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OPTIMIZATION OF ALKALOPHILIC α-AMYLASE PRODUCING *BREVIBACILLUS SP.* IN DIFFERENT MEDIA BY SUBMERGED FERMENTATION (SmF)

SURIBABU K[1*], LALITHA GOVARDHAN T[2] AND HEMALATHA KPJ[1]

1: PG Department Microbiology and Research Centre, Dr. Lankapalli Bullayya Post-graduate College, Visakhapatnam-530 013.A.P, India

2: Department Microbiology, Andhra University, Visakhapatnam-530 003.A.P, India

***Correspondence Author: E Mail: ksuribabu_sda@yahoo.com; Tel: 9440788795**

ABSTRACT

Changes in the external pH will alter the ionization of nutrient molecules thereby reduce their availability to the bacteria. As the plasma membrane relatively impermeable to protons, bacteria often adapt to environmental pH changes to survive. In the current study, α-amylase producing *Brevibacillus borostelensis* R1 was isolated from marine waters of Bay of Bengal, Visakhapatnam by applying different primary screening techniques. The secondary screening of α-amylase production of *B. borostelensis* R1was carried out at varying pH conditions using ten different media. Among ten media taken, Pikovskaya's medium proved to be maximum α-amylase production at pH 9 (2051± 0.5 U/ml) by *B. borostelensis* R1 at 37^0C. The production was absent in Yeast Extract peptone Dextrose Glucose Medium at pH 11. The α-amylase produced by *B. borostelensis* R1 was found to have many applications in starch processing, desizing of textiles, paper sizing, detergent additive, sewage treatment and effluent treatment.

Keywords: *Brevibacillus borostelensis* R1, Alpha-Amylase, pH, Media

INTRODUCTION

Sudden variations in cytoplasmic pH can harm bacteria by disrupting the plasma membrane or inhibiting the activity of enzymes and membrane transport proteins. Changes in the external pH will alter the ionization of nutrient molecules thereby reduce their availability to the bacteria. As the plasma membrane relatively impermeable to

protons, bacteria often adapt to environmental pH changes to survive. Potassium/proton and sodium/proton anti port systems may correct small variations of pH in bacteria. pH is one of the important factors that determine the growth and morphology of microorganisms as they are sensitive to the concentration of hydrogen ions present in the medium. Earlier studies have revealed that fungi require slightly acidic pH and bacteria require neutral pH for optimum growth. pH is known to affect the synthesis and secretion of α-amylase just like its stability **[1]**. The pH value also serves as a valuable indicator of the initiation, successful progression and end of enzyme synthesis **[2]**. Selection of suitable fermentation medium and initial pH is very important for the enhanced α-amylase production **[3]**.

The available data suggests that most of the bacterial α-amylases optimally catalyze a reaction at acidic range, pH 2.0-6.5 **[4-7]**, neutral range pH 6.5-7.5 **[8-11]** and alkaline range pH 7.5-11 **[12-14]**. Therefore, the present study is aimed to optimize the pH in various production media.

MATERIALS AND METHODS

Collection of the Marine Water Samples

Marine water samples were collected from coastal areas of Visakhapatnam across the Bay of Bengal, Rushikonda, Visakhapatnam, Andhra Pradesh, India. The water samples were collected from the above site in sterile BOD bottles (Borosil) and brought to the laboratory for study.

Primary Screening of α-Amylase Producing Bacteria

The collected marine water samples were diluted by serial dilution technique. The diluted samples of 10^{-4} to 10^{-6} (0.1ml) were spreaded with L-shaped glass rod by spread plate technique on the starch agar plates. After incubation at 37^0C for 24hours, the plates were flooded with Lugol solution (1% iodine in 2% potassium iodide w/v) **[15]**. The zone of hydrolysis measuring more than 11mm were selected for further screening of amylase activity.

Optimization of Temperature

Hundred ml of the all ten media: Nutrient Broth (NB), Luria Bertain Broth (LB), Clarks and Lub Medium (CL), Pikovskaya's (PK) Medium, Tendler's Non-synthetic Medium (TNS), Amylase Production Medium (APM), Soluble Starch Beef Extract Medium (SB), Soybean Casein Digest Medium (SCD), Yeast Extract peptone Dextrose Glucose Medium (YPDG) and Tryptone Glucose Beef Extract (TGB) Medium were taken in Erlenmeyer flasks. All the 10 fermentation media were prepared with different pH (2, 5, 7, 9, and 11) by adjusting the pH with NaOH and HCl.

