Medium optimization for the production of amylase by *Bacillus subtilis* RM16 in Shake-flask fermentation

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Abstract: This study was carried out in shaking incubator and covers the optimization of culture conditions of *Bacillus subtilis* for the maximum production of amylase. Optimal activity was found to be 350 Uml^{-1} when soluble starch was used as a substrate. Parameters taken into consideration to observe their effect on the optimum production of amylase include incubation time, incubation temperature, pH, inoculum size, carbon source, nitrogen source and metallic ions. All parameters were monitored in order to obtain high level of the enzyme units in cell-free broth. The established optimized conditions for *Bacillus subtilis*s train RM16 were found to be: incubation time 24 hours, temperature 40°C and pH 8.0. Inoculum size was 5%, starch (1%) as a carbon source while yeast extract (1.5%) as a nitrogen source. Magnesium ions (0.1%) exerted maximum stimulating effect for the production of amylase which can be further used at large scale applications.

Keywords: Media optimization, *Bacillus subtilis*, amylase, starch.

INTRODUCTION

Amylases are classified as degrading enzymes for starch and catalyze the hydrolysis of glycosidic bonds at α-1, 4 position present in starch molecules (Ajayi and Fagade, 2007). It is an extra cellular enzyme which produces various polysaccharides together with α-limit dextrin, maltose and glucose (Riaz *et al*., 2003). Amylases obtained from plants and microorganisms have been used since long time as food additives in different ways (Raul D *et al.,* 2014). Interestingly, the first industrially produced enzyme was an amylase, which was harvested from a fungus and used for the treatment of several digestive disorders. Amylases produced by plants, animals and microbes also have a key role in carbohydrate metabolism (Burhan, *et al*., 2003).

Amylases are produced by wide spectrum of sources (Van der Maarel *et al*., 2002;Aquino *et al*., 2003)but enzymes produced from microbial source may only execute the industrial demand and they also replaced chemical hydrolysis of starch completely (Pandey *et al.*, 2000a; Bhavya, 2007). Amylases are widely use in baking as well as in paper, textile, pharmaceutical and detergent industries. Hence global market for this enzyme is expected to increase for about 3.3% each year (Gangadharan *et al*., 2006). Thus, researchers have keen interest to produce this enzyme on large scale (Gangadharan *et al*., 2006; Gangadharan *et al*., 2009).

Bacillus has been extensively used for the commercial production of alpha and beta amylases (Rasooli *et al*., 2008). The properties of various amylases like pH profile, temperature and metal ions dependency should be matched to its applications (Nielsen and Borchert, 2000; Shafaat *et al*., 2011). Bacillus species are diverse forms of organisms and they are very adaptable to the environment. A variety of factors influence the nature of their metabolic course of action and the enzyme produced (Srivastava and Baruah, 1986).

Medium composition and concentration considerably affect the growth of bacteria, which ultimately affect the production of amylase. For the maximum production of microbial strains, optimization of the culture conditions is of great importance (Božić *et al.*, 2011;Srivastava and Baruah, 1986). In order to get best possible yield of an enzyme, appropriate media composition and suitable conditions must be maintained(Rao and Satyanarayana, 2003). Components of the media especially carbon and nitrogen source as well as the physicochemical conditions such as the temperature, pH, incubation time, inoculum concentration plays significant role for the elevated level of enzyme production (Oshoma *et al*., 2010;Rezaei *et al.*, 2010). The purpose of this study was to optimize the culture conditions for *Bacillus subtilisRM16* and examined the effect of various parameters on the production of amylase.

MATERIALS AND METHODS

Microbial culture maintenance

*Bacillus subtilis*RM16 was grown on Luria Bertani (LB) agar plate at 37°C for overnight. The culture plate was kept at 4°C and was further used and sub cultured.

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Inoculum preparation

100 ml LB broth was taken in 500ml Erlenmeyer flask. 24 hours old loop-full microbial culture was inoculated into the broth and kept at 37°C in rotary shaker. 5ml of this LB broth culture was further used as a inoculum after 18 hours of incubation.

