Research Paper

Concomitant production of detergent compatible enzymes by *Bacillus flexus* XJU-1

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Abstract

A soil screened *Bacillus flexus* XJU-1 was induced to simultaneously produce alkaline amylase, alkaline lipase and alkaline protease at their optimum levels on a common medium under submerged fermentation. The basal cultivation medium consisted of 0.5% casein, 0.5% starch and 0.5% cottonseedoil as an inducer forprotease, amylase, and lipase, respectively. The casein also served as nitrogen source for all 3 enzymes. The starch was also found to act as carbon source additive for both lipase and protease. Maximum enzyme production occurred on fermentation medium with 1.5% casein, 1.5% soluble starch, 2% cottonseed oil, 2% inoculum size, initial pH of 11.0, incubation temperature of 37 °C and 1% soybean meal as a nitrogen source supplement. The analysis of time course study showed that 24 h was optimum incubation time for amylase whereas 48 h was the best time for both lipase and protease. After optimization, a 3.36-, 18.64-, and 27.33-fold increase in protease, amylase and lipase, respectively was recorded. The lipase was produced in higher amounts (37.72 U/mL) than amylase and protease about 1.27 and 5.85 times, respectively. As the 3 enzymes are used in detergent formulations, the bacterium can be commercially exploited to secrete the alkaline enzymes for use in detergent industry. This is the first report for concomitant production of 3 alkaline enzymes by a bacterium.

Key words: *Bacillus flexus*, alkaline enzyme, concomitant production, optimization.

Introduction

The amylase (E.C 3.2.1.1), lipase (EC 3.1.1.3) and protease (EC 3.4.21-24 and 99) are hydrolase enzymes that cleave the glycosidic, ester, and peptide bonds, respectively via the addition of water. The hydrolases account more than 75% of all industrial enzymes. Lipases, amylases and proteases represent for about 3, 18 and 60%, respectively of total worldwide sale of enzymes (Rao *et al.*, 1998; Sharma *et al.*, 2001). The enzymes are mainly added to the detergents to improve the cleaning ability of detergents. When used in detergents, the proteases acted as protein stain removals, amylases remove starch-based food stains whereas lipases remove fatty stains (Gormsen *et al.*, 1991). Crude alkaline lipase and alkaline protease from *Bacillus licheniformis* and *Geobacillus* were able to remove fat and protein stains on clothes in the presence and absence of commercial powder detergent (Amara *et al.*, 2009). A mixture of lipase and protease coproduced from *Serratia marcescens* was also able to remove the blood and grease stains when added to the detergent (Mukesh kumar *et al.*, 2012a).

Amylases, lipases and proteases can be produced from plants, animals and microorganisms. However, they are produced from microorganisms because of reduction of time, labor and cost (Mukesh kumar *et al.*, 2012b; Mukhtar and Haq, 2012). Bacteria especially *Bacillus* species are well known to secrete extracellular enzymes such as amylases, proteases and lipases of potential commercial values (Mukesh kumar *et al.*, 2012b; Mukhtar and Haq, 2012; Sangeetha *et al.*, 2008; Shivakumar, 2012). This is possible because the generation time is shorter, the genetic material can be manipulated easily and the cultural conditions can be optimized easily (Gupta *et al.*, 2004).

The production of enzymes by microorganisms as well as the enzyme yield depends on the nutritional factors especially carbon and nitrogen sources and physicochemical parameters such as initial pH, incubation temperature and incubation time (Mukesh kumar *et al.*, 2012a, 2012b).

All these factors must be optimized for maximal enzyme production. The optimization avoids the use of enzyme substrates in excess, and this reduces the cost of the fermentation process (Hasan and Hameed, 2001; Sangeetha *et al.*, 2008; Anwar *et al.*, 2011). In multienzyme production system, the additives to the basal medium must be chosen with great care since they must allow the production of all enzymes at their optimum levels in a single cultivation. Protease and alpha amylase enzymes are produced under submerged and solid-state fermentations by bacteria, however appreciable amounts of enzymes were recorded with submerged fermentation (Mukhtar and Haq, 2012).

