RESEARCH ARTICLE

Yordan Stefanov
Ivan Iliev
Mariana Marhova
Sonya Kostadinova

Optimization of nutritive medium composition for production of amylase by *Bacillus* strains

Authors' addresses:
Department of Biochemistry and Microbiology, Faculty of Biology, University of Plovdiv „Paisii Hilendarski", 24 Tzar Assen Str., 4000 Plovdiv, Bulgaria.

Correspondence:
Yordan Stefanov
Department of Biochemistry and Microbiology, Faculty of Biology, University of Plovdiv „Paisii Hilendarski", 24 Tzar Assen Str., 4000 Plovdiv, Bulgaria.
Tel.: +359 32 261486
e-mail: iordanstefanov@gmail.com

Article info:
Received: 2018
Accepted: 2018

ABSTRACT
The current study aimed to examine the amylolytic activities of various species of the *Bacillus* genus. A total of 31% of the tested 166 strains showed a positive reaction on starch agar. Their amylolytic activity was in the range of 0.9 to 2.8 U/ml. *Bacillus cereus* №10 showed the highest initial activity, established in the late stationary phase of growth (36 hours of incubation). The effects of different nitrogen sources, metal ions and different type of secondary carbon sources (starch was used as a primary carbon source) on the production of amylase by the screened strain were studied. The enzyme production was significantly influenced by the type and concentration of the secondary carbon source. Replacement of peptone by yeast extract and the addition of 1 mM Ca^{2+} increased initial amylolytic activity by 153% reaching 4.29±0.63 U/ml.

Key words: *Bacillus cereus*, amylase, enzyme production, nutritive medium optimization

Introduction

Amylases are one of the most important industrial enzymes accounting for more than 25% of the world enzyme market (Reddy et al., 2003). They are isolated from a variety of organisms, but the most efficient, cheap and ecologically friendly methods include using bacterial species as main producers (de Souza et al., 2010). The most widely used producers are the species from the genus *Bacillus* – *Bacillus amyloliquefaciens, Bacillus subtilis, Bacillus licheniformis, Bacillus dipsosauri, Bacillus stearothermophilus, Bacillus cereus* and others (de Souza et al., 2010). Species of genus *Bacillus* are preferred as they are rather easy to cultivate. They produce large quantities of different proteins and extracellular enzymes for short periods, which is cost-effective, and they demonstrate favorable properties – wide pH and temperature of activity and stability, which allows them to be used in harsh conditions (Prakash et al., 2010; Vyas et al., 2016). Some of the species are generally regarded as safe (they have GRAS status), allowing the application of the enzymes in the food and pharmaceutical industries (Sewalt et al., 2016).

Amylases, produced by bacilli, are widely used in different industries. In the food and beverage sector, they are used for the production of glucose and glucose-fructose corn syrups, for starch saccharification, for the preparation of different alcoholic beverages. Saccharification is performed in high-temperature conditions and the use of amylases from bacilli is preferable, because they have high thermal stability. Amylolytic enzymes are also one of the most widely used types of enzymes in the detergent industry. This is due to their stability at alkaline conditions (Asad et al., 2011). In food production, bacterial amylases are added to the dough. They hydrolyze the starch to dextrins, di- and monosaccharides which are metabolized by the yeasts. The enzymatic hydrolysis of the starch in the dought increases the quality of the final product and its shelf life without the addition of chemical preservatives (de Souza et al., 2010). In the textile industry amylases are used for the desizing without damaging the textile fibers (Feitkenhauer et al., 2003). For example, amylases are widely applied not only in a food and beverage industry but even in more precise spheres such as clinical diagnostics, analytical laboratory tests, and others. Wide varieties of applications constantly increase the demands of amylolytic enzymes.

In the current research, we aimed to study the amylolytic activity of *Bacillus* strains and to optimize the nutritive medium for the improvement of the enzyme production.

Materials and Methods

Bacterial strains

One hundred and sixty-six *Bacillus* strains from the bacterial culture collection of the Department of "Biochemistry and microbiology", Faculty of Biology,

Plovdiv University, Bulgaria were screened for production of amylases. The collection includes 118 strains *Bacillus cereus*, 36 strains *Bacillus thuringiensis*, and 12 strains *Bacillus sphaericus*. For routine laboratory use the strains were maintained on nutrient agar medium at 4°C. For long-term use, the strains were maintained in nutrient agar under a layer of paraffin at 4°C.

