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PRODUCTION OF ALKALINE PROTEASE FROM A THERMOALKALOPHILIC BACILLUS SP.JB-99 UNDER SOLID STATE FERMENTATION

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ABSTRACT

The production of extracellular thermostable alkaline protease by a thermoalkalophlic *Bacillus sp. JB-99* under solid state fermentation was investigated using various agro wastes as inexpensive solid substrate raw materials. Physical and chemical parameters were optimized. Wheat bran was found as a cheap and chief source of solid substrate to yield maximum amount of alkaline protease (7222 U/g) under the optimum conditions: moisture content at the ratio of 1:2.0 (w/v); Mineral Salt solution (MS1); pH of the medium (10.0) using separately sterilized Na_2CO_3 solution; inoculam concentration of 15% (v/w); incubation temperature at 50^oC under 90-95% relative humidity (provided by sterile distilled water) and 96hours of fermentation. Wheat bran in combination with Sugarcane bagasse showed comparatively better yield of enzyme. Carbon additives like Arabinose, Citric acid, Raffinose, Starch and Fructose; organic nitrogen sources (Casein and yeast extract) and inorganic nitrogen ($NaNO_3$ and KNO_3)improved the enzyme yield, whereas Galactose, Lactose and Glycerol were minimized, but Dextrose, NH_4NO_3 and NH_4Cl were strangely inhibited. The Glucose supplementation has totally repressed the alkaline protease production. This investigation could, therefore, lead to substantial reduction in the overall cost of enzyme production compared to submerged fermentation.

KEY WORDS: Alkaline protease, thermoalkalophilic, solid state fermentation, *Bacillus* sp.JB-99

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INTRODUCTION

Among the industrial enzymes 75% are hydrolytic. Proteases belong to the group of hydrolytic enzymes represent one of the three largest groups of industrial enzymes accounting for 30% of the total worldwide enzyme production[16], constituting more than 65% of the total industrial enzyme market.[18,22] Bacterial proteases are the most significant compared with animal and fungal proteases[46], especially proteases from *Bacillus* sp are more potent.[43] Proteases are ubiquitous in nature, relatively small in size, compact and spherical structures that catalyze peptide bond cleavage in proteins.[28] Proteases are widely used in several industrial sectors such as detergent, food, pharmaceutical, chemical, leather, silk, cleaning contact lenses/dentures, dewooling of animal skin, recovery of soluble proteins and amino acids from chrome leather wastes and waste treatment etc.[23, 34] Proteases play a critical role in many physiological processes including protein catabolism, blood coagulation, cell growth and migration, tissue arrangement, morphogenesis in development, inflammation, tumor growth and metastasis, activation of zymogens[9], release of hormones and pharmacologically active peptides from precursor proteins and transport of secretory proteins across membranes.[36] This has created an increasing attention towards the exploitation of exotic microbial strains for the production of alkaline proteases from novel sources.

Solid-state fermentation (SSF) has shown much promise in the development of bioprocesses and products. SSF has been known for centuries and used effectively for the production of oriental foods. Recently, it has gained importance in the production of microbial enzymes owing to several economic advantages over conventional submerged fermentation. Proteases produced by SSF process have greater economic feasibility. It is estimated that approximately 160 billion tons of organic biomass are produced annually worldwide by photosynthesis.[17] Most agricultural wastes contain three major components; cellulose (30-50%), hemicellulose (20-35%) and 4-35% lignin.[8] Among the various agro wastes, *wheat bran* was the preferred choice.[23] In SSF, the solid substrate not only supplies the nutrient to the culture but also serves as an anchorage for the microbial cells, where cost and availability are important considerations. Solid State Fermentation was shown to have good potential for the production of many enzymes, subtilisins[44] and other fine biochemicals which result in much greater productivity than the submerged fermentation.[14] SSF offers numerous advantages over submerged fermentation (SmF) system, including high volumetric productivity, relatively higher concentration of the products, less effluent generation, no requirement for complex machinery, equipments and control systems[3], use of an inexpensive substrate, simpler downstream processing and lower energy requirements.[21] The aim of the present research was to devise a simpler SSF process with a new novel *Bacillus* sp. JB-99 for its capacity to grow rapidly on a solid support and extract large quantities of extra cellular proteases.

MATERIALS AND METHODS

Microorganism and culture conditions

The bacterial strain thermoalkalophilic *Bacillus* sp. JB-99[19] was used for the production of thermostable alkaline protease under solid state fermentation. The strain was routinely maintained on chemically defined medium (CD), consisting of (g/l) citric acid: 10.0, $NaNO_3$: 10.0, K_2HPO_4: 5.0, $MgSO_4.7H_2O$: 0.3, $CaCl_2.2H_2O$: 0.2, NaCl: 5.0, the pH (10.5) was adjusted by the addition of separately sterilized Na_2CO_3 (1%) just before inoculation. Media was inoculated with 1% (v/v) of fresh culture and incubated in Rotary incubator shaker with agitation (180 rpm) at 50^o C. 24h old culture was used as an inoculam. Alkaline protease

production was determined by skimmed milk/casein agar methods.

