Microbiology Research Journal International

27(3): 1-7, 2019; Article no.MRJI.24572
ISSN: 2456-7043
(Past name: British Microbiology Research Journal, Past ISSN: 2231-0886, NLM ID: 101608140)

Effect of Agitation Speed and Incubation Time on Amylase Production by *Bacillus* Species Isolated from Malted and Fermented Maize (*Zea mays*)

Effiom Henshaw[1*] and Sherifat M. Wakil[2]

[1]Bioremediation and Ecology Research Group, Department of Microbiology, Faculty of Biological Sciences, University of Calabar, Nigeria.
[2]Department of Microbiology, University of Ibadan, Nigeria.

Authors' contributions

This work was carried out in collaboration between both authors. Both authors were equally contributed to design, write and revise the manuscript. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/MRJI/2019/v27i330097
Editor(s):
(1) Raúl Rodríguez-Herrera, Professor of Genetics and Food Biotechnology School of Chemistry, Universidad Autónoma de Coahuila, México.
Reviewers:
(1) Chen-Chin Chang, University of Kang Ning, Taiwan (ROC).
(2) Marcela Bianchessi da Cunha Santino, Universidade Federal de São Carlos, Brazil.
(3) Olaolu Oyedeji, Obafemi Awolowo University, Nigeria.
(4) Elif Demirkan, Uludag University, Turkey.
Complete Peer review History: http://www.sdiarticle3.com/review-history/24572

Original Research Article

Received 14 March 2016
Accepted 25 August 2016
Published 05 April 2019

ABSTRACT

Aims: The effect of agitation speed and incubation time on *Bacillus* species growth and amylase production isolated from malted and fermented maize were investigated.
Study Design: An experimental study.
Methodology: *Bacillus* species were screened for amylase production using starch hydrolysis method on starch agar. *Bacillus* sp.IBM21 was observed to exhibit highest hydrolytic activity with zone of clearance of diameter 41.6mm. Process parameters optimizations were evaluated using submerge fermentation techniques.
Results: The effect of agitation speed on growth and amylase production was varied from 100-250 rpm. It was observed that, the amylolytic *Bacillus* species were able to grow optimally at agitation speed of 100 rpm while their highest amylase activities were recorded at speed of 150 rpm for

**Corresponding author: E-mail: henzefilo@yahoo.co.uk;*

Bacillus sp.IBM22, 10.16Um^{-1} and 200 rpm for *Bacillus* sp.IBM21, 5.59Um^{-1}. The effect of incubation time was varied from 12-72h, amylase activities as well as bacterial growth increases with increase in incubation time until peaks were attained at 36h (10.16Uml^{-1}, OD_{540nm}, 2.98) for *Bacillus* sp.IBM22 and 48h (5.5Uml^{-1}, OD_{540nm} 1.55) for *Bacillus* sp.IBM21.

Conclusion: The results of this study indicate that *Bacillus* species isolated from malted/fermented maize produced amylase maximally at optimal agitation speed and incubation time ranges of 150-200rpm and 36-48h respectively.

Keywords: Agitation; incubation time; amylase production; Bacillus species; malted and fermented.

1. INTRODUCTION

Amylase is among the most important enzymes in present-day biotechnology. Although amylase can be derived from several sources; including plants, animals and microorganisms, microbial enzymes generally meet industrial demands. Currently, a large number of microbial amylase are available commercially and they have almost completely replaced the chemical hydrolyses of starch in the starch processing industry [1]. Starch is a major source of carbohydrate in nature, and an abundant carbon source. The annual sale of α -amylase in the global market is estimated to be $ 11million [2]. In spite of the wide distribution of amylase, microbial sources, namely fungal and bacterial amylases are used for industrial production due to advantages such as cost effectiveness, production with ease of process modification and optimization [3]. Microbes are easy to manipulate to obtain enzymes of desired characteristic. Many microorganisms produce amylase but the most commonly used organisms for industrial production of amylase are *Bacillus licheniformis, Bacillus amyloliquifaciens*.

Incubation time and agitation speeds affect the rate of amylase production and growth of amylolytic microorganisms [4]. Incubation time is governed by the characteristics of the culture and is based on growth rate and enzyme production.