Enzyme production medium

Enzyme production was carried out in the medium of following per liter composition

Tryptone 10g, Yeast extract 5g, sodium chloride 10g and soluble starch 10g.pH of the medium was adjusted to 7.0 and sterilized at 121°C for 20min. 500ml Erlenmeyer flasks (containing 100ml medium) was inoculated with 5 ml of the overnight starter culture, and incubated at 40°C (variable in case of temperature optimization parameter) in a rotary shaker at 150 rpm. At regular interval of 24 hours till 3 days, aliquots of 10 ml were harvested and the bacterial cells were separated by centrifugation (5000 rpm for 15 min). The supernatant was then used for enzyme assay.

Estimation of amylase

Enzyme assay was done by taking 100µl of starch solution (1%) and 20µl of cell-free broth having the enzyme with 80µl of 20mM Tris-HCl of pH 8.0. Reaction mixture was then incubated at 37°C for 10min. The reaction was stopped by the addition of 200µl DNS solution (3, 5-dinitrosalicyclic acid) and placed in the boiling water bath for 5 minutes (Bernfeld, 1955). Reaction mixture was diluted with 2ml distilled water and finally absorbance was measured at 492 nm via colorimeter. One unit of amylase activity is defined as the amount of enzyme that releases one µmol of reducing sugar in a minute.

Optimization of the parameters

The current study was designed to find out the effect of various process parameters on the production of large quantities of amylase in the appropriate medium. All parameters were optimized independently and enzyme activity was measured at 24, 48 and 72 hours for each of the parameter separately. The optimized parameters were the incubation time i.e. 24, 48, 72 hours, incubation temperature i.e. 40°C, 50°C, 60°C and 70°C and pH of the medium i.e. 5.0, 6.0, 7.0, 8.0, 9.0. For further studies, Carbon source (1%) i.e., starch, glucose, sucrose and maltose were supplemented. Nitrogen source (1%) i.e., (the organic source: tryptone, yeast extract) and (the inorganic source: Ammonium sulphate, Ammonium nitrate, Sodium nitrite) were used. Inoculum size was also tested (2.5%, 5%, 7.5% and 10% by mass per volume). The effect of the metallic ions i.e. copper sulphate, magnesium sulphate, calcium chloride was determined for the high level production of amylase.

STATISTICAL ANALYSIS

All the experiments were conducted twice in duplicate and standard deviation was determined. Analyses of the data were performed by SPSS software (version 17.0) using ANOVA (analysis of variance).

RESULTS

The effect of various parameters that were evaluated for the maximum production of amylase from *Bacillus subtilis* RM16 include:

Effect of incubation temperature

The result from fig. 1 clearly shows the maximum yield of amylase (368 Uml^{-1}) in the medium at 40°C. The range from 40-50°C is obligatory for optimum production of enzyme from *Bacillus subtilis* RM16. At 50°C, the enzyme yield dropped down more quickly with the increase in incubation period. At 70°C, activity of enzyme dropped down up to 40% as compared to the optimum enzyme activity at 40°C (fig. 1).

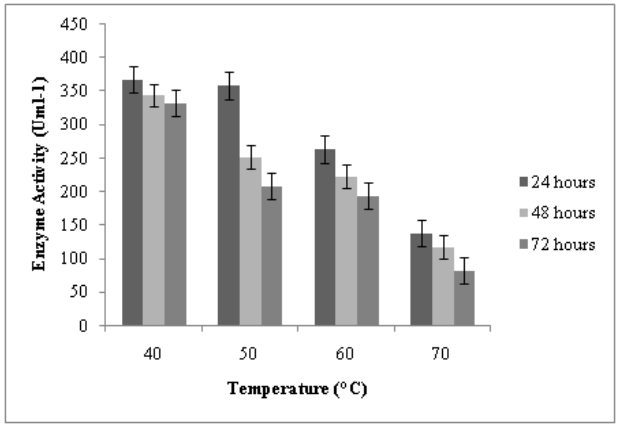

Fig. 1: Effect of incubation temperature on the production of amylase by *Bacillus subtilis* RM16 in shaking incubator using 1% starch as a substrate and inoculum size of 5%

Effect of pH

The amylase production in this study was found to be maximum at the range of pH 7.0-8.0 (295-300Uml^{-1}). Increase or decrease from this pH range showed significant drop in the production of enzyme (fig. 2).