Solid state fermentation for the production of thermostable alkaline protease

Chemicals, Agro wastes (solid substrates) and their utilization

All the chemicals used were of analytical grade and various Agro wastes (solid substrates) were obtained from the *Gulbarga* market (India) and the wheat bran from '*Raichur flour mill'* (Karnataka, India).

Growth determination and enzyme production

The thermoalkalophilic *Bacillus* sp. JB-99 was grown in Erlenmeyer flasks (250ml) containing 10gm of agro waste (solid substrate) moistened with an appropriate volume of chemically defined medium; suitably sterilized, cooled and the alkaline pH was adjusted by the addition of separately sterilized Na_2CO_3 (w/v) (as the final moisture content of the medium), just before inoculation using 24h old culture of *Bacillus* sp. JB-99. The flasks were incubated in an incubator chamber (humidified with sterile distilled water) at 45-50^oC for 3-5 days.

At different interval of incubation, a known concentration of bacterial bran was withdrawn from the flask under aseptic conditions and moistened with 50mM Glycine NaOH buffer pH 10.5 (100ml/10gm substrate), thoroughly mixed upon continuous agitation for 30 min at room temperature. The moldy bran was squeezed through fresh sterile muslin cloth and the enzyme extract was centrifuged at 10,000 rpm for 10min at 25^oC. The clear supernatant obtained was used for the enzyme assays.

Enzyme assay

The alkaline protease activity of the cell-free culture supernatant was determined using the modified Kembhavi et al., method.[20] The reaction volume (2 ml) contains 1ml of casein 1 % (w/v) (dissolved in 50mM Glycine NaOH buffer, pH-10.5) and 0.95 ml of Glycine NaOH buffer. The reaction was initiated by the addition of 0.05 ml of suitably diluted enzyme solution and incubated at 70^oC for 20 min. The reaction was arrested by the addition of 2 ml of 10 % TCA (w/v) and allowed to stand for 1h at room temperature. The reaction mixture was centrifuged at 12,000 X g for 10 min. The absorbance of the supernatant was determined at 280 nm. One unit of alkaline protease activity is defined, as the amount of enzyme required to liberate 1μg of tyrosine per minute under the experimental conditions.

Optimization of fermentation process under SSF

Various process parameters influencing enzyme production during SSF were optimized. The strategy followed was to optimize each parameter, independent of the others and, subsequently, optimal conditions were employed in all experiments. In a sequential order, the various process parameters were optimized for maximal enzyme production as follows:

various substrates as shown in Table 1 [(Rice bran (RB), wheat bran (WB), maize bran (MB), red gram bran (RgB), black gram bran (BgG), white gram bran (WgB), sugarcane bagasse (SB), dry sugarcane leaves (DSL), rice husk (RH), sorghum waste (SW), wheat straw (WS), rice straw (RS), fruit wastes from pineapple (PW), apple pomace (AP), orange peel (OpW), oil cakes from sunflower (SoC) and safflower (SfoC)]; different combination of substrates as shown in fig.4 (RS+SB, SB+WS, RB+WS, RB+SB, WB+SB, WB+RS & WB+RB); initial moisture content (1:1.5, 1:2.0, 1:2.5 and 1:3.0 w/v); incubation period (24-144 hours); moistening agents such as Tap water (TP), Chemically defined medium (CD), distilled water (DW) and different mineral salt solutions (MS) [**MS-1:** K_2HPO_4-0.1, $MgSO_4$ 0.03, $ZnCl_2$-0.03 and $CaCl_2$-0.03; **MS-2**: KH_2PO_4-0.1, $MgSO_4$ 0.05, KCl-0.05, $FeSO_4$-trace, $ZnSO_4$-trace; **MS-3:** KH_2PO_4-0.2, NaCl-0.5. $MgSO_4$-0.03, $CaCl_2$-0.01; **MS-4:** NaCl-0.5, $MgSO_4$-0.02, $CaCl_2.2H_2O$-0.01, KH_2PO_4-0.10; **MS-5:** $NaNO_3$-0.25, KH_2PO_4-

0.10, $MgSO_4$-0.05, KCl-0.05; **MS-6:** NH_4NO_3-0.30, KH_2PO_4-0.10, $MgSO_4$-0.10, $FeSO_4$-0.001; **MS-7:** NaH_2PO_4-1.28, KH_2PO_4-0.3, NaCl 0.05, NH_4Cl-0.1, $MgSO_4$-0.05, $CaCl_2$-0.001; **MS-8:** KH_2PO_4-0.4, $MgSO_4$-0.05; **MS-9:** Calcium acetate (CH_3COONa)-0.01; **MS-10:** KH_2PO_4-0.1, $MgSO_4$-0.01, $CaCl_2$-0.01; **MS-11:** KH_2PO_4-0.3, $MgSO_4$-0.002, $FeSO_4$-0.005, $MnCl_2$-0.002, $CaCl_2$-0.002 and Na_2MoO_4-0.0001]; solid substrate medium pH (7-12), adjusted with separately sterilized Na_2CO_3 solution; incubation temperature (30-60^oC); inoculam level 5-50% (v/w); supplementary additives such as carbon sources (Arabinose, Citric acid, Dextrose, Sucrose, Maltodextrin, Fructose, Galactose, Glycerol, Lactose, Mannose, Raffinose, Starch and Gelatine), nitrogen sources, metal ions [(organic: casein, peptone, tryptone, yeast extract & meat extract) (inorganic:$NaNO_3$, NH_4NO_3, KNO_3 & NH_4Cl)] and effect of Glucose on enzyme production (1-11%). The cultivation process, enzyme harvesting & extraction followed by its assay (in triplicates) were conducted as described earlier.