Growth conditions

The cultivation was carried out in a basal medium of a following composition (in % (w/v)): soluble starch 1; Na_2HPO_4 0.25; peptone 0.2; KH_2PO_4 0.1; NaCl 0.1; glucose 0.05; $MgSO_4.H_20$ 0.005; $CaCl_2$ 0.005; pH 7. The medium was inoculated with 10% of 24 h cell cultures and incubated at 37°C on a rotary shaker at 100 rpm for 48 h. On every two hours, 5-ml samples of culture suspension were withdrawn and were assayed for enzyme activity to determine the optimal duration. The pH of the culture was determined. The cell density was determined using McFarland Densitometer, Grant-Bio (Grant Instruments, England). After the incubation, the culture was centrifuged at 10 000 g at 4°C for 20 minutes. The cell-free supernatants were collected and used for further analysis.

Qualitative amylase assay

All strains were plated on starch agar (0.3% beef extract; 1% soluble starch; 1.5% agar; the medium is sterilized at 121°C for 15 minutes) and incubated at 37 ± 2°C. After 48 h of incubation, plates were flooded with Lugol's iodine solution.

The formation of transparent halo around the colony indicated its starch hydrolyzing ability. The colonies with the largest halo were selected for quantitative amylase assay.

Quantitative enzyme assay

Amylase activity was measured quantitatively using soluble starch as a substrate by the method of Bernfeld et al. (1955). The reaction mixture containing 1% soluble starch dissolved in 20 mM phosphate buffer (pH 6.9) and 1 ml of an appropriately diluted enzyme is incubated at 20°C for 3 minutes. After incubation to the mixture is added 1 ml 96 mM 3,5-dinitrosalicylic acid. The reaction mixture is placed in a boiling water bath for 15 minutes. To each sample is added 9 ml pure water after cooling. The absorbance of the sample is measured against blank control at λ=540 nm. The activity is expressed in units per milliliter (U/ml). One unit of amylase activity is defined as the quantity of enzyme that liberated 1.0 mg of maltose from starch for 3 minutes at pH 6.9 at 20°C.

Optimization of the enzyme production

Effects of carbon and nitrogen sources on amylase production

Effects of secondary organic carbon sources were determined by substitution of the glucose in the initial medium with fructose, ribose, and maltose respectively at a concentration of 0.1%. To study the effects of different nitrogen sources, 0.2% of yeast extract, peptone, casein, KNO_3 and $(NH_4)_2SO_4$, respectively were added to the optimized nutritive medium in separate experiments.

Effects of metal ions on extracellular amylase activity

To determine the effects of the metal ions on the production of amylolytic enzymes, the initial medium was supplemented with Ca^{2+}, Fe^{2+}, Mg^{2+}, Zn^{2+} and Mn^{2+} ions in 1 mM concentration. Medium containing (w/v): 0.1% KH_2PO_4; 0.25% Na_2HPO_4; 0.1% NaCl; 0.2% yeast extract; 0.1% ribose; 1% soluble starch, was used as a control.

Results

Screening for amylase producers

We have established that 31% of the studied *Bacillus* strains showed amylolytic activity after cultivation on a starch agar (61% of *Bacillus cereus*, 36% of *Bacillus thuringiensis* and 3% of *Bacillus sphaericus*) (Figure 1a). Twenty-three strains forming a halo of over 5 mm were further studied for the production of extracellular amylolytic enzymes on the initial medium containing 1% starch after 48 h incubation in order to establish a baseline. The strains` amylase activity was in the range of 0.86 – 2.80 U/ml (Figure 1b). The strain *Bacillus cereus* №10 showed 2.80 U/ml activity - 25 to 125% higher of the rest of the studied strains.