The ingredients of ten fermentation media used for the optimization of α-amylase production were in (g/l) **NB** (NaCl 5.0, Beef extract 3.0 and Peptic digest of animal tissue 5.0), **LB** (Tryptone 10.0, Yeast extract 5.0 and NaCl 10.0), **CL** (Glucose 5.0, Peptone 5.0 and K_2HPO_4 5.0), **PK** (Glucose 10.00, $Ca_3(PO_4)_2$ 5.0, $(NH_4)_2SO_4$ 0.50, $MgSO_4.7H_2O$ 0.10, $MnSO_4.7H_2O$ 0.01, $FeSO_4$ 0.01, KCl 0.20 and Yeast extract 0.50), **TNS** (Tryptone 10.0, Yeast extract 4.0, Sodium citrate 0.5, Ammonium nitrate 1.0, K_2HPO_4 0.3, $MgSO_4$ 0.5 and Starch 2.0), **APM** (Starch 2.0, Na_2HPO_4 3.0, KH_2PO_4 6.0, NH_4Cl 1.0, $CaCl_2$ 0.15, $MgSO_4.7H_2O$ 0.25, Casein hydrolyte 0.20 and Yeast Extract 0.10), **SB** (Soluble Starch 2%, Beef Extract 1%, Yeast Extract 0.2%, $CaCl_2$ 0.02% and $MgSO_4.7H_2O$ 0.01 %), **SCD** (Pancreatic digest of casein 17.00, Soybean meal 3.00, NaCl 5.00, KH_2PO_4 2.50 and Dextrose 2.50), **YPDG** (Yeast extract 10.00, Peptone 20.00 , Glycerol 30.0ml and Dextrose 1.00), **TGB** (Tryptone 5.00, Glucose 3.0 and Beef extract 1.00). The final pH was adjusted to 7.0 with 0.1N HCl and 0.1N NaOH before autoclaving.

Submerged Fermentation (SmF)

Two percent of pure culture of *Brevibacillus borostelensis* R1 isolated from coastal waters of Bay of Bengal, Rushikonda, Visakhapatnam from pre-incubated pure strain was inoculated to each of 250ml Erlenmeyer flask (Borosil). All the ten media were incubated in rotary incubator (Remi) at 120rpm, 37^0C for 24 hours. After incubation, each sample was subjected for centrifugation at 5,000 rpm for 15 minutes at room temperature. The supernatant was collected in sterile test tubes for α-amylase assay and the pellet was discarded.

Assay of α-Amylase

Estimation of α-amylase activity was carried out according to the dinitro salicylic acid (DNS) method **[16]**. The starch substrate [0.5ml of 0.5% in 0.1M phosphate buffer (pH 6.8)] was mixed with 1% (0.2ml) NaCl in a test tube and pre incubated at 37^0C for 10 minutes. The supernatant collected from the centrifugation of the production media was used as enzyme source, 0.5ml of this was added to the reaction mixture. The reaction was terminated by the addition of 1.0 ml of 3, 5-dinitrosalicylic acid reagent [1.0 gm DNS in 0.8% NaOH, 60% Na K tartrate] after incubation at 37^0C for 15 minutes. The contents were mixed well and kept in boiling water bath for 10 minutes. Then they were cooled and diluted with 10 ml of distilled H_2O. The color developed was read at 520nm. One unit of enzyme activity was defined as the amount of enzyme that that releases one

micromole of maltose per minute under the standard assay conditions.

All the above experiments were carried out in quadrant sets and standard deviation was calculated.

RESULTS

The potent α-amylase producing *Brevibacillus borostelensis* R1 was isolated during primary screening. The *B.borostelensis* R1 was identified by colony, morphological, biochemical and 16S gene sequencing. *B.borostelensis* R1 colonies were small, rough, dull, rubbery, whitish grey, serrate, flat, irregular, translucent, sediment growth in broth and filiform growth on Nutrient agar. The cells were Gram positive, *Streptobacilli*, measuring 1.838 ± 0.123µm x 0.774 ± 0.048µm in size. The scanning electron micrograph of *B. borostelensis* R1 was shown in **Figure 1**.