Agitation intensity influences the mixing and oxygen transfer rate during fermentation especially when using submerged technique, there by resulting in the distribution of nutrient and air evenly in the culture medium which may enhanced microorganism metabolic activities culminating in the production of primary metabolic such as amylase. Fermented foods of cereal origin are widely consumed in south and western Africa countries as traditional food stuffs [5]. Maize is the principal cereal produced in sub-Saharan Africa with an annual production currently at approximately 1.4 million metric tons [6]. Considerable work has been carried out on amylase production [7,8,9,10,11,12]. However information on the use of amylolytic bacterial strains isolated from malted and fermented maize in the study of the effect of agitation speed along with incubation time on amylase production and growth of microorganism is scanty.

2. MATERIALS AND METHODS

2.1 Sample Collection and Processing

Maize (*Zea mays*) used for this study was purchased from Ojoo market, Ibadan, Nigeria in a clean sterile polythene bag and brought to the laboratory. The grains were kept in the refrigerator until use. The maize grains were picked cleaned separately and 150g each were weighed. The weighed grains were washed with distilled water, and then steeped in 500ml sterile distilled water in a cleaned sterile Erlenmeyer flask for 24 hours at 30±2°C. After 24 hours, the water was drained and the soaked grains were spread on a sterile jute bag for germination at 30±2°C for 48 hours and wetted twice daily.

2.2 Fermentation and Isolation of Microorganism from Samples

Rootlets from malted maize was removed, wet milled with 300ml sterile distilled water using a sterilized blender (sterilize with 70% ethanol). The milled samples were kept in sterile conical flasks covered with clean aluminum foil to allow spontaneous fermentation for 72 hours at 30±2°C. Samplings were carried out at 12 hourly intervals (12h, 24h, 36h, 48h, 60h, and 72h) for microbiological analysis. At 12 hours interval, 10ml aliquots of the fermenting slurry were drawn under aseptic conditions mixed thoroughly with 90ml sterile distilled water and used for the isolation of microorganism by pour-plate method [8].

2.3 Isolation Medium

Nutrient agar (NA) (Oxoid) (Difco, USA) was used for bacterial isolation.

2.4 Screening for Amylase-producing *Bacillus* Species

Bacilli isolates were subjected to amylase screening using the method of [5,9].

2.5 Identification of Amylolytic *Bacillus* Species

Pure cultures of bacterial isolates were identified with reference to Bergey's manual of determinative bacteriology [9].

2.6 Amylase Production by Amylolytic *Bacillus* Species

2.6.1 Inoculum preparation

Twenty milliliters of basal medium containing soluble starch, 6g; peptone, 6g; $MgSO_4.7H_2O$, 0.5g; H_2HPO_4, 2g; Na_2HPO_4, 5g; NaCl, 2g; $(NH_4)_2SO_4$, 4g; $FeCl_3$.$6H_2O$, 0.05g; $CaCl_2.2H_2O$, 0.05g; distilled water 1000ml, pH adjusted to 6.6 was transferred to each 150ml cotton plugged conical flasks. Flasks were sterilized in an autoclave at 121°C for 15min, cooled and then inoculated aseptically with a loop-full of bacteria culture. The flasks were subsequently rotated in an orbital incubator (Model: 10X400.XX2.C, Sanyo Gallenkamp PLC, UK) at 200 r.p.m at 37°C for 18h.

2.6.2 Basal medium for amylase production

The medium used consisted of soluble starch, 6g; peptone, 6g; Mg $SO_4.7H_2O$, 0.5g; K_2HPO_4, 2g; Na_2HPO_4, 5g; NaCl, 2g; $(NH_4)_2SO_4$, 4g; $Fecl_3$.$6H_2O$, 0.05g; $CaCl_2.2H_2O$, 0.05g; distilled water 1000ml, pH adjusted to 6.6 [4].

2.6.3 Optimization of amylase production conditions

Amylase production was optimized with two parameters (agitation speed and incubation time).

2.6.4 Effect of agitation speed on growth and amylase production

The effect of agitation speed on growth and amylase production by amylolytic *Bacillus* species were determined using the medium as stated above [4]. Thirty milliliters (30ml) of the basal medium was dispensed into 100ml conical flasks, autoclaved at 121°C for 15mins cooled and inoculated aseptically with 2ml of amylolytic isolate inoculums (bacterial culture from the stock grown in the basal medium 18h prior to the experiment) using a sterile pipette. Flasks (in triplicate for each isolate) were incubated at 37°C for 72h in an orbital incubator (Model: 10X400.XX2.C, Sanyo Gallenkamp PLC, UK) at agitation speed of 100 rpm. At 12 hour interval, flasks (in triplicate for each isolate) were removed, and growth (O.D) measurement was determined at 610nm using Perkin-Elmer lambda UV/VIS spectrophotometer. Subsequent agitation were carried out at 150, 200, and 250 rpm respectively and at each agitation speed growth (O.D) were determined following the same procedure as mentioned above.