Effect of carbon source

The effect of 1% carbon sources added in the medium such as starch, maltose, glucose and sucrose has been studied. As shown in fig. 3, glucose showed greater enzyme activity in 24 hours but a greater decrease has been observed in 48 and 72 hours. Starch and maltose showed enhanced amylase production (380 Uml^{-1} and 400 Uml^{-1}, respectively) after 24 hours but maltose supplied medium showed a moderate decrease in enzyme activity

after 48 and 72 hours. Only starch has been shown to biosynthesized the enzyme with lesser decrease till 72 hours of incubation.1% sucrose like in glucose, showed much quicker decline in the production of enzyme as the incubation period increases to 72 hours.

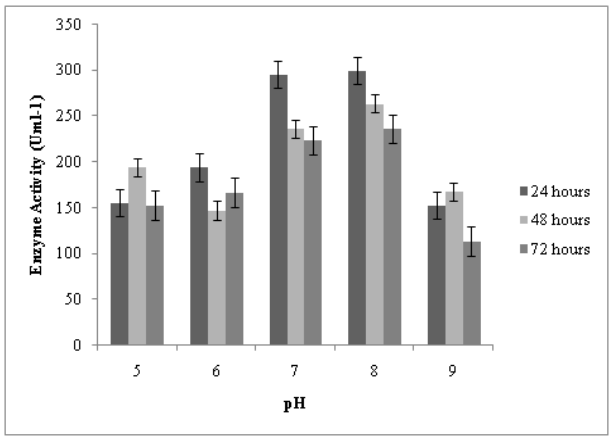

Fig. 2: Effect of pH on the production of amylase by *Bacillus subtilis* RM16 in shaking incubator using 1% starch as a substrate, inoculum size of 5% and temperature 40πC.

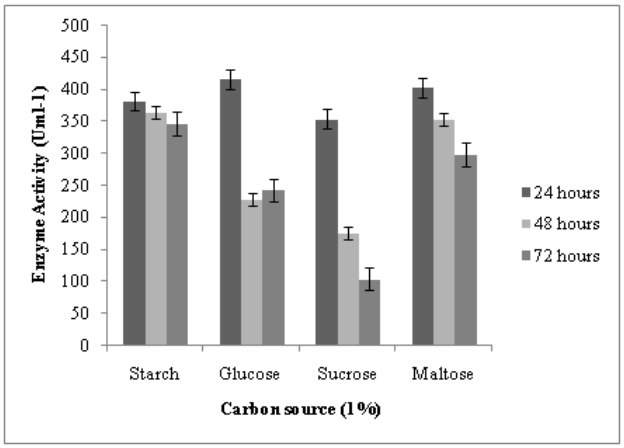

Fig. 3: Effect of different substrates on the production of amylase by *Bacillus subtilis* RM16 in shaking incubator using inoculum size of 5% and temperature 40πC.

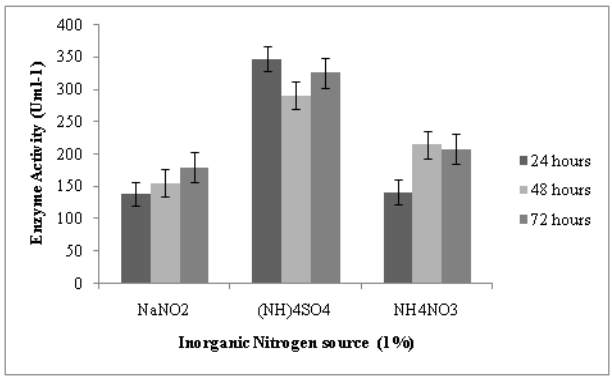

Fig. 4: Effect of 1% inorganic nitrogen sources on the production of amylase by *Bacillus subtilis* RM16 in shaking incubator using 1% starch as a substrate, inoculum size of 5% and temperature 40°C.