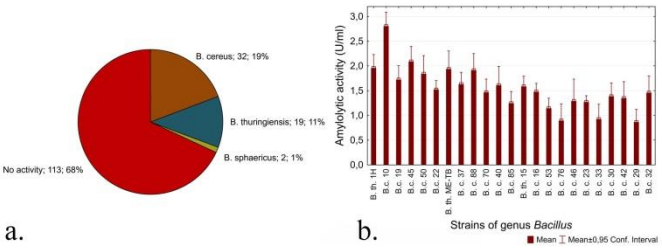

Figure 1. *Amylolytic activity of strains of genus Bacillus – qualitative (a) and quantitative (b) analysis.*

Dynamics of the enzyme production during growth

The production of amylolytic enzymes began at the second hour of cultivation and slowly increased. The activity reaches 1 U/ml at the eighth hour. It remains stable until the fourteenth hour of cultivation, followed by a steady slow increase to 1.6 U/ml at the 20-22 h and 2.6 U/ml at the 26-28 h. The peak of the enzyme activity of 3.14 U/ml was established at the thirty-sixth hour. The prolonged enzyme

production, the presence of three plateaus of activity (8-14 hours, 20-22 hours, 26-28 hours), and the maintenance of relatively high activity over the incubation period suggest a synthesis of different types of amylases that are secreted into the culture medium during the different phases of growth. No sporulation was detected for the 48 h study period.

The pH remained relatively constant during the experiment. There was an initial pH increase of the cultivation medium to pH 6.9 after six hours of cultivation followed by a steady slow decrease to pH 6.5 at the 48 h. The peak of the enzyme activity was at pH 6.6 (Figure 2). The cell density analysis showed that the exponential growth starts immediately after inoculation and ended at the 14 h. Maximal enzyme production was established during the late stationary phase.

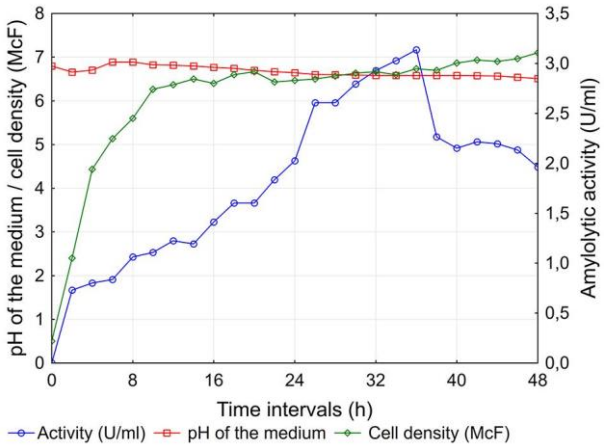

Figure 2. *Dynamics of the amylolytic enzymes activity, pH and cell density during growth of Bacillus cereus.*

Effects of different carbon sources

To determine the effects of different carbohydrates on the amylolytic production, the basic medium containing only starch as a sole carbon source was supplemented with fructose, glucose, ribose, and maltose, respectively, at a concentration of 0.1%. The control medium contained no secondary carbohydrates (Figure 3).

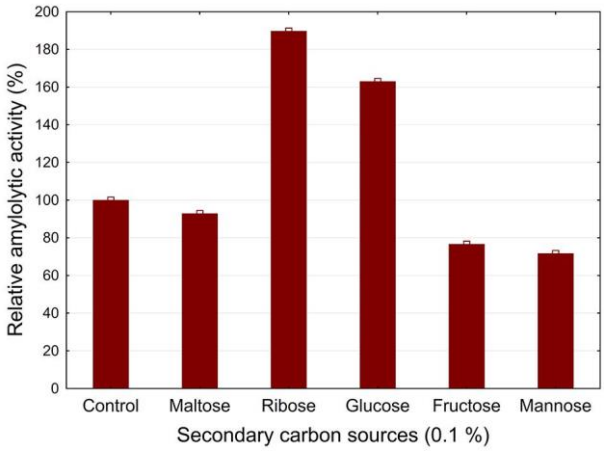

Figure 3. *Effects of different secondary carbon sources on the amylolytic activity of Bacillus cereus strain №10.*

The maximum relative amylolytic activity of 190% compared to the control test was recorded in a ribose-containing medium. The addition of glucose leads to an increase in relative activity to 163%. The maltose, fructose, and mannose had an inhibitory effect on the enzyme production resulting in the activity of 93%, 77%, 72% respectively.

We have established that changes in the concertation of the ribose have a significant effect on the end amylolytic activity. The results are shown in Figure 4.