RESULTS AND DISCUSSION

The strain thermoalkalophilic *Bacillus* sp. JB-99 was previously isolated and studied under submerged fermentation.[19] In this investigation, we have optimized the conditions for the production of thermostable alkaline protease from the same strain under solid state fermentation using inexpensive solid substrate, *wheat bran* as raw material. We have not analyzed the nutrient levels of wheat bran. Skimmed milk/casein agar plates showed clear zones of substrate hydrolysis around culture (Fig 1a & b). A profuse growth of *Bacillus* sp.JB99 on wheat bran in the flask (Fig 2) and plates containing biomass on wheat bran showed starch utilization, a blue colour was developed on control plate upon treated with Gram's Iodine and no color on test plate (Fig 3).

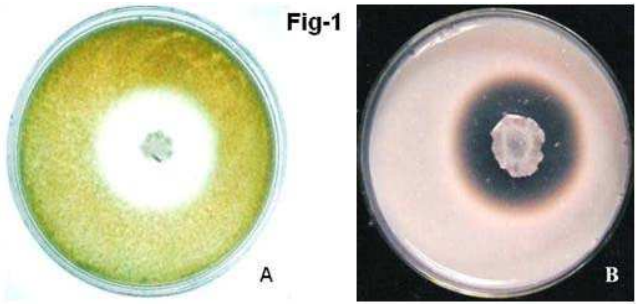

Figure 1
Protease hydrolysis
(A) Skim milk agar plate (B) Casein agar plate

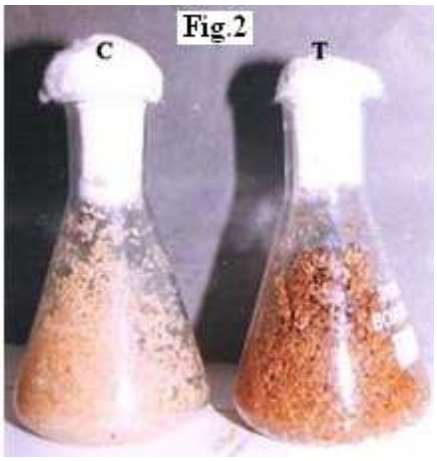

Figure 2
Biomass of Bacillus sp. JB-99 on wheat bran [C.Control; T.Test]

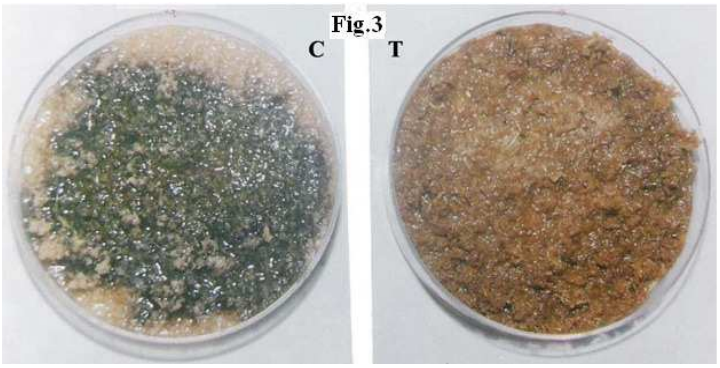

Figure 3
Starch utilization upon fermented wheat bran using Gram's iodine
C. Control-Negative starch utilization
T. Test-Positive starch utilization

Screening of various agro wastes for alkaline protease production

Proteases production from *Bacillus* sp. JB-99 was attempted using various agro industrial lignocellulosic substrates (Table 1). In the trials of different types of substrates on enzyme production, only wheat bran was emerged as an optimized solid substrate for the luxuriant growth of *Bacillus* sp.JB99, which produced a maximum of 3153 U/g of alkaline protease followed by wheat straw (2835 U/g), pineapple waste (2806 U/g) and rice bran (2736 U/g). Sugarcane bagasse, Rice straw, Rice husk, black gram and safflower oil cake yielded a moderate level of protease and maize bran and red gram with moderately less enzyme production whereas dried sugarcane leaves showed a negligible result (Table 1).