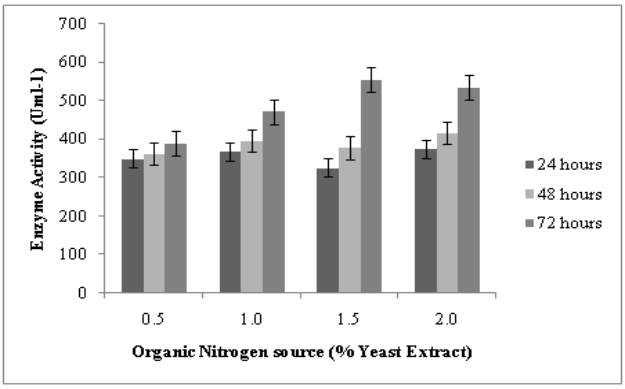

Fig. 5: Effect of different concentration of Yeast extract as a source of organic nitrogen on the production of amylase by *Bacillus subtilis* RM16 in shaking incubator using 1% starch as a substrate, inoculum size of 5% and temperature 40°C.

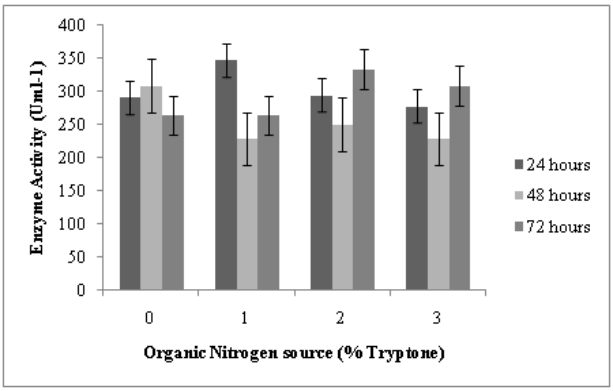

Fig. 6: Effect of different concentration of tryptone as an organic nitrogen source on the production of amylase by *Bacillus subtilis*RM16 in shaking incubator using 1% starch as a substrate, inoculum size of 5% and temperature 40°C.

Effect of nitrogen source

Studies have been done for the supplementation of organic and inorganic sources of nitrogen in the medium that showed various effects on enzyme production by *B. subtilis* RM16. 1% sodium nitrite, ammonium sulphate and ammonium nitrate were tested as inorganic source of nitrogen while tryptone and yeast extract were used as the organic source of nitrogen.

Among the sources provided for inorganic nitrogen, ammonium sulphate significantly improved the production of amylase i.e. 345Uml^{-1} (fig. 4) while sodium nitrite and ammonium nitrate showed fairly low enzyme yield. According to Coleman & Elliott (1962). (Krishna and Chandrasekaran, 1996; Sodhi *et al.*, 2005).

For organic sources of nitrogen, the effect of different concentrations of yeast extract and tryptone was also

studied. In fig. 5, 1.5% yeast extract showed the best enzyme activity at 72 hours of the incubation period (500 Uml^{-1}) while enzyme activities at 0.5, 1.0 and 2.0 are comparable. Among different concentrations of tryptone, 1% showed better production of amylase (340Uml^{-1}) followed by 2% (294Uml^{-1}) after 24 hours as described in fig. 6. Enzyme activity was also detected in the culture broth having yeast extract as a single nitrogen source i.e. notryptone was added (290Uml^{-1}).