The highest amylase activity was observed in different media at acidic conditions in CL at pH 2 (1703 ± 3 U/ml), at pH 5 in NB (195 ±3U/ml), LB (320U/ml), TGB (340U/ml), in neutral condition at pH 7 in LB (320U/ml), TNS (915 ±3 U/ml), APM (653 ± 3 U/ml), SCD (840 U/ml) and YPDG (338 ±3 U/ml) and at alkaline conditions at pH 9 in PK (2051± 0.5 U/ml) and SB (1378± 3 U/ml).

The lowest amylase activity was observed in different media at different pH: NB at pH 11(40U/ml), LB at pH 2 (160U/ml), CL at pH 7(800U/ml), PK at pH 11 (500U/ml), TNS at pH 2(120U/ml), APM at pH 11(190 U/ml), SB at pH 11(230U/ml), SCD at pH 9 (410U/ml), YPDG at pH 11(0 U/ml) and TGB at pH 2(140U/ml).

The range of amylase activity observed in different media at different pH (2-11): NB (40-200U/ml) was shown in **Figure 2**, LB (160-320U/ml) was shown in **Figure 3**, CL (800-1710U/ml) was shown in **Figure 4**, PK (500-2060U/ml) was shown in **Figure 5**, TNS (120-920U/ml) was shown in **Figure 6**, APM (190-660U/ml) was shown in **Figure 7**, SB (230-1380U/ml) was shown in **Figure 8**, SCD (410-840U/ml) was shown in **Figure 9**, YPDG(0-340U/ml) was shown in **Figure 10** and TGB (140-340U/ml) was shown in **Figure 11**.

DISCUSSION

The amylase produced by *Brevibacillus borostelensis* R1 at various pH conditions of 2, 4, 7, 9, and 11 in ten different media was estimated. The outcome showed that ideal production of amylase occurred in acidic conditions (pH 2) grown in CL medium, (pH 5) in NB, LB and TGB Media. In neutral conditions (pH 7) cultured in LB, TNS, APM, SCD and YPDG Media and in alkaline conditions (pH 9) in PK and SB Media. However, the production was highest in

Pikovskaya's Medium at pH- 9. In the literature survey the optimum amylase production was delineated by several authors in different media in acidic conditions (pH 4.0 - 6.5) in *Bacillus sps.***[15,17-22]** neutral conditions (pH 6.5 - 7.5) in *Bacillus sp.* **[13, 23-30]** and alkaline conditions (pH 7.5 - 11) in *Bacillus sp.* **[12-14, 31-33].**

CONCLUSION

The present studies were carried out to optimize the α-amylase production of *Brevibacillus borstelensis* R1 using ten different media. Among ten media taken, Pikovskaya's medium proved to be maximum α-amylase production at pH 9 (2051± 0.5 U/ml) by *B. borostelensis* R1 at 37^0C. The production was absent in Yeast Extract peptone Dextrose Glucose Medium at pH 11.

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REFERNCES

[1] Fogarty MW, Microbial Amylases, In; Microbial Enzymes and Biotechnology, W.M. Fogarty Applied Science Publishers Ltd., London, UK, 1983, 1-92.

[2] Gigras P, Sahai V and Gupta R, Statistical media optimization and production of ITS alpha amylase from *Aspergillus oryzae* in a bioreactor, Cur. Microbio., 45, 2002, 203-208.

[3] Hughes MN and Poole RK, Metal specification and microbial growth-the hard (and soft) facts, J. Gen. Microbiol., 137, 1991, 725-734.

[4] Mabrouk, Production of β-mannanase by *Bacillus amyloliquifacians* 10A1 cultured on potato peels, Afr. J. of Biotechnol., 7, 2008, 1123-1128.

[5] Rajagopalan G and Krishnan C, Alpha-amylase production from catabolite derepressed *Bacillus subtilis* KCC103 utilizing sugarcane bagasse hydrolysate, Bioresour. Technol., 99, 2008, 3044-3050.

[6] Schwab K, Bader J, Brokamp C, Popovic MK, Bajpai R and Berovic M, Dual feeding strategy for the production of alpha-amylase by *Bacillus caldolyticus* using complex media, N. Biotechnol., 26, 2009, 68-74.

[7] Asoodeh A, Chamani J and Lagzian M, A novel thermostable, acidophilic alpha-amylase

from a new thermophilic *Bacillus sp. Ferdowsicous* isolated from Ferdows hot mineral
spring in Iran: Purification and biochemical characterization, Int. J. Biol. Macromol., 46, 2010, 289-297.