The production of enzymes with better properties is a continuous exercise. Since the production of detergent compatible enzymes using different fermentation processes is quite expensive (Sharma *et al.*, 2001), the concomitant production of industrial enzymes in a single fermentation medium is a new challenge. The production of alkaline amylase, alkaline lipase and alkaline protease by *Bacillus* species using separate fermentation media have been extensively reported. Few reports are also available for *Bacillus* species concomitantly producing amylase and lipase (Anwar *et al.*, 2011), amylase and protease (Mukesh kumar *et al.*, 2012b; Mukhtar and Haq, 2012; Shivakumar, 2012) and lipase and protease (Sangeetha *et al.*, 2008, 2010; Valasange and Bagewadi, 2012). But there are no reports for the concomitant production of all 3 enzymes of *Bacillus* species in a single fermentation medium. Bacillus flexus XJU-1 was recently shown to be an alkaline lipase producer. In the present study, the bacterium was induced to concomitantly produce 3 alkaline enzymes in a single production medium and the nutritional and physicochemical parameters were optimized for maximal enzyme production under submerged fermentation.

Materials and Methods

Chemicals and reagents

p-nitrophenylpalmitate (pNPP, CAS No. 1492-30-4) was procured from Sigma-Aldrich, Co, St Louis, MO (USA). Rhodamine B (CAS No. 81-88-9) and gum acacia (CAS No.9000-01-5) were purchased from HiMedia Laboratories (Mumbai, India). The other chemicals and reagents were brought from Merck Specialities Private Limited (Mumbai, India), Sd fine-Chem limited (Mumbai, India) and Qualigens fine chemicals (Mumbai, India).

Bacterial strain

The bacterial strain used in the present study was recently screened from the Bangalore potato field using serial dilution and spread plate techniques and identified as *Bacillus flexus* XJU-1 based on morphological, cultural, biochemical characteristics and 16S rDNA sequencing (Niyonzima and More, 2013b).

Detection of amylolytic, lipolytic and proteolytic activities on agar plates

The medium proposed by Kumar *et al.* (2012) was used for detecting enzymes with minor modification. It consisted of 0.5% (w/v) casein, 2% (v/v) olive oil, 0.2% (w/v) NH$_4$NO$_3$, 0.2% (w/v) MgSO$_4$, 0.006% (w/v) CuSO$_4$, 0.001% (w/v) Rhodamine B and 1.5% (w/v) agar. It was adjusted to pH 10.0 with 2 M sodium carbonate. The plates were inoculated with a *B. flexus* XJU-1 culture and incubated at 37 °C for 48 h. The protease production was indicated by a clear zone of casein hydrolysis around the bacterial colony while the lipase was shown by the formation of orange halos around the organism upon irradiation with UV transilluminator (Model MD-25, Wealtec, India) at 350 nm. The casein was replaced by 0.5% (w/v) soluble starch in the above medium and the bacterium was grown in the same conditions. The formation of clear zone of starch hydrolysis after addition of iodine solution was an indication of the presence of amylase.

Inoculum preparation, submerged fermentation and crude enzyme preparation

Nutrient agar slants were streaked with *B. flexus* XJU-1 culture and incubated for 48 h. One loop of the culture from the agar slant was taken and used to inoculate 25 mL of nutrient broth contained in 50-mL conical flask. The incubation was carried out in an orbital incubator (Model S150, Stuart, India) at 100 rpm for 24 h at 37 °C. The resulted inoculum was adjusted to 10^7 colony forming units/mL with nutrient broth prepared and sterilized in the same conditions. The standardized inoculum was used to carry out submerged fermentation.

Submerged fermentation was carried out in 250-mL Erlenmeyer flask containing 100 mL of the medium consisted of 0.5% (w/v) casein, 0.5% (w/v) starch, 0.5% (v/v) cottonseed oil, 0.2% (w/v) NaCl and 0.01% (w/v) CaCl$_2$, pH 10.0. The medium was inoculated with 1% inoculum and the incubation was carried out in an orbital incubator (Model SI50, Stuart, India) at agitation speed of 100 rpm for 48 h at 37 °C. After the incubation period, the bacterial cells were removed by centrifugation (Model C-30 BL, Cooling centrifuge, Remi, India) at 10,000 rpm for 10 min at 4 °C. The clear supernatant was used as a crude enzyme source.

Enzyme assays

Determination of alkaline amylase activity

Amylase activity of the culture supernatant was estimated as per the modified method of Bernfeld (1955) using soluble starch as substrate. 0.5 mL of 0.2 M Tris-HCl buffer (pH 8.5), 1% (w/v) mL of soluble starch and 0.5 mL of crude enzyme were mixed together, and incubated for 10 min at 37 °C. After incubation period, 2 mL of DNS reagent was added and shaken well to arrest the reaction. The

contents of test tubes were then incubated in a water bath for 15 min. After incubation, the test tubes were allowed to cool down and 7 mL of distilled water was added into each test tube. The absorbance was recorded at 540 nm with a UV spectrophotometer (Model SL-159, Elico, India). The blank samples were without crude enzymes. One unit of alkaline amylase was defined as the amount of enzyme required to release 1 μmol of glucose as reducing sugar per min per ml under the assay conditions.