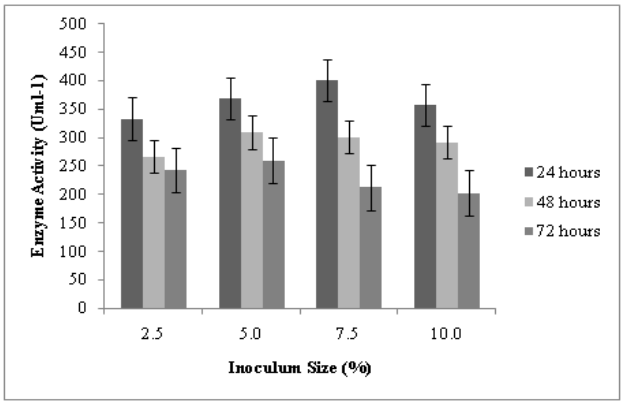

Fig. 7: Effect of the Inoculum size on the production of amylase by *Bacillus subtilis* RM16 in shaking incubator using 1% starch as a substrate and temperature 40πC.

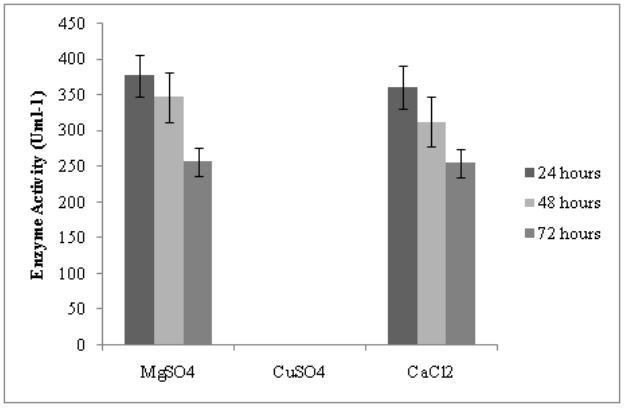

Fig. 8: Effect of 0.1% metal ion concentrations on the production of amylase by *Bacillus subtilis* RM16 in shaking incubator using 1% starch as a substrate, inoculum size of 5% and temperature 40πC. $CuSO_4$ inhibited the growth of *Bacillussubtilis* RM16.

Effect of inoculum size

The lowest enzyme production was obtained at 2.5% while maximum enzyme activity was obtained at 7.5% inoculum i.e. 400Uml^{-1} (fig. 7) but a constant decrease has been observed after 48 and 72 hours. Enzyme activity at 5% inoculums size has shown to be the most stable.

Effect of Metal ions

Among metal ions, 0.1% $MgSO_4$ and $CaCl_2$ stimulated the production of amylase in the medium i.e. 370 Uml^{-1} while $CuSO_4$ inhibited it completely (fig. 8).

Effect of incubation time

The above figures clearly show the time period for the best production of amylase in the basal medium having 1% starch as a substrate. The optimum time achieved for the production of amylase was 24 hours for each of the experiment. From 24 to 72 hours of incubation period, a gradual decrease in enzyme production was observed.

DISCUSSION

Fermentation medium is said to be optimized or improved if it showed enhance production of the target enzyme. In the recent study, *Bacillus subtilis* RM16 was found to produce maximum enzyme unit (amylase) at temperature of 40°C with pH ranges from 7.0-8.0 after 24 hours of the incubation time period. Regarding carbon and nitrogen sources, 1% starch as well as 1.5% yeast extract showed maximum enzyme activity. Magnesium sulphate (0.1%) and inoculums size (5%) has shown to increase amylase production in cell-free broth.

Regarding optimal temperature requirement, *B. subtilis* RM16 showed mesophilic character showing maximum enzyme production at 40°C. The growth of the organism inhibited with an increase in the temperature of the basal medium (Pandey *et al*., 2000b). The initial pH of the fermentation medium also plays significant role for the best possible production of amylase (Pedersen and Nielsen, 2000). *B. subtilis* RM16 showed maximum enzyme activity at a range of pH 7.0-8.0. However, several researchers have reported the optimum activities of Bacillus genus from low to high pH values i.e. 3.5 to 12 (Konsula and Liakopoulou-Kyriakides, 2004; Asgher *et al*., 2007). Many of the previous studies revealed an optimum pH range between 6.0 and 7.0 for both the bacterial growth and production of enzyme (Banargee and Bhattacharya, 1992).