[8] Coronado MJ, Vargas C, Hofemeister J, Ventosa A and Nieto JJ, Production and biochemical characterization of an α-amylase from the moderate halophile *Halomonas meridian*, FEMS Microb. Lett., 183, 2000, 67-71.

[9] Thaddeus C, Ezeji AW and Bahl H, Isolation, characterization and identification of *Geobacillus thermodenitrificans* HRO 10, an α-amylase and α-glusosidase production thermophile, Can. J. of Microbio., 51, 2005, 685-693.

[10] Asgher M, Javaid Asad M, Rahman SU and Legge RL, A thermostable α-amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing, J. Food Eng., 79, 2007, 950-955.

[11] Smita HP, Mnas RS, Shaktimay K, Ramesh CR and Dider M, Statistical optimization of α-amylase production from probiotic *Lactobacillus plantarum* MTCC1407 in submerged fermentation, Polish J. Microbiol., 57, 2008, 149-155.

[12] Hagihara H, Igarashi K, Hayashi Y, Endo K, Ikawa-Kitayama K, Ozaki K, Kawai S and Ito S, Novel alpha-amylase that is highly resistant to chelating reagents and chemical oxidants from the alkaliphilic *Bacillus* isolate KSM-K 38, Appl. Environ. Microbiol., 67, 2001, 1744-1750.

[13] Burhan A, Nisa U, Gökhan C, Ömer C, Ashabil A and Osman G, Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic *Bacillus* sp. Isolate ANT-6, Proc. Biochem., 38, 2003, 1397-1403.

[14] Saxena L, lyer BK and Ananthanarayan L, Three phase-partitioning as a novel method for purification of ragi (*Eleusine coracana*) bifunctional amylase /protease inhibitor, Process Biochem., 42, 2007, 491-595.

[15] Amoozegar MA, Malekzadeh F and Malik KA, Production of amylase by newly isolated moderate halophile, *Halobacillus sp.* Strain MA-2, J. Microbio. Meth., 52, 2003, 353-359.

[16] Miller GL, Use of Dinitro salicylic acid reagent for determination of reducing sugar, Analy. Chem., 31, 1959, 426 - 429.

[17] Malhotra R, Noorwez SM and Satyanarayana T, Production and partial characterization of thermostable and calcium-independent α-amylase of an extreme thermophile *Bacillus thermooleovorans* NP54, Lett. Appl. Microbiol., 31, 2000, 378-384.

[18] Deutch CE, Characterization of a salt-tolerant extracellular α-amylase from *Bacillus dipsosauri*, Lett. Appl. Microbiol., 35, 2002, 78-84.

[19] Soni SK, Kaur A and Gupta JK, A solid state fermentation based bacterial α-amylase and fungal glucoamylase system and its suitability for the hydrolysis of wheat starch, Proc. Biochem., 39, 2003, 185-192.

[20] Konsula Z and Liakopoulou-Kyriakides M, Hydrolysis of starches by the action of an α-amylase from *Bacillus subtilis*, Proc. Biochem., 39, 2004, 1745-1749.

[21] Kirani Z, Uoului UM and Arikan B, Effects of Carbon Sources and Various Chemicals on the Production of a Novel Amylase from a Thermophilic *Bacillus sp.* K-12, Turk. J. Biol., 29, 2005, 99-103.

[22] Sivaramakrishan S, Gangadharan D, Nampoothiri KM, Sccol CR and Pandey A, α-Amylases from Microbial sources-An Overview on Recent Developments, Food Technol. Biotechnol., 4, 2006, 173-184.

[23] Ramesh MV and Lonsane BK, Ability of a solid state fermentation technique tosignificantly minimise catabolite repression of amylase production by *Bacillus licheni formis* M27, Appl. Microbiol. Biotechnol., 35, 1991, 591-593.

[24] Castro GR, Garcia GF and Sineriz F, Extracellular isoamylase produced by *Bacillus circulans* MIR-137, J. Appl. Microbiol., 73, 1992, 520-523.

[25] Lealem F and Gashe BA, Amylase production by a gram positive bacterium isolated from fermenting, J. Appl. Bacterial., 77, 1994, 348-352.