Determination of alkaline lipase activity

The alkaline lipase activity of the crude enzymes was determined spectrophotometerically by the method reported by Sumathi and Meerabai (2012) with slight modification using pNPP in the emulsion. 1 mg of pNPP was dissolved in 1 mL of 2-propanol (solution A) with a cyclo-mixer (Model CM 101, Remi, India) for 5 min at room temperature. 0.01 g of gum acacia and 0.04 mL of Triton X-100 were dissolved in 9 mL of 50 mM Tris HCl buffer (pH 8.5) (solution B) with a magnetic stirrer (Model 2MLH, Remi, India). The solution A was added one drop at a time to the solution B with vigorous stirring using a magnetic stirrer. The resulted emulsion served as a substrate solution. 0.9 mL of the substrate solution was mixed with 0.1 mL of crude enzyme, shaken well and incubated at 37 °C for 20 min. The reaction was arrested by incubating in boiling water at 95 °C for 5 min. 3 mL of 50 mM Tris HCl buffer (pH 8.5) was added and mixed well. The enzyme solution was omitted in the control tube and was replaced with the same amount of buffer. The produced p-nitrophenol was measured using a UV-VIS spectrophotometer (Model SL-159, Elico, India) at 410 nm. The unknown concentration of p-nitrophenol produced was deduced from a p-nitrophenol standard curve constructed in the 1 mg to 5 mg range. One unit (U) of lipolytic activity was defined as the amount of enzyme required to produce 1 μmol of p-nitrophenol from pNPP per min per ml under the assay conditions.

Determination of alkaline protease activity

The alkaline protease activity was assayed as per Niyonzima and More (2013a) method. The amount of amino acids released was deduced from a standard curve constructed from known concentrations of tyrosine. One unit of alkaline protease was defined as the amount of enzyme required to release 1 μmol of tyrosine per min per ml under the assay conditions.

Optimization of nutritional and physicochemical factors

The carbon and nitrogen sources as nutritional factors and inoculum level, initial pH and incubation temperature as physicochemical factors were optimized in this study. The time course of maximal enzyme production was also studied. The strategy used was to optimize one factor each time keeping all other factors unchanged and use the opti-

mized condition in the subsequent experiments. The influence of casein and starch concentrations in the range 0.5% to 3% (w/v) on the enzyme secretion by the bacterium was studied. The effect of cottonseed oil concentration was also analyzed in the range 0.5 to 3.5% (v/v). The cultivation media were inoculated with different inoculum levels (from 1 to 5%, v/v). The pH of the cultivation medium was adjusted from 7.0 to 13.0 to investigate the effect of initial pH on enzyme production. Four temperatures viz. 30, 37, 45 and 55 °C were used to study the effect of incubation temperature on the production of alkaline enzymes. The effect of nitrogen source (0.5%, w/v) as additive to the cultivation medium was studied. The effect of best nitrogen source concentration (soybean meal) was also analyzed in the 0.5 to 3% (w/v) range. The submerged fermentation, crude enzyme preparation and enzyme activity determination were carried out as described earlier.

The time course of alkaline enzyme production

After optimization of various nutritional and physicochemical parameters, time course was analyzed in order to know the optimum time for enzyme production. The medium of pH 11.0 composed of 1.5% casein, 1.5% starch, 2% cottonseed oil, 0.2% (w/v) NaCl, 0.01 (w/v) $CaCl_2$ and 1% soybean meal was inoculated with 2% inoculum. The culture flasks were incubated in an orbital shaker incubator with 100 rpm as agitation speed at 37 °C. The activity was recorded at regular intervals after exactly 12 h for a period of 72 h.

Statistical analysis

All the experiments were carried out independently with each treatment replicated three times. The significant differences among the means for each parameter were given by one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) at the 5% significance level with the help of SPSS software.

Results and Discussion

A bacterium used in this work was screened from the potato field soil of Bangalore (India). It was identified as *B. flexus* XJU-1 based on cultural, microscopic, biochemical and molecular techniques (Niyonzima and More, 2013b). The strain was able to secrete alkaline lipase with cottonseed oil as the best lipidic carbon source (data not shown). In the present study, the bacterium was also induced to secrete alkaline protease and alkaline amylase by cultivating it on a basal medium consisting of casein, starch and cottonseed oil. The casein and starch acted as a carbon source for protease and amylase production, respectively. In addition, the casein served as nitrogen source for the production of all enzymes.