In the previous studies, starch is also known to enhance the production of amylases in *Bacillus* sp. PS-7, *B. subtilis* CBTK 106, *B. subtilis* IMG 22 and *Bacillus* sp. I-3 (Sodhi *et al*., 2005; Kumar R and Mehta A, 2013). In this study, 1% starch showed improved production of amylase as compared to the other carbon sources utilized i.e. maltose, glucose and sucrose. Whereas 1% ammonium sulphate (inorganic nitrogen source) and 1.5% tryptone (organic nitrogen source) showed greater enzyme production from *B. subtilis* RM16. According to Coleman & Elliott (1962) ammonium salts were stimulators of B. subtilis amylase synthesis. It has also been reported previously that the supplementation of ammonium sulphate in the medium increased enzyme production in various microorganisms (Krishna and Chandrasekaran, 1996; Sodhi *et al.*, 2005). Previous reports revealed that yeast extract also served as good organic nitrogen source for α-amylase synthesis from *Bacillus amyloliquefaciens* (Sharma *et al*. 2012). The nature and relative

concentration of different complex nitrogenous sources in the growth medium are both important in the synthesis of amylase. Lower levels of nitrogen and also excess nitrogen are equally detrimental causing enzyme inhibition (Sharma *et al*., 2012).

Longer incubation period is requiring for microbial cells if added in small inoculums size so that they can multiply up to a certain number that can consume substrate and produce the desired product. A balance should be maintained between the proliferating biomass and the nutrients of the medium so that enzyme synthesis would be maximum (Ramachandran *et al*., 2004; Özdemir *et al*., 2009). In this study, 5% inoculums size was used to monitor maximum amylase synthesis. The production of enzyme decreased as the inoculum level increases. Exhaustion of nutrients in the fermentation medium could be the reason as mentioned elsewhere (Mahadik *et al*., 2002).

Copper sulphate (0.1%) in this study, strongly repressed the growth of *B. subtilis* as described previously (Konsula and Liakopoulou-Kyriakides, 2004). The inhibition of the growth by Cu^{2+} ions could be because of the competition among the given cation and the protein-associated cations that result in decreased metallo-enzyme activity (Lévêque *et al*., 2000).

Time of incubation depend upon the various characteristics of the microbial culture, its multiplication rate and enzyme production (Baysal *et al*., 2003). *B. subtilis* RM16 showed enhanced production of amylase after 24 hours of incubation and decreased subsequently when incubated for longer period (till 72 hours). In order to check and confirm this decrease of enzyme activity, 24, 48 and 72 hours were used in all experiments for optimization of each parameter. The decline in enzyme yield could be the result of decomposition or denaturation of the enzyme due to the possible interaction with other components of the medium used (Ramesh and Lonsane, 1987).

CONCLUSION

In the present study, effect of different fermentation parameters such as incubation time, incubation temperature, pH, carbon source, nitrogen source, inoculum size and metal ions for amylase production by *Bacillussubtilis* strain RM16 in shaking incubator were evaluated. From the results, we conclude that enzyme production was found to be maximum when 1% starch was supplemented in the production medium. 1.5% yeast extract and 1% ammonium sulphate were the best components among nitrogen sources that provide the necessary nutrients for *Bacillus subtilis* RM16 to synthesize the enzyme sufficiently. 40°C was found to be the most suitable temperature along with 8.0pH value for the production of amylase by *B. subtilis* RM16. Findings of pH range propose that amylase is active at fairly higher pH in the medium, making this enzyme attractive for the detergent industries. Maximum enzyme yield was obtained after 24 hours of incubation in each subsequent experiment along with 5% of the inoculum size. Addition of Magnesium sulphate (0.1%) also enhanced the production of amylase in cell-free broth. *Bacillus subtilis* RM16 can be a potent producer of amylase. Due to importance of these results, further investigations need to be carried out in order to use this strain for the development of biotechnological processes.