Table 1
Production of thermostable alkaline protease from thermoalkalophilic Bacillus JB-99 using various agro industrial wastes as substrates

Substrates	Enzyme activity (U/g)
Wheat bran (WB)	3153
Wheat straw (WS)	2835
Pineapple waste (PW)	2806
Rice bran (RB)	2736
Orange peel waste (OpW)	2700
Apple pomace (AP)	2600
Sugarcane bagasse (SB)	2569
Rice straw (RS)	2200
Rice husk (RH)	1800
Black gram bran (BgB)	1541
White gram bran (WgB)	1516
Safflower oil cake (SfoC)	1350
Sorghum waste (SW)	1200
Sunflower oil cake (SoC)	1180
Red gram bran (RgB)	750
Dry sugarcane leaves (DSL)	220

Wheat bran was found to be the most preferred choice for the production of proteases and the proteins associated with the starch in wheat bran serve as inducers, which stimulate enzyme activity[23], and as good source of carbon as starch.[39] The universal suitability of wheat bran may be due to the fact that it contains sufficient nutrients and is able to remain loose even in moist conditions, thus providing a large surface area[11]. In our investigation, we used an intermediate size of wheat bran, because solid substrate particle size is the most important one to influence the process.[15] Generally smaller substrate particles provide larger surface area for microbial growth, however, too small substrate particle may result in substrate agumulation, which may interfere with microbial respiration/aeration, and therefore result in poor growth. In contrast, larger particles provide better respiration/aeration efficiency (due to increased inter-particle space) but provide limited surface for microbial growth, this necessitates a compromised particle's size for a particular process.[33] Our results have shown similarity with various other reports shown; wheat bran was found to be the best in terms of maximum enhancement of protease yield when compared with other substrates[38], coffee byproducts[31], rice mill wastes[27], pigeon pea[17], soybean products[29] and green gram husk.[30]

Effect of combination of substrates for alkaline protease production

Among the high alkaline protease yielding substrates tested in two different combinations, almost all combinations were found to be equally efficient in enzyme yield. Wheat bran and Sugarcane bagasse (WB+SB) combination emerged with maximum activity (2700 U/g) followed by (WB+RB), (WB+RS), (RB+WS), (WB+RS), (RS+SB) and (RB+SB) as shown in Fig 4. In other reports, supplementation of substrates with wheat bran, such as soybean cake, casein and rice, enhanced the protease production[3]. The combination of wheat bran (WB) with chopped date stones (CDS) proved to be an efficient mixture for protease production[13]. Mustard oil cake, wheat bran, rice bran, *Imperata cylidrica* grass, banana leaves, potato peels and used tea leaves support for the production of alkaline protease by a thermophilic strain of *Bacillus subtilis* DM-04.[4]

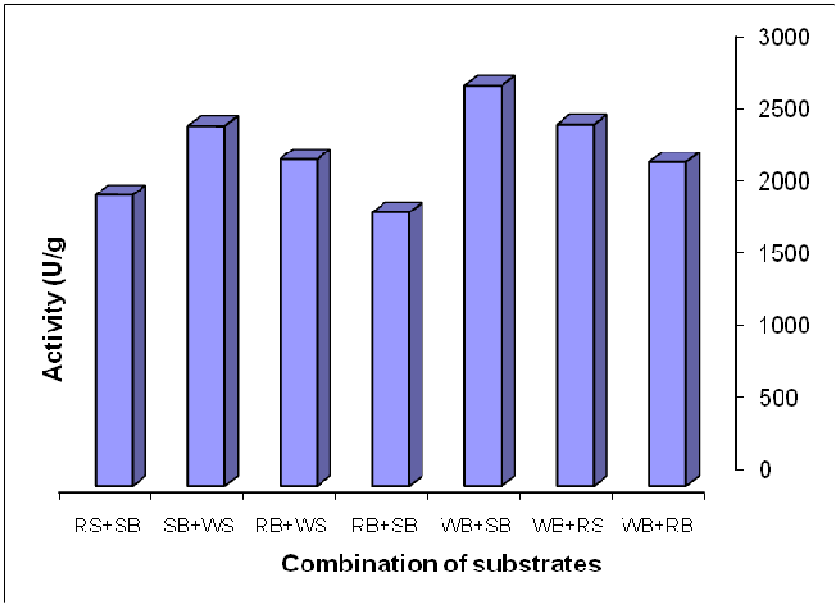

Figure 4
Effect of different combination of substrates for the production of thermostable alkaline protease from a thermoalkalophilic Bacillus sp. JB-99
Wheat bran –WB; Rice bran –RB; Rice straw –RS; Sugarcane bagasse –SB; Rice straw-RS; Wheat straw-WS