[26] Syu MJ and Chen YH, A study on the α-amylase fermentation performed by *Bacillus amyloliquefaciens*, Chem. Engg. J., 65, 1997, 237-247.

[27] Castro GR, Baigori MD and Sineriz F, Studies on α-Amylase Production

by *Bacillus licheniformis* MIR-61, Acta Biotechnol., 19, 1999, 263-272.

[28] Cordeiro CAM, Martins MLL and Luciano AB, Production and properties of α-amylase from thermophilic *Bacillus sp.*, Braz. J. Microbiol., 33, 2002, 57- 61.

[29] Tanyildizi MS, Ozer D and Elibol M, Optimization of α-amylase production by *Bacillus* sp. using response surface methodology, Proc. Biochem., 40, 2005, 2291-2296.

[30] Nagarajan M, Deborah Paripuranam T and Umamaheswari S, Efficient production of Alpha–amylase from agro residues using *Bacillus subtilis*, J. Chem. Pharm. Res. 2, 2010, 442-448.

[31] Mc Tigue MA, Kelly CT, Fogarty WM and Doyle EM, Production studies on the alkaline amylases of three alkalophilic *Bacillus spp*., Biotechnol. lett., 16, 1994, 569-574.

[32] Horikoshi K, Alkaliphiles from an industrial point of view, FEMS Microbiol. Rev., 18, 1996, 259-270.

[33] Igarashi K, Hatada Y, Hagihara H, Saeki H, Takaiwa M, Uemura T, Ara K, Olali K, Kawai S, Kobayashi T and Ito S, Enzymatic properties of a novel liquefying α-amylase from an alkaliphilic *Bacillus* isolate and entire nucleotide and amino acid sequences, App. and Env. Microbio., 64, 1998, 3282–3289.

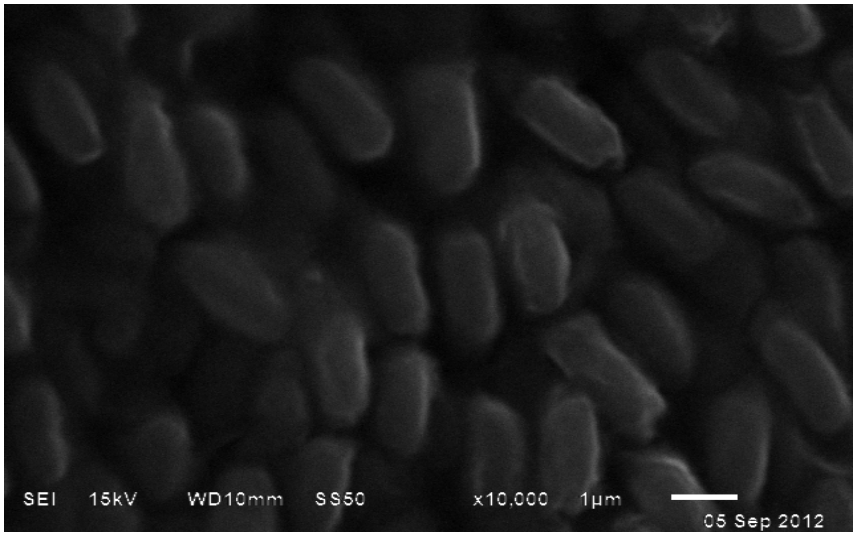

Figure 1: Scanning Electron Micrograph of *Brevibacillus borostelensis* R1

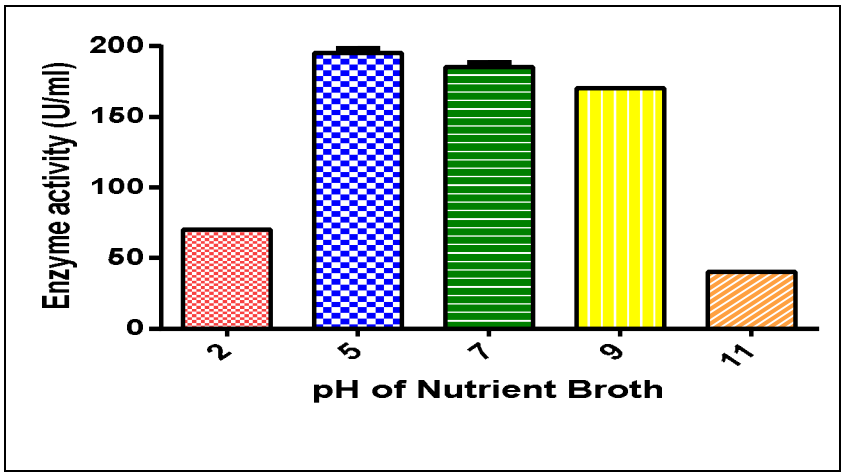

Figure 2: Effect of pH on the Production of α-Amylase by of *B. borstelensis* R1 in Nutrient Broth (NB)

