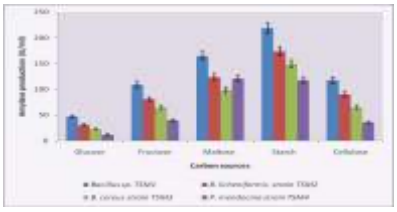
Effect of different nitrogen sources on product amylase on by *Bacillus sp.* TSM1, *Bacillus licheniformis* TSM2, *Bacillus cereus* TSM3 and *Pseudomonas mendocina* TSM4



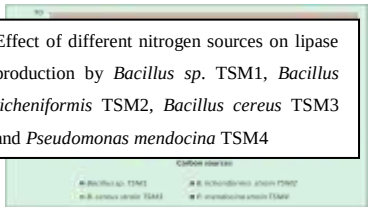
Effect of different nitrogen sources on cellulase production by *Bacillus sp.* TSM1, *Bacillus licheniformis* TSM2, *Bacillus cereus* TSM3 and *Pseudomonas mendocina* TSM4

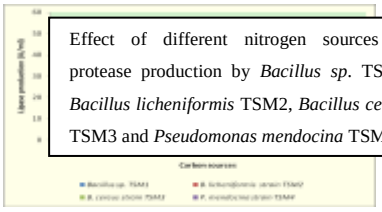






Effect of different nitrogen sources on lipase production by *Bacillus sp.* TSM1, *Bacillus licheniformis* TSM2, *Bacillus cereus* TSM3 and *Pseudomonas mendocina* TSM4





Effect of different nitrogen sources on protease production by *Bacillus sp.* TSM1, *Bacillus licheniformis* TSM2, *Bacillus cereus* TSM3 and *Pseudomonas mendocina* TSM4

III.RESULT & DISCUSSION

Temperature is an effective parameter for production of amylase enzyme by bacteria. To study the effect of temperature on amylase activity, the experimental flask containing starch medium were incubated at different temperatures. Production of amylase was increased at 35ºC but further increase in the temperature showed decrease in amylase production. Results are supported by earlier studies carried out for maximum production of amylase at temperature 37ºC (Mishra *et al*., 2005; Elizabeth *et al*., 2006; Sajitha *et al*., 2011). pH

can significantly affect the growth of microorganisms because change in pH can influence fermentation by change in growth pattern of the culture as a result it will influence the metabolic activities. Thus, to check the effect of different pH on enzyme production, the experimental design with production medium adjusted to different pH, inoculated with the culture and was incubated at 30°C. The maximum enzyme production was found at pH 7.0 by all the strains used. Similar results have been reported by earlier studies carried out for production of amylase (Vidyalakshmi *et al*., 2009).The effect of carbon sources at 1% (w/v) level was determined on amylase production. The four isolated bacteria showed efficient growth in all the substrates and amylase production but maximum amylase production was found in production media containing starch as carbon source and the maximum amount of amylase was produced by the strain *Bacillus sp*. TSM1 followed by *B. licheniformis* TSM2 and *B. cereus* TSM3. For the strain *P.mendocina* TSM4 maltose exerted maximum amylase activity. Results are supported by earlier studies carried out for production of amylase with starch as carbon source for *Bacillus sp*. (Ashwini *et al*., 2011; Suribabu *et al*., 2014). The nitrogen source in the medium also influences the lipase titers in production broth. Generally, organic nitrogen sources, such as peptone and yeast extract are preferred, which have been used as a major source for lipase production by various *Bacillus sp*. In the present study, the maximum lipase was produced in yeast extract supplemented medium by *Bacillus sp*. TSM1, *Bacillus licheniformis* TSM2 and *Bacillus cereus* TSM3; whereas, *P.mendocina* TSM4 exhibited maximum production in peptone substituted medium than the other tested organic and inorganic nitrogen sources. Thomas *et al*., (2003)

IV.CONCLUSION

The present study demonstrates that, the Bacillus *sp*. TSM1, *Bacillus licheniformis* TSM2, *Bacillus cereus* TSM3 and *P. mendocina* TSM4 isolated from the fish gut showed amylase, cellulase, lipase and protease production which were non conventional source. The results obtained for media optimization for better enzyme production needs to be done using statistical design analysis tool for better enzyme production which will in turn shows the industrial potentiality of each enzymes, which would lead to the significant improvement of enzymatic hydrolysis of those enzymes. Thus the four producer strains obtained would be helpful for enzyme hydrolysis on large scale.

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