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REFERENCES

Ajayi AO and Fagade OE (2007). Heat activation and stability of amylases from Bacillus species. *Afr. J. Biotechnol.,* **6**(10): 1181-1184.

Aquino A, Jorge J, Terenzi H and Polizeli M (2003). Studies on a thermos table α-amylase from the thermophilic fungus *Scytalidium thermophilum*. *Appl. Microbiol. Biotechnol.,* **61**(4): 323-328.

Asgher M, Asad MJ, Rahman S and Legge R (2007). A thermostable α-amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing. *J. Food Eng.,* **79**(3): 950-955.

Banargee R and Bhattacharya BC (1992). Extra cellular alkaline protease of newly isolated *Rhizopus oryzae*. *Biotechnol. let.,* **14**: 301-304.

Baysal Z, Uyar F and Aytekin C (2003). Solid state fermentation for production of α-amylase by a thermo tolerant *Bacillus subtilis* from hot-spring water. *Process Biochem.,* **38**(12): 1665-1668.

Bernfeld P (1955). Amylases, α and β. *Methods in enzymology*, **1**: 149-158.

Bhavya D (2007). Production and characterization of fungal amylase enzyme isolated from *Aspergillus* sp. JGI 12 in solid state culture. *Afr. J. Biotechnol.,* **6**(5): 576-581.

Božić N, Ruiz J, López-Santín J and Vujčić Z (2011). Optimization of the growth of and α-amylase production by *Bacillus subtilis* IP 5832 in shake flask and laboratory fermenter batch cultures. *J. Serb. Chem. Soc.,* **76**(7): 965-972.

Burhan A, Nisa U, Gökhan C, Ömer C, Ashabil A and Osman G (2003). Enzymatic properties of a novel thermos table, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic *Bacillus* sp. isolate ANT-6. *Process Biochem.,* **38**(10): 1397-1403.

Coleman G and Elliott WH (1962). Studies on α-amylase formation by *Bacillus subtilis.Biochem. J.,* **83**(2): 256-263.

Gangadharan D, Madhavan Nampoothiri K, Sivaramakrishnan S and Pandey A (2009). Immobilized bacterial α-amylase for effective hydrolysis of raw and soluble starch. *Food Res. Int.,* **42**(4): 436-442.

Gangadharan D, Sivaramakrishnan S, Nampoothiri KM and Pandey A (2006). Solid culturing of *Bacillus amyloliquefaciens* for alpha amylase production. *Food Technol. Biotechnol.,* **44**(2): 269-274.

Konsula Z and Liakopoulou-Kyriakides M (2004). Hydrolysis of starches by the action of an α-amylase from *Bacillus subtilis*. *Process Biochem.,* **39**(11): 1745-1749.

Krishna C and Chandrasekaran M (1996). Banana waste as substrate for α-amylase production by *Bacillus subtilis* (CBTK 106) under solid-state fermentation. *Appl. Microbiol. Biotechnol.,* **46**(2): 106-111.

Kumar R and Mehta A (2013) Isolation, Optimization and Characterization of α-Amylase from *Bacillus alcalophilus.Int. J. Sci. Res.,* **2**(7): 171-174.

Lévêque E, Janeček Š, Haye B and Belarbi A (2000). Thermophilic archaeal amylolytic enzymes. *Enzyme Microb. Technol.,* **26**(1): 3-14.

Mahadik ND, Puntambekar US, Bastawde KB, Khire JM and Gokhale DV (2002). Production of acidic lipase by *Aspergillus niger* in solid state fermentation. *Process Biochem.,* **38**(5): 715-721.

Nielsen JE and Borchert TV (2000). Protein engineering of bacterial α-amylases. *Biochim. Biophysica. Acta.,* **1543**(2): 253-274.