Effect of Moisture content on alkaline protease production

A marked improvement of alkaline protease production was achieved by the moisture content in the medium. Initially the enzyme production began from 1:1 (w/v) with 2000 U/g of activity and gradually showed increase (1:1.5 w/v yielded 3700 U/g) upto 1:2.0 (w/v) an optimized ratio which yielded a maximum of 4200 U/g, thereafter there was a sudden decrease (1:2.5 w/v yielded 3000 U/g). From Fig 5, it is clear that moisture ratio of 1:1.5 to 1:2.5 was found to be suitable level for growth of organism, as well as production of enzyme. The moisture content of the fermentation medium is one of the main factors in SSF and often determines the success of a process.[24, 21] It is reported that lower moisture content leads to poor growth due to lower mass transfer resulting in slower conversion of substrate to biomass.[37] The higher moisture content also retards growth due to substrate agglomeration, poor aeration and decreased available area for growth.[33] It is known that the water content of medium has a profound influence on the growth and production, greatly influenced by absorbing capacity and capillary forces of the substrate, the growth temperature, the amount of metabolic heat generated the quality of moisture evolved and the growth requirements of the organism. *Bacillus amyloliquifaciens* also produced higher protease at 1:2.0 moisture levels.[12] In case of alkaline protease production by *Aspergillus flavus,* a moisture content of 52-63% favored maximum production. Moisture content of 50-63% was sufficient for acid protease production by *Aspergillus ochraceous* studied under SSF conditions.[45] Moisture is a factor that is intimately related with the definitions of SSF and with the characteristics of the biological material. In a general sense it has been established that in the case of bacteria the moisture of the solid matrix must be higher than 70%. For yeasts, the moisture range can be a little wide as 60-70% and in the case of fungi the range could be as wide as 20-70%.

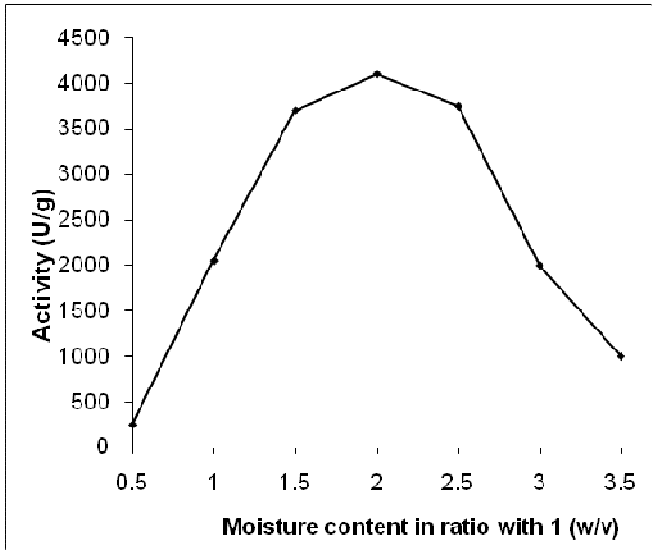

Figure 5
Effect of different moisture contents on production of thermostable Alkaline protease from a thermoalkalophilic Bacillus sp. JB-99

Effect of incubation period on alkaline protease production

A low level of alkaline protease appeared in early stages (2000 U/g at 48h and 3000 U/g at 72h) of incubation and enzyme levels steadily reached to a maximum (4250 U/g) by 96 hours. A prolonged incubation time beyond this period did not help to further increase in the yield (Fig 6). Our results have shown better than *Rhizopus oryzae* yielded alkaline protease upon 9 days of incubation under solid state fermentation.[1] *Aspergillus fumigatus* produced maximum amount of alkaline protease after 72h of incubation.[37] Fibrinolytic enzyme produced at 4 days of incubation[40], beyond this level, the bran turned semi-solid and hence reduces the growth and enzyme production.[12] The incubation time was governed by the characteristics of the culture, based on the growth rate and enzyme production pattern.[26]

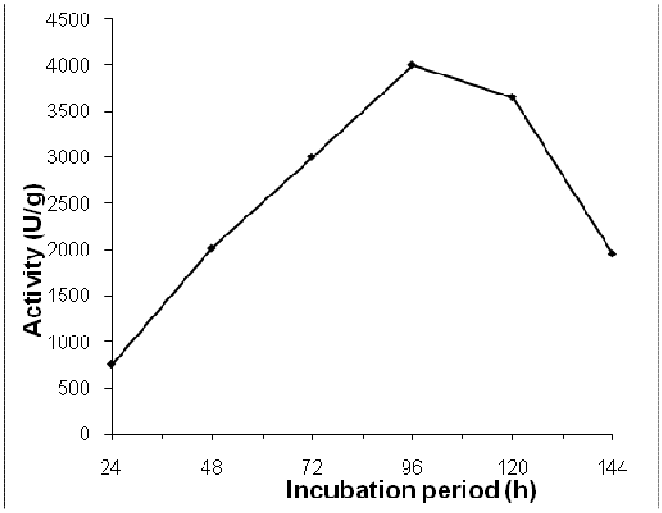

Figure 6
The time course of enzyme production from a thermoalkalophilic Bacillus sp. JB-99

Effect of Mineral Salt solutions on alkaline protease production

A variety of mineral salt solutions have been employed as moistening agents. MS-1 has produced maximum amount of alkaline protease (5200 U/g) followed by MS-9 (4900 U/g), tap water (TP) and chemically defined medium (CD) gave similar result (4700 U/g), but MS-6 and MS-11 has shown less productivity, whereas MS-7 has totally inhibited the synthesis, as shown in Fig 7. In other reports, chemically defined medium[19] and distilled water[7] yielded superior alkaline protease. Wheat bran with tap water as moistening agent shown amylase production.[5] While dealing with the media formulations, it is necessary to take into account the biomass composition; cellular biomass presents an average 40-50% carbon, 30-50% oxygen, 6-8% hydrogen and 3-12% nitrogen. The other elements such as phosphorous, sulphur and metals are important, as part of the cell and its metabolism but are present in such small quantities that need to be considered for their particular significance. The present study further reveals that the composition of inorganic salts has distinct effect on protease production in the presence of MS-1 salt solution followed by MS-9. Carbon and nitrogen sources are usually supplied as the complex mixtures of cheap natural products, but the trace elements are normally added.