Production of alkaline enzymes on agar plates

When the casein olive oil Rhodamine agar plates were streaked with *B. flexus* XJU-1 culture, a clear zone of casein hydrolysis around the bacterial colony was seen and indicates the proteolytic activity. An orange halo around the organism was also observed upon irradiation with UV transilluminator at 350 nm and indicates lipolytic activity (Figure 1A). When starch olive oil Rhodamine agar plates were streaked with *B. flexus* XJU-1 colonies, an amylolytic activity was shown by a clear zone of starch hydrolysis around the organism. The zone was clearly seen after iodine solution addition (Figure 1B). The bacterium is thus producing 3 alkaline enzymes. Similarly, amylolytic and proteolytic activities were also seen when *Bacillus* species were streaked on starch casein agar plates (Mukesh kumar *et al.*, 2012b; Shivakumar, 2012). The formation of orange fluorescent halo zones around the *Bacillus* species was a condition used by Niyonzima and More (2013) to screen for true lipase producers. The fluorescent zones are seen due to the formation of fluorescent compounds resulted from the complexation reaction between Rhodamine B dimers and fatty acids, monoglycerides and diglycerides released from oil in the growth medium (Hou and Johnston, 1992).

Optimization of nutritional and physicochemical factors

Effect of varying casein concentration on the production of alkaline enzymes

In the living systems including bacteria, carbon sources have two important roles. They provide raw material for the structure and act as an energy source (Hasan and Hameed, 2001). When different amounts of casein were added to the basal medium, the maximum activity of all alkaline enzymes was observed at 1.5% although statistically at par with 2% (Figure 2). The 1.5% casein concentration was used in the optimization of next parameter as it is more economical. A gradual decrease in enzyme activities was seen with higher casein levels (Figure 2). Casein served as a specific inducer at appropriate concentration, whereas at higher concentration, it acted as substrate repressor. In yeast cells, the decrease in enzyme production at higher casein concentrations was ascribed to substrate repression (Ogrydziak, 1993). In addition to serve as a carbon source for protease production, casein acted as a nitrogen source for the production of all the 3 enzymes. Supportively, in the concomitant production of lipase and protease by *Bacillus pumilus* SG2, casein was a better nitrogen source than others tested, namely gelatin, yeast extract and beef extract (Sangeetha *et al.*, 2008).

Effect of varying starch concentration on the production of alkaline enzymes

The influence of different concentrations of soluble starch was analyzed. An 8.25-fold increase in amylolytic
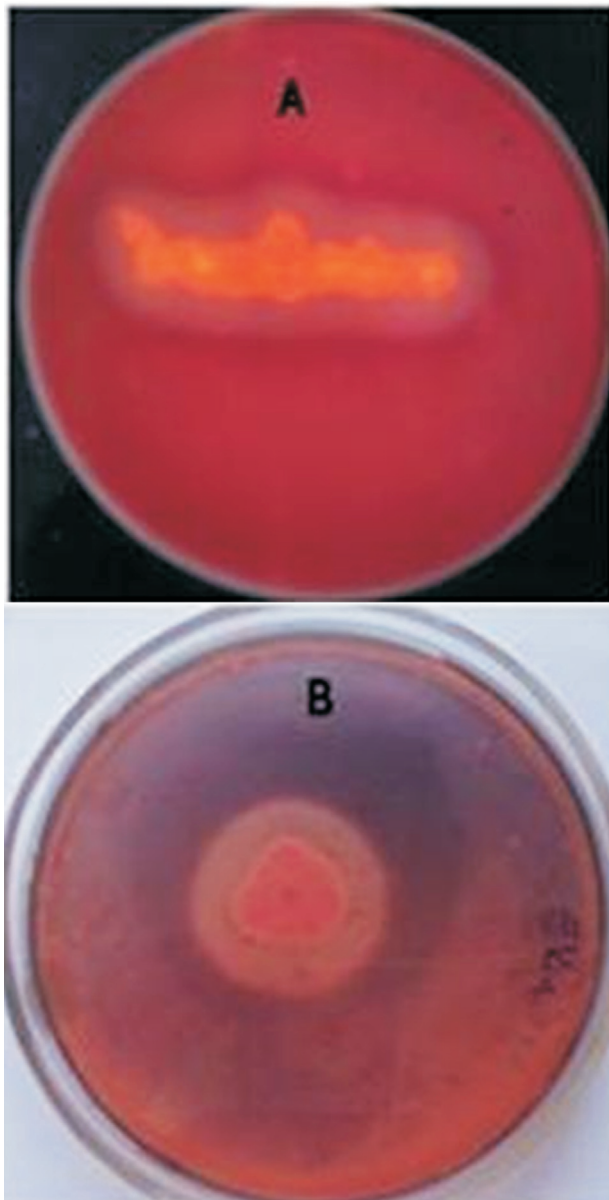