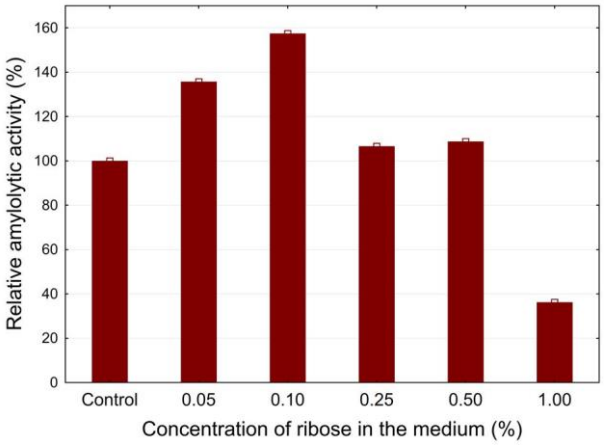

Figure 4. *Effects of different concentrations of ribose on the amylolytic activity of Bacillus cereus №10.*

The highest relative amylolytic activity of 157% was observed when 0.1% ribose was used as an additional source of organic carbon and energy. Lower as well as higher concentrations of the added ribose resulted in a reduction of the amylolytic activity of the studied strain. The lowest relative activity was observed at 1% concentration: 36%.

Effects of nitrogen sources

The effects of the substitution of the initial nitrogen source (peptone) with other organic and inorganic nitrogen sources on the amylase production are shown in Figure 5.

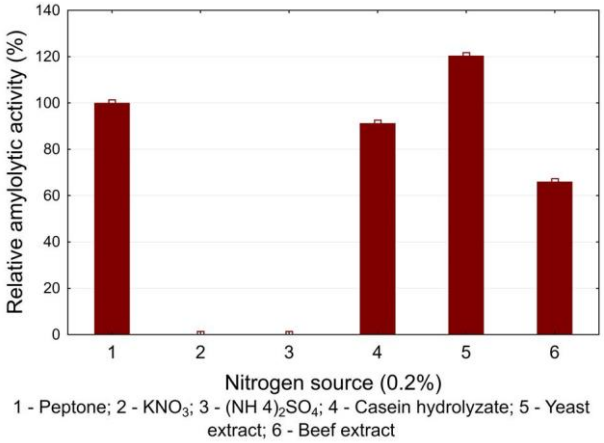

Figure 5. *Effect of the nitrogen source on the amylolytic activity of Bacillus cereus strain №10.*

We`ve established that yeast extract yielded better results than the initially used peptone, while the substitution with casein hydrolysate showed similar to the control results. The strain did not grow in the presence of an inorganic nitrogen source only.

Effects of metal ions

Amylolytic enzymes are metalloproteins and they require a source of metal ions in order to function properly. We have enriched the culture medium with sources of metal ions. Highest stimulation of extracellular amylase activity was obtained by the addition of calcium ions – 130% of the control ($p<0.005$). (Figure 6). The ions of magnesium, manganese, and iron also increased the enzyme production, while zinc ions had no significant effect, with a relative activity of 95%, close to the control test.

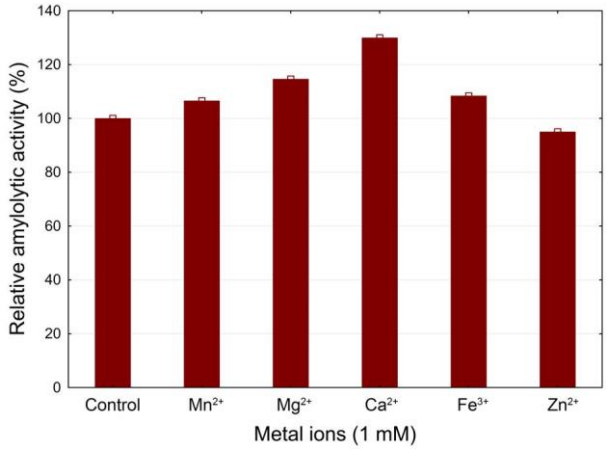

Figure 6. *Effects of different metal ions on the amylolytic activity of Bacillus cereus №10.*

Discussion

Many factors affect the growth of the producers and the synthesis of the desired metabolite. Different species and strains of the genus *Bacillus* demonstrate a different preference for the environmental conditions and the composition of the growth medium. To reach maximum enzyme production it is very important to optimize nutritive medium composition and culture conditions (Nanganuru et al., 2012).