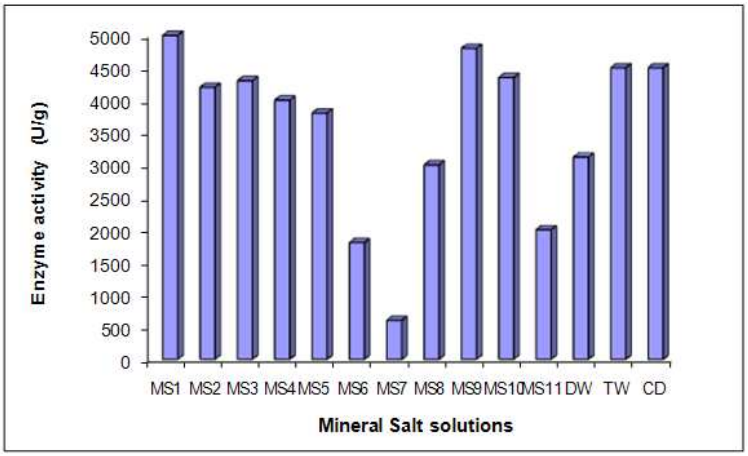

Figure 7
Effect of different Mineral Salt solutions on the production of thermostable Alkaline protease from a thermoalkalophilic Bacillus sp. JB-99

Effect of temperature on alkaline protease production

The maximum cell growth and highest protease yield (5400 U/g) of *Bacillus* sp. JB-99 was coincided at a temp of 50^{o}C, in a humidified incubator chamber (Fig 8). Any temperature beyond this range is found to have some adverse effect on the metabolic activities of the microorganisms. The enzyme level declined with prolonged incubation. This could be due to the loss of moisture after 50^{o}C. The reports on protease and α-amylase production by *Aspergillus oryzae* observed at 38^{o}C are on similar lines.[24] Biological processes are characterized by the fact that they are developed in relatively very narrow range of temperature.

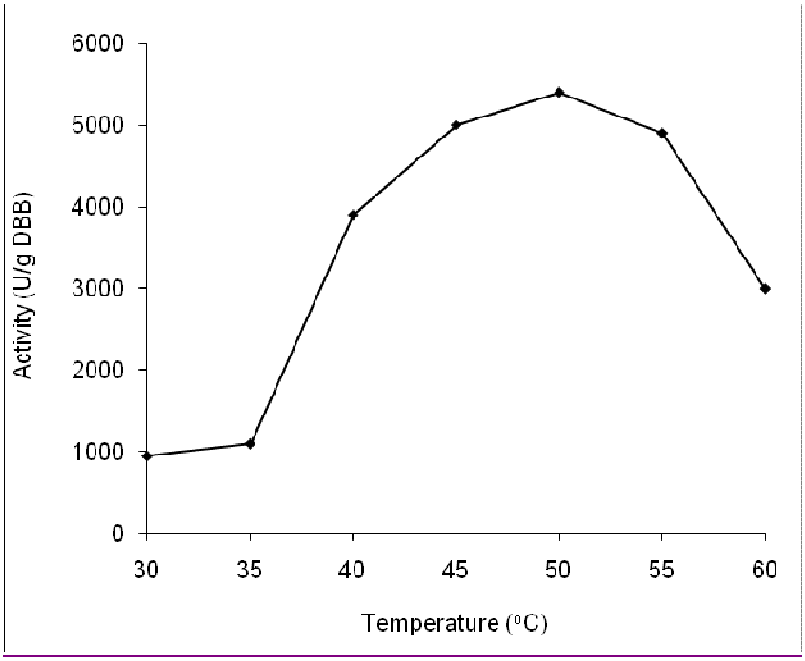

Figure 8
Effect of temperature on the production of thermostable alkaline protease from a thermoalkalophilic Bacillus sp. JB-99

Effect of pH on alkaline protease production

The present strain is able to grow over a wide range of pH 7-12. The enzyme secretion begins with pH 7.0 (without Na_2CO_3) yielded 3000 U/g. The improvement in enzyme titers was observed from pH 8.0 (adjusted with Na_2CO_3) in mineral salt solution. The maximum enzyme titers could be seen at pH 10.5 (5600 U/g); thereafter the activity gradually decreases (Fig 9). As the metabolic activities of the microorganisms are very much sensitive to the pH change, protease production by *Bacillus* sp. JB-99 is found to be affected if pH level is higher or lower compared to the optimum value. As the organism is a thermoalkalophilic, it shows luxuriant growth and enzyme yield at higher alkaline pH conditions (10-11).