For extracellular amylase activities assay, the culture fluid was centrifuged using a refrigerated high speed ultra-centrifuge at 10,000xg (4°C) for 20mins. Thereafter, the filtrates (supernatants) were assayed for extracellular amylase using the DNSA reagent method [5]. The boiled culture filtrate served as control. A standard curve was prepared with 0.1- 1.0mg of glucose. The calibration curve so established was used to convert the spectrophotometer values to glucose equivalents. Amylase activity was calculated using the formula [8].

> Enzyme activity = [(mg/ml of glucose released x 0.36) / (Volume of enzyme taken x incubation time)]

2.6.5 Effect of incubation time on growth and amylase production

The effect of incubation time on growth and amylase production by amylolytic microorganism was determined using the medium as stated above. The same procedures as described above were followed and flasks (in triplicate for each isolate) were incubated at 37°C at different agitation rate as mentioned above. Here the effect of incubation time on growth and amylase production by amylolytic organism were evaluated at 12h, 24h, 36h, 48h, 60h and 72h respectively. At the end of incubation, flasks (in triplicate for each isolate) were removed, growth (O.D) measurement was determined at 610nm using Perkin-Elmer lambda UV/VIS spectrophotometer. The culture fluid was centrifuged using a refrigerated high speed ultra-centrifuge at 10,000 X g (4°C) for 20mins.

Thereafter the filtrate was assayed for extracellular amylase using DNSA reagents method [5].

3. RESULTS AND DISCUSSION

The amylolytic *Bacillus* species isolated following days of fermentation of malted maize are *Bacillus* sp.IBM21 and *Bacillus* sp.IBM22. Process parameters were optimized with agitation speed varying from 100-250 rpm to evaluate their effect on growth and amylase activity by amylolytic strains as shown on Figs. 1, 2, 3 and 4. In general, it was observed that *Bacillus* sp.IBM22 exhibited highest amylase activity (10.16 Uml^{-1}) at 150 rpm and *Bacillus* sp.IBM21 at 200 rpm (5.42 Uml^{-1}). Both strains grew optimally at speed of 100 rpm (OD_{540nm}, 1.55 for *Bacillus* sp.IBM21 and 2.98 for *Bacillus* sp.IBM22), and was observed as the best agitation speed for optimum growth. There was a gradual accumulation of amylase by strains with increase in agitation speed from 100 rpm with peaks attained at 150 and 200 rpm for *Bacillus* sp.IBM22 and *Bacillus* sp.IBM21 respectively, after which a decrease in amylase activity was recorded. Other observations for varied speed; 100 rpm, 250 rpm and their corresponding amylase activities and growth are: 100 rpm; 0.81Uml^{-1}, OD_{540nm}2.98 for *Bacillus* sp.IBM22 and 0.84Uml^{-1}, OD_{540nm}1.55 for *Bacillus* sp.IBM21, 250rpm; 2.07Uml^{-1}, OD_{540nm} 2.02 for *Bacillus* sp.IBM22 and 1.69Uml^{-1} ,OD_{540nm} 0.34 for *Bacillus* sp.IBM21. The results of maximum amylase activity at speed of 150 rpm by *Bacillus* sp.IBM22 is in harmony with earlier reports by [10], who noticed that highest α-amylase production was obtained at speed of 150 rpm by *B. amyloliquefaciens*. Similar observations were reported by [12] that maximum enzyme production by *B. amyloliquefaciens*, *Bacillus sp.* and *B. subtilis* were at speed of 160, 170 and 180 rpm respectively. Further increase in speed beyond optimum (150 rpm for *Bacillus* sp.IBM22 and 200 rpm for *Bacillus* sp.IBM21 showed decrease in amylase activities. This may be due to the fact that higher speed results in mechanical and oxidative stress disruption and physiological disturbance of cells [13]. Also at high speed excessive aeration could lead to cell lyses and increase permeability [14].

Growth of species were also monitored at the above stated conditions and they were observed to grow optimally at 100 rpm after which further increase in speed led to a decrease in growth. This finding was in harmony with the report of [2] who reported that amylase production by thermophilic *Bacillus sphaericus* increase along with growth of the organism.