The results obtained showed that over 30% of the studied strains showed amylolytic activity and confirm the importance of the *Bacillus* species as enzyme producers as stated by de Souza et al. (2010). The initial enzyme activity of 2.80±0.29 U/ml in the *B. cereus* №10 supernatant is comparable with the results of Pavithra et al. (2014) for *Bacillus subtilis* and Divakaran et al. (2011) for *Bacillus licheniformis.*

The results showed that the production of amylolytic enzymes by *Bacillus cereus №10* is strongly dependent on the duration of the fermentation process, reaching maximum activity at 36^{-th} hour of the cultivation, which corresponds to the late stationary phase of growth. The pH of the culture medium varied within short limits during the study period, suggesting relatively high buffer stability of the medium. Amylases are typically secreted in the late stationary phase of growth. They are induced by the presence of oligo and polysaccharides, containing alpha-1, 4-; beta-1, 4; beta-1, 6 glucosyl bonds (Saito et al., 1975). The duration of the fermentation process is species and strain-specific property and it can vary greatly. Nanganuru et al. (2012) have shown that a strain of *Bacillus subtilis* reaches its pick of enzyme production after 50 hours of the cultivation, while Salman et al. (2016) report a maximal activity after 24 hours. The results are highly heterogenic. The decline of enzyme activity may be due to inactivation as results of the changing conditions and composition of the surrounding environment or even due to the production of proteolytic enzymes (Unakal et al., 2012). The studied strain *B. cereus* has no proteolytic activity (Stefanov et al., 2018), favoring the maintenance of an amylolytic activity of 2 U/ml for a long period of time.

The presence of exogenous glucose or other low-molecular-weight metabolites has an inhibitory effect over the synthesis of the enzymes (Saito et al., 1975). The stimulatory effect of the addition of low concentrations of carbohydrates in our study could be explained with the fact that the bacterial culture may use the additional carbon source to boost its growth. After the depletion of the secondary carbon source, the synthesis of amylases may begin, which is induced by the presence of starch or starch containing materials. Santos et al., 2003 are reporting similar catabolite repressing effect when simple sugars are added to the medium as a secondary carbon source.

The present study revealed that the addition of 0.1% glucose, maltose, mannose, fructose, and ribose, as a secondary carbon source, lead to the increase of the enzyme activity compared to the control medium. The addition of ribose yielded the highest relative activity (190 %). Both the increase and the decrease of the ribose concentration had a negative effect. Similar positive effect on the enzyme activity after addition of rhamnose, glucose, lactose, and galactose, is demonstrated by other authors (Salman et al., 2016; Jana et al., 1998; Sudharhsan et al., 2007). They also report that the addition of sorbose and fructose inhibits the enzyme activity.

The substitution of the initial organic nitrogen source with inorganic, such as NO_3^- and NH_4^+ inhibited the bacterial growth. The amylolytic activity was enhanced by the addition of yeast extract to the medium, while the addition of beef extract or casein hydrolysate resulted in reduced activity. Our results confirm previous findings that the addition of complex organic sources of nitrogen to the medium stimulates the production of amylolytic enzymes (Thippeswamy et al., 2006). Organic nitrogen sources may not directly stimulate

the production of amylases, but it rather increases the growth rate of the organism in the culture (Sreekanth et al., 2013). On the other hand, it must be taken into account that yeast extract not only stimulates the cell growth but it also strongly influenced the pH of culture medium. The acidification of the culture medium caused by yeast extract can repress amylase production (Santos et al., 2003).

We`ve established that the presence of metal ion also affects the enzyme activity. The addition of Ca^{2+} demonstrated the highest effect and increased the activity with 130% compared to the control. The presence of Zn^{2+} had an inhibitory effect. Sudha et al. (2012) describe similar results. Most of the known amylases are metal-dependent enzymes. The enhancement of the amylolytic activity may be due to the interaction the metal ions with the negatively charged amino acids in the proteins. Higher concentrations of metal ions are salting out hydrophobic amino acid residues, which results in a more compact and stable enzyme structure (Sudha et al., 2012).

Conclusion

The selected *Bacillus cereus №10* strain proved to be a good producer of extracellular amylolytic enzymes. The highest enzyme activity was established in the late stationary phase of growth. The enzyme production was significantly influenced by the substitution of the secondary carbon sources with ribose in a concentration of 0.1%, the replacement of the peptone with yeast extract and the addition of 1 mM Ca^{2+} ions. These changes in the composition of the nutritive medium increased the initial amylolytic activity by 153% reaching 4.29±0.63 U/ml. The stable amylase production makes the studied strain *B. cereus* a potential producer, but further analysis of the enzymes and their properties are needed.