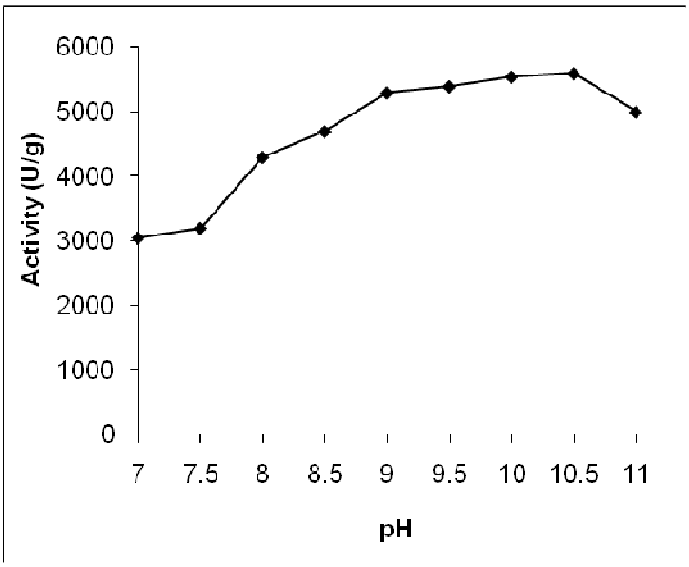

Figure 9
Effect of different pH range on the production of thermostable alkaline protease from a thermoalkalophilic Bacillus sp. JB-99

Effect of inoculam size on alkaline protease production

Importance of inoculam size on microbial fermentation process is widely accepted. Out of eight inoculam sizes tested (5-40 % v/w), a 15% inoculam (based on the initial weight of wheat bran) was found to be the most suitable to attain a high production of alkaline protease (6000 U/g) by *Bacillus* sp. JB-99 in SSF. From Fig 10, it is clear that the protease production steadily increased with the increase in size of inoculam until it reached the magnitude when enzyme productivity became maximum. Thereafter no appreciable change in enzyme activity with high inoculam size could be observed, this indicates that the inoculam density does not have unlimited effect on fermentation processes. It has some optimum value depending on the microbial species and fermentation system.[41] Our result correlates with xylanase production[2], α-amylase produced by *Bacillus coagulans* B-149 was maximum at 10% (v/w) inoculam level.[5] Protein synthesis in *Aspergillus niger* was best at $4x10^6$-$4x10^7$ spores g^{-1} wheat bran, but declined at $4x10^8$ spores counts.[33] Fungal species *Fusarium oxysporium* produced maximum fibrinolytic enzymes at an inoculam concentration of 5%, and beyond this range enzyme activity decreases.[40]

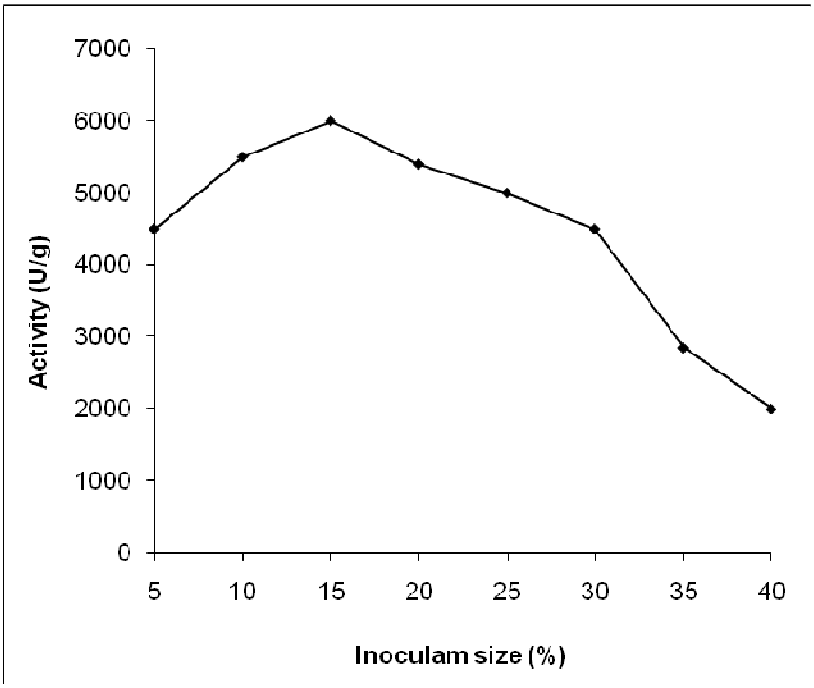

Figure 10
Effect of different concentrations of inoculam sizes for the Production of thermostable alkaline protease from a Thermoalkalophilic Bacillus sp. JB-99