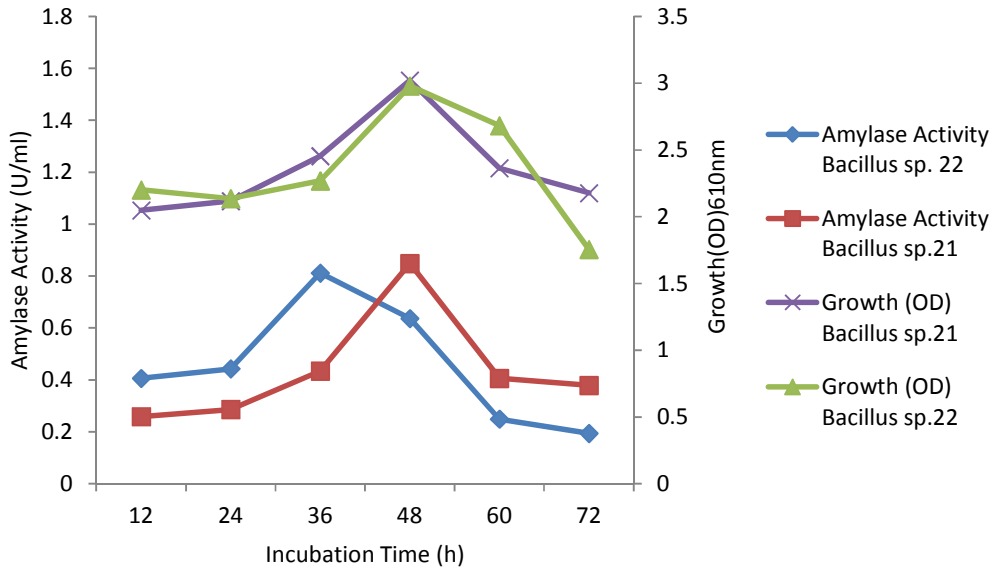

Fig. 1. Bacterial growth and amylase activity of *Bacillus* sp.22 and *Bacillus* sp.21 at agitation speed of 100 rpm

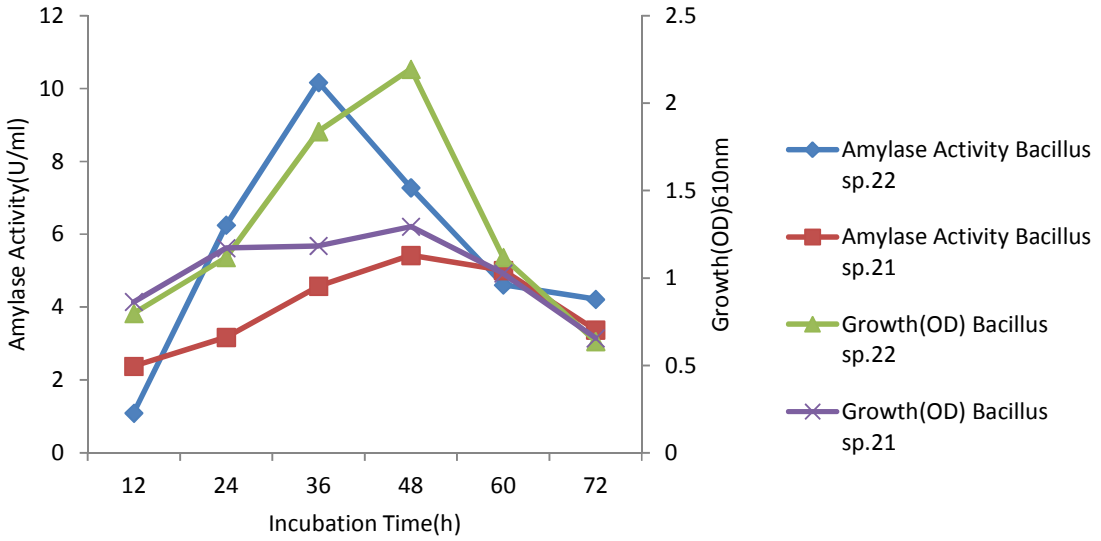

Fig. 2. Bacterial growth and amylase activity of *Bacillus* sp.22 and *Bacillus* sp.21 at agitation speed of 150 rpm