Oshoma C, Imarhiagbe E, Ikenebomeh M and Eigbaredon H (2010). Nitrogen supplements effect on amylase production by *Aspergillus niger* using cassava whey medium. *Afr. J. Biotechnol.,* **9**(5): 682-686.

Özdemir S, Güven K, Baysal Z and Uyar F (2009). Screening of various organic substrates and the development of a suitable low-cost fermentation medium for the production of a-amylase by *Bacillus subtilis*. *Food Technol. Biotechnol.,* **47**: 364-369.

Pandey A, Nigam P, Soccol C, Soccol V, Singh D and Mohan R (2000a). Advances in microbial amylases. *Biotechnol. Appl. Biochem.,* **31**: 135-152.

Pandey A, Soccol CR and Mitchell D (2000b). New developments in solid state fermentation: I-bioprocesses and products. *Process Biochem.,* **35**(10): 1153-1169.

Pedersen H and Nielsen J (2000). The influence of nitrogen sources on the α-amylase productivity of *Aspergillus oryzae* in continuous cultures. *Appl. microbiol. Biotechnol.,* **53**(3): 278-281.

Ramachandran S, Patel AK, Nampoothiri KM, Francis F, Nagy V, Szakacs G and Pandey A (2004). Coconut oil cake a potential raw material for the production of α-amylase. *Bioresource. Technol.,* **93**(2): 169-174.

Ramesh M and Lonsane B (1987). A novel bacterial thermos table alpha-amylase system produced under solid-state fermentation. *Biotechnol. Letters,* **9**(7): 501-504.

Rao JUM and Satyanarayana T (2003). Statistical optimization of a high maltose-forming, hyper thermos table and Ca^{2+}-independent alpha-amylase production by an extreme thermophile *Geobacillus thermoleovorans* using response surface methodology. *J. Appl. Microbiol.,* **95**(4): 712-718.

Rasooli I, Astaneh SD, Borna H and Barchini KA (2008). A thermostable α-Amylase producing natural variant of *Bacillus*spp. Isolated from soil in Iran. *Am. J. Agri. Biol. Sci.,* **3**(3): 591-596.

Raul D, Biswas T, Mukhopadhyay S, Das SK and Gupta S (2014). Production and partial purification of alpha amylase from *Bacillus subtilis* (MTCC 121) using solid state fermentation. *Biochem. Res. Int.,* http://dx.doi.org/10.1155/2014/568141.

Rezaei PS, Darzi GN and Shafaghat H (2010). Optimization of the fermentation conditions and partial characterization for acido-thermophilic α-amylase from *Aspergillus niger* NCIM 548.*Korean J. Chem. Eng.,* **27**(3): 919-924.

Riaz *N*, Haq I and Qadeer M (2003). Characterization of α-amylase by *Bacillus subtilis*. *Int. J. Agr. Biol.,* **5**(3): 249-252.

Shafaat S, Akram M and Rehman A (2011). Isolation and characterization of a thermos table-amylase from *Bacillus subtilis*. *Afr. J. Microbiol. Res.,* **5**(20): 3334-3338.

Sharma N, Vamil R, Ahmad S and Agarwal R (2012). Effect of different carbon and nitrogen sources on α-amylase production from *Bacillius amyloliquefaciens*. *Int. J. Pharm. Sci. Res.,* **3**(4): 1161-1163.

Sodhi HK, Sharma K, Gupta JK and Soni SK (2005). Production of a thermos table α-amylase from *Bacillus* sp. PS-7 by solid-state fermentation and its synergistic use in the hydrolysis of malt starch for alcohol production. *Process Biochem.,* **40**(2): 525-534.

Srivastava R and Baruah J (1986). Culture conditions for production of thermostable amylase by *Bacillus stearothermophilus*. *Appl. env. Microbiol.,* **52**(1): 179-184.

Van Der Maarel MJ, Van Der Veen B, Uitdehaag J, Leemhuis H and Dijkhuizen L (2002). Properties and applications of starch-converting enzymes of the α-amylase family. *J. Biotechnol.,* **94**(2): 137-155.