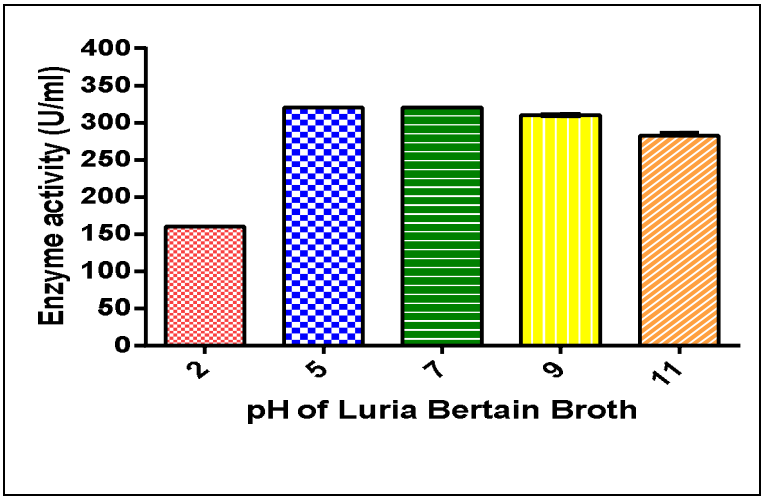

Figure 3: Effect of pH on the production of α-Amylase by of *B. borstelensis* R1 in Luria Bertain Broth (LB)

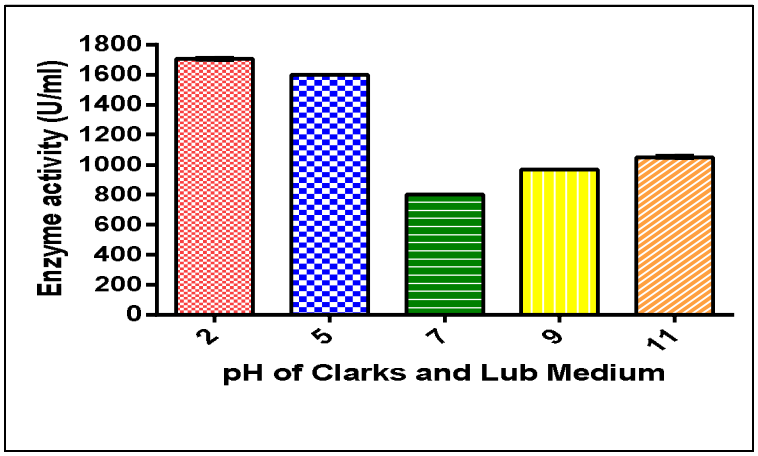

Figure 4: Effect of pH on the Production of α-amylase by of *B. borstelensis* r1 in Clarks and Lub Medium (CL)

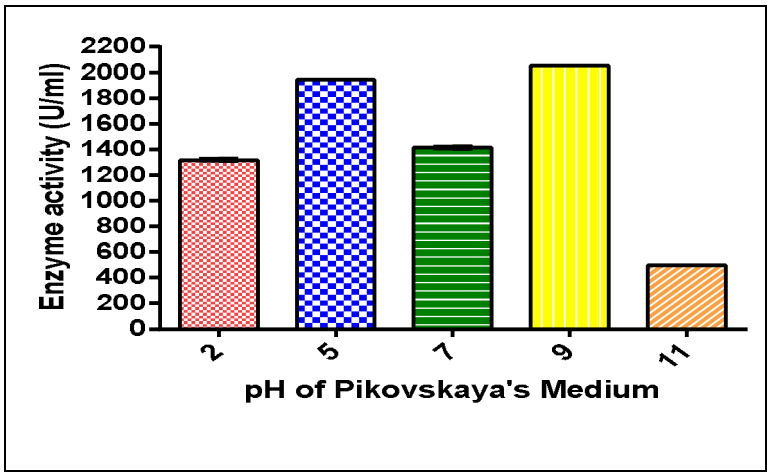

Figure 5: Effect of pH on the Production of α-Amylase by of *B. borstelensis* R1 in Pikovskaya's (PK) Medium