Figure 1 - *Bacillus flexus* grown on casein cottonseed oil Rhodamine B agar plate (A) and on starch cottonseed oil Rhodamine B agar plate (B).

activity was obtained with 1.5% soluble starch. The protease and lipase activities were also optimum at this concentration with 1.57- and 10.96-fold increase, respectively (Figure 3). This is evident for alkaline amylase because soluble starch acted as its inducer. However, for alkaline protease and lipase, the starch may be acting as a carbon source additive for enzyme production. Similarly, the soluble starch was among the chief ingredients in the fermentation medium for protease (and amylase) production by *Bacillus subtilis* IIB-26 (Mukhtar and Haq, 2012). Likewise, soluble starch was the best carbon source supplement for lipase production by *Bacillus subtilis* JPBW-9 (Anwar *et al.*, 2011). Furthermore, 1% casein and 1%
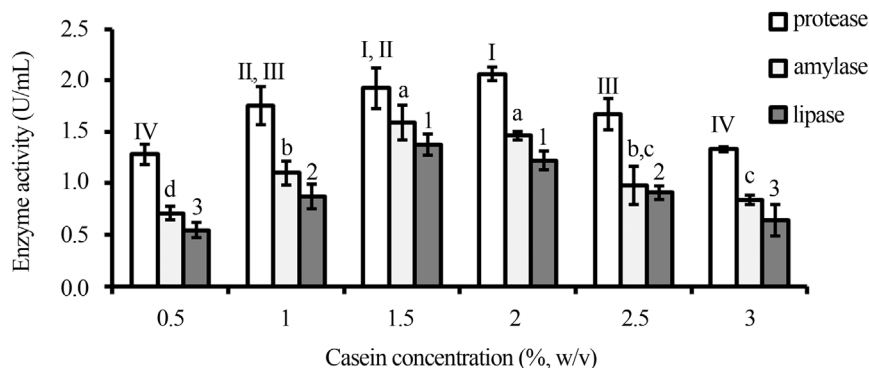
Figure 2 - Effect of different casein concentrations on the production of alkaline enzymes by *Bacillus flexus* XJU-1. 0.5% casein was control. The significant difference at $P_{0.05}$ is indicated by different letters or numerals or Roman numerals on the error bars.
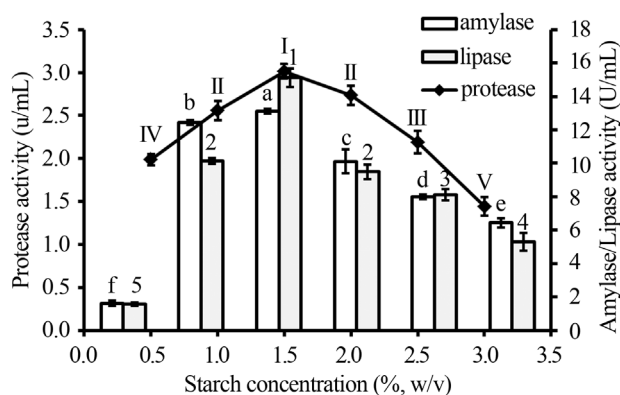
Figure 3 - Effect of different starch concentrations on the production of alkaline enzymes by *Bacillus flexus* XJU-1. 0.5% starch was control. The significant difference at $P_{0.05}$ is indicated by different letters or numerals or Roman numerals on the error bars.
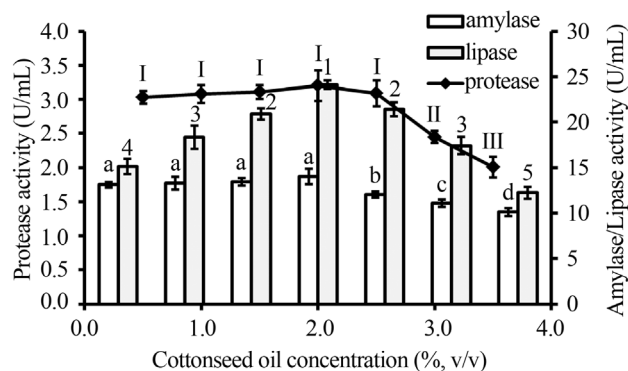
Figure 4 - Effect of different concentrations of cottonseed oil in the production of alkaline enzymes by *Bacillus flexus* XJU-1. 0.5% cottonseed oil was a control. The significant difference at $P_{0.05}$ is indicated by different letters or numerals or Roman numerals on the error bars.