References

Asad W, Asif M, Rasool SA. 2011. Extracellular enzyme production by indigenous thermophilic bacteria: partial purification and characterization of α-amylase by Bacillus sp. WA21. Pak. J. Bot., 43(2):1045–1052.

Bernfeld P. 1955. Amylases, α and β. Methods Enzymol., 1:149-158.

de Souza PM. 2010. Application of microbial α-amylase in industry - A review. Braz. J. Microbiol., 41(4):850-61.

Divakaran D, Chandran A, Pratap Chandran R. 2011. Comparative study on production of a-Amylase from Bacillus licheniformis strains. Braz. J. Microbiol., 42(4):1397-404.

Feitkenhauer H. 2003. Anaerobic digestion of desizing wastewater: influence of pretreatment and anionic surfactant on degradation and intermediate accumulation. Enzyme Microb. Technol., 33:250–258.

Jana M, Chattopadhyay DJ, Pati BR. 1998. Effect of different carbon sources on the production of amylase by Bacillus sp. MD 124. Acta Microbiol. Imm. H., 45(2):229-37.

Nanganuru HY, Korrapati N, Mutyala S. 2012. Studies on the production of α- amylase by *Bacillus subtilis*. Iosr-Jen., 2(5): 1053-1055.

Pavithra S, Ramesh R, Aarthy M, Ayyadurai N, Gowthaman MK, Kamini NR. 2014. Starchy substrates for production and characterization of Bacillus subtilis amylase and its efficacy in detergent and breadmaking formulations. Starch-Starke, 66(12):976–984.

Prakash O, Jaiswal N. 2010. Review alpha-Amylase: an ideal representative of thermostable enzymes. Appl. Biochem. Biotech., 160(8):2401-14.

Reddy NS, Nimmagadda A, Sambasiva RKRS. 2003. An overview of the microbial α-amylase family. Afr. J. Biotechnol., 2:645–648.

Saito N, Yamamoto K. 1975. Regulatory factors affecting alpha-amylase production in Bacillus licheniformis. J. Bacteriol., 121(3):848-56.

Salman T, Kamal M, Ahmed M, Mariam SS, Khan R, Hassan A. 2016. Medium optimization for the production of amylase by *Bacillus subtilis* RM16 in Shake-flask fermentation. Pak. J. Pharm. Sci., 29:439-444.

Santos EO, Martins MLL. 2003. Effect of the medium composition on formation of amylase by *Bacillus sp.* Braz. Arch. Biol. Technol., 46(1):129-134.

Sewalt V, Shanahan D, Gregg L, La Marta J, Carillo R. 2016. The Generally Recognized as Safe (GRAS) Process for Industrial Microbial Enzymes. Ind. Biotechnol., 12:295-302.

Sreekanth MS, Vijayendra SVN, Joshi GJ, Shamala TR. 2013. Effect of carbon and nitrogen sources on simultaneous production of α-amylase and green food packaging polymer by Bacillus sp. CFR 67 J. Food Sci. Tech. Mys., 50(2): 404–408.

Stefanov YM, Iliev IZ, Marhova M, Kostadinova S. 2018. Isolation and purification of proteolytic enzymes, produced by strains of genus *Bacillus*. Ecologia Balcanica., 10(2): in press.

Sudha, 2012. Effect of different concentrations of metal ions on alpha amylase production by Bacillus amyloliquefaciens. Res. J. Biotechnol., 3(4): 67-71.

Sudharhsan S, Senthilkumar S, Ranjith K. 2007. Physical and nutritional factors affecting the production of amylase from species of Bacillus isolated from spoiled food waste. Afr. J. Biotechnol., 6:430–435.

Thippeswamy S, Girigowda K, Mulimani VH. 2006. Isolation and identification of alpha-amylase producing Bacillus sp. from dhal industry waste. Indian J. Biochem. Bio., 43(5):295-8.

Unakal C, Kallur RI, Kallur BB. 2012. Production of α-amylase using banana waste by *Bacillus subtilis* under solid state fermentation. Euro. J. Exp. Bio., 2 (4):1044-1052.

Vyas P, Shirsat M. 2016. Biotechnological aspects of α-amylase production from *Bacillus subtilis* and *Bacillus licheniformis* - A Review. ARJMCS, 2(7): 1 – 6.