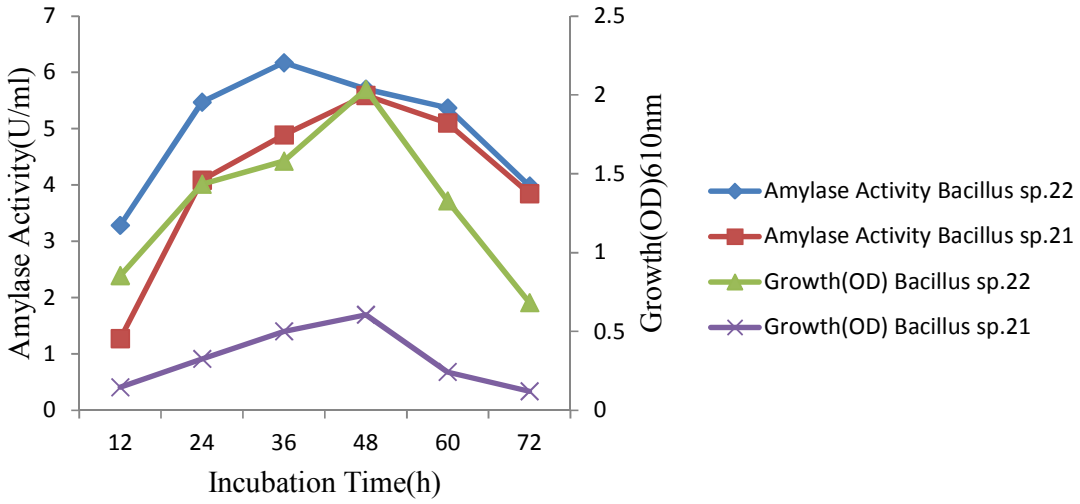

Fig. 3. Bacterial growth and amylase activity of *Bacillus* sp.22, *Bacillus* sp.21 at agitation speed of 200 rpm

Results of the effect of incubation time on growth and amylase production showed that, these species had optimal growth and maximum activities at range of 36-48h of incubation time (Figs. 1, 2, 3 and 4), which happen to be the stationary phase of organisms' growth cycle where metabolites such as antibiotics, enzymes are produced. This observations agrees with earlier reports that maximum amylase activities and growth optimum for *Bacillus* spp was between the range of 36-48h of incubation time for *B. subtilis* [3]; *B. amyloliquefacien* [4]; *B. thermooleovorans* [11]; *B. subtilis* GCUCM-25 [15]; *B. subtilis* [13] and *Bacillus licheniformis* TSI-14 [16]. On the contrary, Bozic et al. [17] reported that maximum amylase production by *Bacillus* sp. R2 was observed at 72h of incubation. It was also observed that growth of these strains increases along with amylase activities at the above stated incubation time. Devi and Khaund [6] had earlier reported that growth of *B. cereus* and *B. subtilis* increase

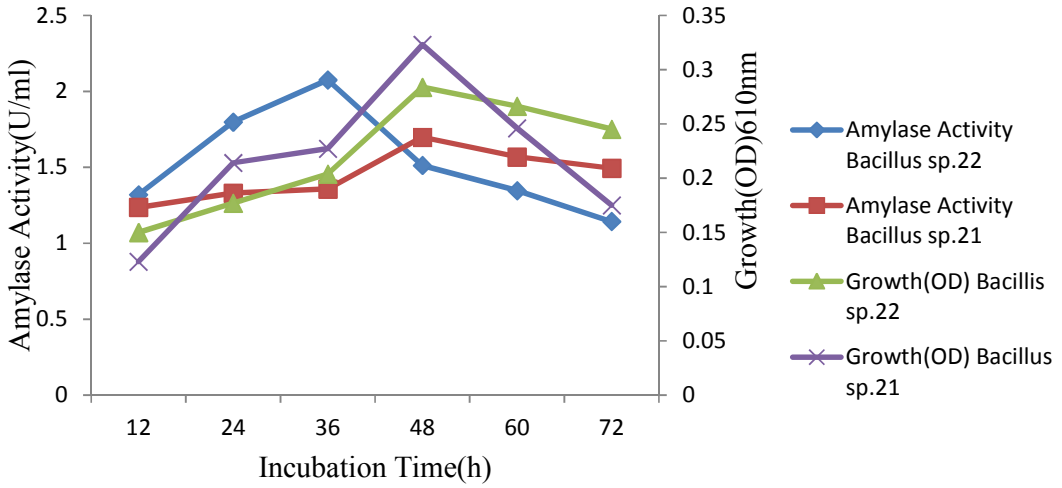

Fig. 4. Bacterial growth and amylase activity of *Bacillus* sp.22 and *Bacillus* sp.21 at agitation speed of 250 rpm

along with enzyme production. Further increase in incubation time beyond that above stated range resulted in reduced growth and amylase activities. This may be due to that fact that after maximum production of amylase, the production of other by products and depletion of nutrients set in [7]. It may also be due to the denaturation or proteolysis of enzymes because of interaction with other compounds in the fermentation medium [10,14].