starch in a single cultivation medium enhanced protease (3.73 fold) and amylase (2.07 fold) production in *Aspergillus awamori* (Negi and Banerjee, 2010). In *Serratia rubidaea*, starch was an appropriate substrate for maximal lipase production (Immanuel *et al.*, 2008).

Effect of varying cottonseed oil concentration on the production of enzymes

Lipidic carbon sources especially natural oils were experimentally reported by many researchers to be essential for obtaining a high lipase yield as acted as inducers (Bora and Bora, 2012). The ability of the isolate to grow in different concentrations of cottonseed oil was tested. The amylase and protease showed important activities in 0.5 to 2% cottonseed oil. However, the maximum activities for all 3 enzymes were obtained at 2% cottonseed oil concentration, after which a decrease in activities was observed (Figure 4). This decrease in enzyme activity may be ascribed to the accumulation of higher oil concentrations preventing proper bacterial culture aeration, affecting bacterial growth and thus enzyme production. Similarly, a delay in mycelial growth was seen due to poor aeration resulting in little enzyme yield (Maia *et al.*, 1999).

Effect of inoculum size on the production of alkaline enzymes

The inoculum size is one of the important factors for bacterial growth and enzyme production, especially under submerged fermentation. Different inoculum concentrations in the 1 to 5% range were investigated for the production of alkaline enzymes by *B. flexus* XJU-1. Maximum yield for all 3 enzymes were observed at 2% inoculum size after which the activities gradually decreased (data not shown). 1% inoculum also gave a significant amylase yield. The gradual decline in enzyme activities observed with higher inoculum levels may be probably attributed to the insufficiency of some nutrients owing to fast bacterial growth. A higher inoculum size of 10% was the best for lipase and α-amylase production in *B. subtilis* JPBW-9 (Anwar *et al.*, 2011). Therefore, the effect of inoculum size on enzyme production may depend on the bacterial efficiency, stability, type and size.
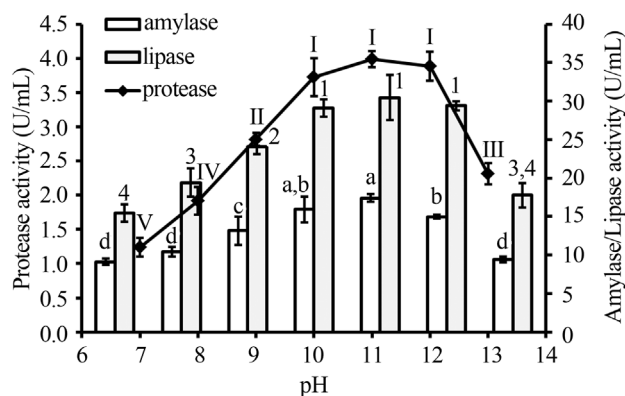
Effect of initial pH on the production of alkaline enzymes

The initial pH plays a crucial role in bacterial growth and thus in enzyme production. If the bacteria are screened and isolated at alkaline pH, the enzymes produced may

have higher activities in the alkaline region (Hasan and Hameed, 2001). After investigating the effect of the initial pH of the fermentation media, the enzyme productions appeared to occur at a range of optimum pH viz. 10.0, 11.0 and 12.0. The maximum yield for all the enzymes however was at pH 11.0, beyond which the activities declined (Figure 5). This clearly shows the alkalophilic nature of this strain. Similarly, the optimum pH was the same for amylase and protease (Shivakumar, 2012) or lipase and protease (Amara *et al.*, 2009; Sangeetha *et al.*, 2010; Valasange and Bagewadi, 2012) secreted by *Bacillus* species. The similarity in pH optima of enzymes produced by *Bacillus* species has been ascribed to genetic similarity in enzyme sequences (Anwar *et al.*, 2011). The concomitant production of amylase and lipase, amylase and protease or lipase and protease at optimum level of different *Bacillus* species in the alkaline region from 7.5 to 10.0 has been reported (Sangeetha *et al.*, 2008, 2010; Anwar *et al.*, 2011; Mukesh kumar *et al.*, 2012b; Mukhtar and Haq, 2012; Shivakumar, 2012; Valasange and Bagewadi, 2012). A decrease in enzyme activities was noted after optimum pH. This decline has been ascribed to the alteration of transport mechanisms across the microbial membrane that may prevent release of enzymes (Hasan and Hameed, 2001).