Effect of Additives on alkaline protease production

The additives Arabinose 5% (w/w) yielded a maximum amount of protease (6222 U/g). Citric acid (6111 U/g) and $NaNO_3$ (7000 U/g) also enhanced the alkaline protease production. Raffinose, Starch, Fructose, Maltodextrin, Gelatin, Sucrose and Mannose have shown better yield enzyme, but Glycerol, Galactose and Lactose have shown moderate enzyme production, whereas Dextrose has strongly inhibited the enzyme yield, which shows 440 U/g (Table 2). 1% Arabinose was most effective substrate concentration for protease production.[47] Among the organic nitrogen sources casein produced maximum enzyme (6470 U/g), followed by yeast extract (5000 U/g), tryptone (4805 U/g) and peptone (4250 U/g), but Meat extract had minute influence on enzyme yield (2880 U/g). Among inorganic nitrogen sources $NaNO_3$ yielded maximum enzyme (7000 U/g) followed by KNO_3 (5200 U/g) and with less enzyme by NH_4NO_3, whereas NH_4Cl has completely repressed the enzyme activity (Table 3). *Bacillus stearothermophilus* F1 has also produced alkaline protease using $NaNO_3$ as nitrogen source.[32] Tween-80, dioctyl sodium sulfosuccinate, biotin, Ca^{2+} and 1-Naphthyl acetic acid are used as additives for the production of protease under solid state fermentation which is also reported.[42] The type, source and nature of carbon and nitrogen are among the most important factors for any fermentation process. Carbon source represents the energetic source as simple as a pure monosaccharide compound such as glucose or as complex as polymeric molecules such as cellulose or starch. Nitrogen is the next most crucial factor that determines the growth of microorganism and needs to be calculated/ determined to obtain maximum biomass. The other two components, oxygen and hydrogen are obtained through the organic carbon source.[25]

Table 2
Effect of additives on production of thermostable alkaline Protease from thermoalkalophilic Bacillus sp. JB-99

Carbon Sources	Enzyme Activity (U/g)
Arabinose	6222.0
Citric acid	6111.0
Raffinose	5780.0
Starch	5570.0
Fructose	5420.0
Maltodextrin	4888.0
Gelatin	4610.0
Sucrose	4200.0
Mannose	4088.0
Glycerol	3445.0
Galactose	3222.0
Lactose	2166.0
Dextrose	0440.0

Table 3
Effect of various organic and inorganic nitrogen sources on production of Alkaline protease by thermo alkalophilic Bacillus sp. JB-99

Organic Nitrogen sources	Enzyme activity (U/g)	Inorganic Nitrogen sources	Enzyme activity (U/g)
Casein	6470	$NaNO_3$	7000
Yeast extract	5000	KNO_3	5200
Tryptone	4805	NH_4NO_3	0666
Peptone	4250	NH_4Cl	000
Meat extract	2880		

Effect of Glucose on repression of alkaline protease production

In this study under Solid State Fermentation, the effect of Glucose on repression of alkaline protease depicted tolerance up to 10%. Catabolic repression by glucose was virtually absent in this system, in which only 25% of reduction in protease titer was recorded even at concentrations as high as 10% (Fig 11). It is emphasized that wheat bran itself contains about 8.5% starch on a dry weight basis, which becomes saccharified to glucose due to the action of enzyme elaborated by the culture during the course of fermentation. Thus, the effective concentration of glucose in the SSF medium will be substantially higher than the value of the supplemented concentration. Our results also support the catabolic repression of α-amylase biosynthesis in SSF reported in *Bacillus licheniformis.*[35] The protease of the strain *Aspergillus oryzae* NRRL 2160 was catabolically repressed.[6] The catabolic repression of exopectinase activity was observed in submerged fermentation but improved in SSF, but the protease levels are minimal in SSF with the addition of sucrose.[10]

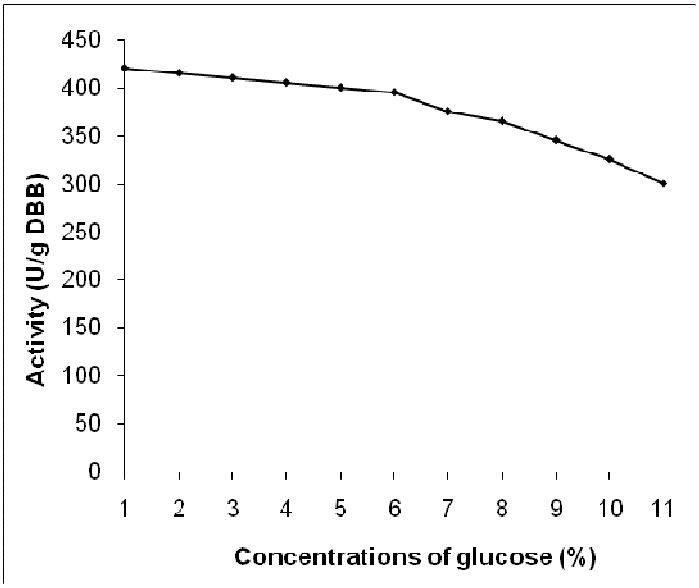

Figure 11
Effect of different concentrations of glucose on the production of thermostable alkaline protease from a Thermoalkalophilic Bacillus sp. JB-99

CONCLUSION

The present study is a new investigation on optimization of fermentation conditions and factors responsible for higher production of thermostable alkaline protease under solid state fermentation (SSF) using novel strain *Bacillus* sp. JB-99 which was found highly potent during submerged fermentation (Smf) while using chemically defined medium. Further utilization of wheat bran for the production of enzyme in SSF would reduce the cost considerably. The results will be useful for industrial production of this important enzyme with low input cost.

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