4. CONCLUSION

Observations recorded in this study indicate that *Bacillus* species isolated from malted and fermented maize produced amylase with peaks at agitation speed of 150-200rpm and incubation time of 36-48h.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. Bergeys manual of determinative bacteriology. Ninth edition. Lippincott Williams, Wilkins, Philadelphia, USA; 2000.
2. Nusrat A, Rahman SR. Comparative studies on the production of extracellular alpha-amylase by three mesophilic *Bacillus* isolates. Bangladesh Journal of Microbiology. 2007;24:192-132.
3. Baig MA, Pazlarova J, Votruba J. Kinetics of α-amylase production in a batch and fed-batch culture of *Bacillus subtilis*. Folia Microbiol. 1984;29:359-364.
4. Slimane C, Omar K, Ben C. *Bacillus* sp. R2 α-amylase production optimization: Pasta cooking water as medium of amylase production. Afri J of Biotech. 2015;14(47):3184-3189.
5. Capuccino JC, Sherman N. Microbiology. A laboratory manual, 6th edition Pearson education, Dorling Kindersley Publisher, New Delhi 2003;107.
6. Devi LS, Khaund P, Joshi SR. Thermostable alpha amylase from natural variants of *Bacillus spp*. prevalent in eastern Himalayan range. Afri J of Microbiol Res. 2010;4(23):2534-2542.
7. Riaz N, Ikram-ul-Haq N, Qadar MA. Characterizat ion of α - amylase by *Bacillus subtilis*. Int J of Agric and Biol 2003;5(3):249-252.
8. Haq I, Ali S, Javed MM, Hammed U, Saleem A, Adrain F, Qadeer A. Production of α-amylase from randomly induced mutant strain of *Bacillus amyloliquefaciens* and its application as a desizer in textile industry. Pak J of Bot. 2010;42(1):473-484.
9. Abdullah R, Haq I, Javid M. Optimization of cultural conditions for the production of α-amylase by wild and mutant strains of *Aspergillus oryzae* in stirred fermenter. Pak J of Bot. 2011;43(1):715-723.

10. Krishna C, Chandrasekaran M. Banana waste as substrate for α-amylase production by *Bacillus subtilis* (CTBK-106) under solid state fermentation. Appl. Microbiol. Biotechnol. 1996;46:106-11.
11. Malhotra R, Noorwez SM, Satyanarayana T. Production and partial characterization of the thermostable and calcium independent α-amylase of extreme thermophile *Bacillus thermooleovorans* NP54. Lett. Appl. Microbiol. 2000;31:378-384.
12. Al-Qodah Z, Daghstani H, Geopal P, Lafi W. Determination of Kinetic parameters of alpha-amylase producing thermophile *Bacillus sphaericus*. A J of Biotech. 2007;6(6):699-706.
13. Prabakaran G, Pugalvendhan R. Production and immobilization of alpha-amylase by using *Bacillus subtilis*. Recents Res in Sci and Tech. 2009;1(4):189-94.
14. Ramesh MV, Lonsane S. Critical importance of moisture content in amylase production by *Bacillus licheniformis* M27 in solid state fermentation. Appl. Microbiol. Biotechnol. 1990;33:501-505.
15. Duochaun L, Yijun Y, Youliang P, Chongyao S, Peijin Z, Yicum H. Purification and properties of thermostable α-amylase from thermophilic fungus *Thermomyces lanuginosus*. Acta Microbiol. Sin. 1997;37:107-117.
16. Rushit S, Satya S. Production optimization, purification and characterization of amylase from thermophilic *Bacillus licheniformis* TSI-14. Starch. 2015;67(8): 629-639.
17. Bozic N, Ruiz J, Lopez- Santin J, Vujcic Z. Production and properties of the highly efficient raw starch digesting amylase from a *Bacillus licheniformis* ATCC 9945a. Biochem Eng J. 2011;53:203-209.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle3.com/review-history/24572