Effect of incubation temperature on the production of alkaline enzymes

The physical factors like incubation temperature, initial pH and aeration play a vital role in enzyme production as they modulate bacterial growth (Gupta *et al.*, 2004). Enzyme production was observed in all the temperatures tested with optimum for all enzymes at 37 °C although statistically at par with 30 and 45 °C (data not shown). This shows the mesophilic nature of the *Bacillus flexus* XJU-1. The incubation temperature of 37 °C was also good for the coproduction of amylase and protease (Mukhtar and Haq, 2012; Shivakumar, 2012), and lipase and protease (Sangeetha *et al.*, 2008; Valasange and Bagewadi, 2012) by

Bacillus species. The production of all enzymes decreased significantly at 55 °C. The low value recorded at 55 °C may be due to the partial denaturation of enzymes at higher temperatures.

Effect of different nitrogen sources for the production of alkaline enzymes

For any organism including bacteria, a nitrogen source served as a secondary energy source for growth and enzyme secretion (Kumar *et al.*, 2012). The effect of nitrogen sources as additives on the fermentation medium was studied. Among the different nitrogen sources used, a significant enzyme yield was observed with soybean meal and peptone for alkaline protease. However, for alkaline amylase and alkaline lipase, soybean meal, peptone and yeast extract were best nitrogen sources (Figure 6). Since the soybean meal is a cheap nitrogen source; it was chosen as the appropriate nitrogen source. The casein present in the cultivation medium served as a carbon source for alkaline protease and organic nitrogen source for all alkaline enzymes. Supportively, a combination of both soybean meal and casein was the appropriate nitrogen source for lipase production in *S. rubidaea* (Immanuel *et al.*, 2008). Addition of inorganic nitrogen sources did not have a pronounced effect on all alkaline enzymes when compared to organic sources (Figure 6). Likewise, low enzyme yields were noted when inorganic nitrogen sources were used as nitrogen sources in the production of lipase and protease by *B. pumilus* SG2 (Sangeetha *et al.*, 2008). Therefore, unlike inorganic nitrogen sources, most of the organic nitrogen sources, in addition to acting as a nitrogen source, supply vitamins, minerals and accessory growth factors in the cultivation medium that enhance bacterial growth and enzyme production (Gupta *et al.*, 2004).
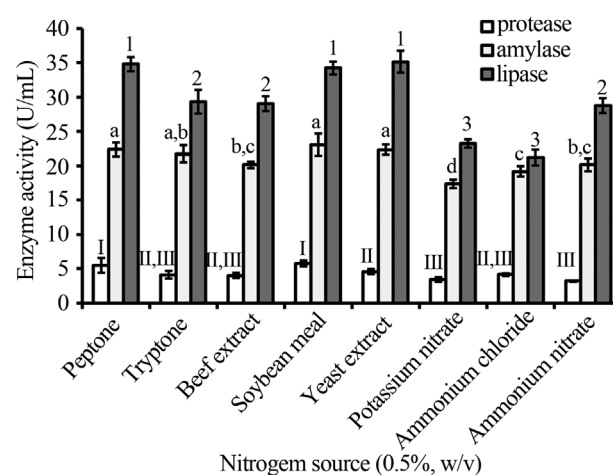

Figure 5 - Effect of different initial pHs on the production of alkaline enzymes by *Bacillus flexus* XJU-1 under submerged fermentation. The pH 10.0 was a control. The significant difference at $P_{0.05}$ is indicated by different letters or numerals or Roman numerals on the error bars.

Figure 6 - Effect of nitrogen sources as supplements on enzymes production by *B. flexus* XJU-1. A medium without any nitrogen source addition was taken as the control. The significant difference at $P_{0.05}$ is indicated by different letters or numerals or Roman numerals on the error bars.
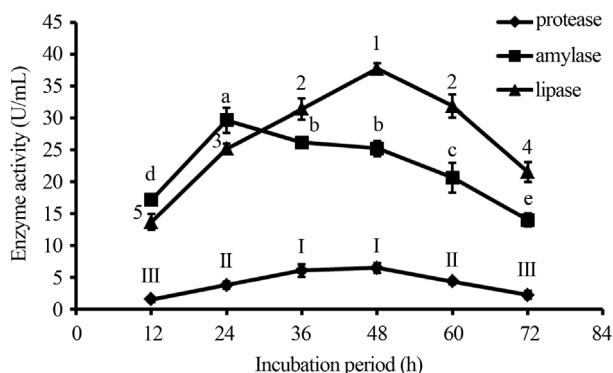
Effect of varying soybean meal concentration on the production of enzymes

A 0.5 to 3% range of soybean meal was tested in order to analyze its effect on alkaline enzyme production. A slight increase in enzyme activities was observed at 1% concentration although statistically at par with 1.5% when compared to control (data not shown). However, in the optimization of next parameter, 1% was used as it is more economical. 2% soybean meal in combination with 1% polypeptone was appropriate for the coproduction of alkaline protease and alpha amylase by *B. subtilis* IIB-26 (Mukhtar and Haq, 2012). At concentrations greater than 1.5%, a decline in enzyme activities was noticed. This decrease may be probably ascribed to nitrogen metabolite repression. Ogrydziak (1993) reported same repression in the production of protease by various types of yeast.

Time course study for optimal production of enzymes by Bacillus flexus XJU-1

After optimization of nutritional and physicochemical factors, the optimized medium for all the 3 alkaline enzymes was used in order to know the optimal time for harvesting enzymes. The optimum incubation period recorded for amylase was 24 h while 48 h was registered for protease and lipase (Figure 7). Likewise, 24 h and 44 h were appropriate time for maximal amylase and protease production by *Bacillus* sp. Y, respectively (Shivakumar, 2012) while incubation period of 48 h gave higher lipase and protease yields in *B. pumilus* (Valasange and Bagewadi, 2012). Contrastingly, a higher incubation period of 120 h was recorded in the coproduction of amylase and protease by *Bacillus* sp. HPE10 (Mukesh kumar *et al.*, 2012b). Sangeetha *et al.* (2008) recorded 36 h (in mid log phase of growth) and 63 h (late log phase) as the optimal time for a protease and lipase, respectively by *B. pumilus* SG2. They concluded that the bacterium uses first a protein as carbon source and then uses a lipidic substrate after the exhaustion of the protein. When optimal incubation period was attained, further increase in incubation time led to a decrease in enzyme ac-

tivities (Figure 7). The decrease seen after optimum incubation time has been ascribed to the shortage of some nutrients in the fermentation medium (Shivakumar, 2012). Although the decrease in lipase activity was attributed to proteolytic degradation by some authors (Maia *et al.*, 1999; Bora and Bora, 2012), the present study showed that the produced lipase is stable in presence protease since they have same optimum incubation time of 48 h (Figure 7). The alkaline lipase was produced in higher amounts (37.72 U/mL) than alkaline amylase (29.63 U/l) and alkaline protease (6.45 U/mL) in this work. Similarly, in the coproduction of amylase and protease by *Bacillus* species, the amylase was produced in higher amounts (Mukesh kumar *et al.*, 2012b; Shivakumar, 2012). Likewise, the lipase was produced in higher amounts than protease by *B. pumilus* SG2 (Sangeetha *et al.*, 2008). However, in the concomitant production of lipase and amylase by *B. licheniformis*, the lipase yield was less than that of protease (Amara *et al.*, 2009). This difference in enzyme production by the *Bacillus* species may depend on the genetic differences.

Conclusion

In the present study, alkaline α-amylase, alkaline lipase and alkaline protease were secreted in higher amounts by *B. flexus* XJU-1 in a single cultivation under the same conditions at flask level. The production of 3 alkaline enzymes in a single cultivation medium, stability of 3 enzymes in a single mixture, the short incubation periods and use of cheap substrates (cottonseed oil and soybean meal) make the submerged fermentation cost-effective. The fermentation can be exploited at fermentor and industrial levels since the crude preparation of a mixture of these enzymes finds applications in detergent industries and other biotechnological applications. During the process, time, labor, energy and money can be saved. We are investigating further the use of crude enzyme in detergent industries.

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Figure 7 - Time course of alkaline enzyme production by *Bacillus flexus* XJU-1. The significant difference at $P_{0.05}$ is indicated by different letters or numerals or Roman numerals on the error bars.

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