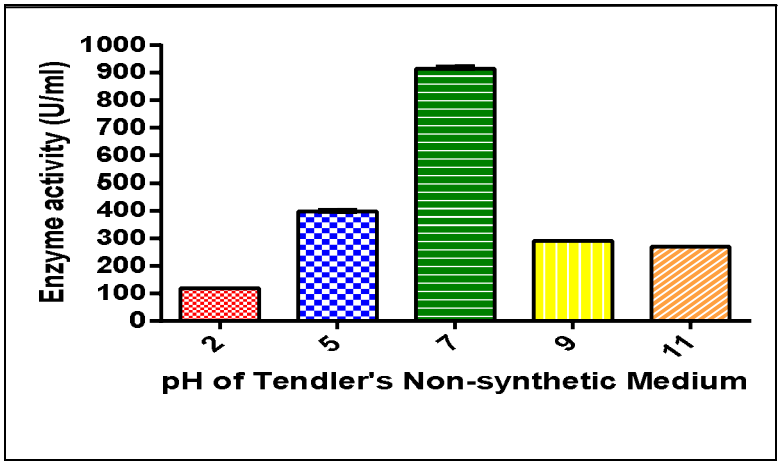

Figure 6: Effect of pH on the Production of α-Amylase by of *B. borstelensis* R1 in Tendler's Non-synthetic Medium (TNS)

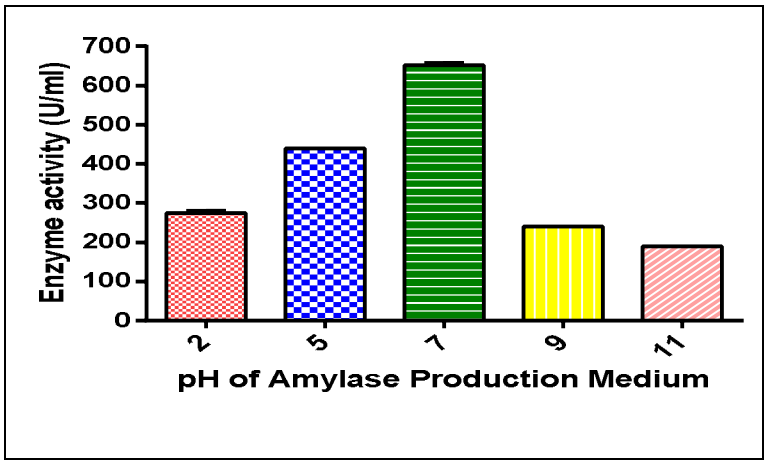

Figure 7: Effect of pH on the Production of α-Amylase by of *B. borstelensis* R1 in Amylase Production Medium (APM)

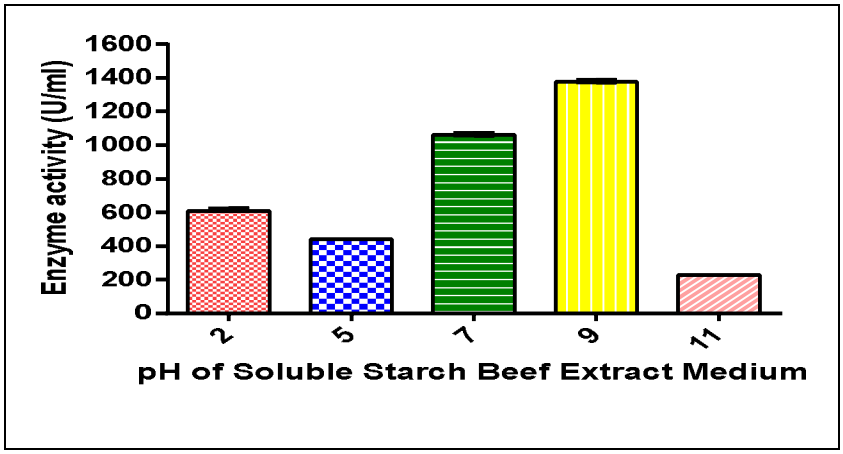

Figure 8: Effect of pH on the Production of α-Amylase by of *B. borstelensis* R1 in Soluble Starch Beef Extract Medium (SB)

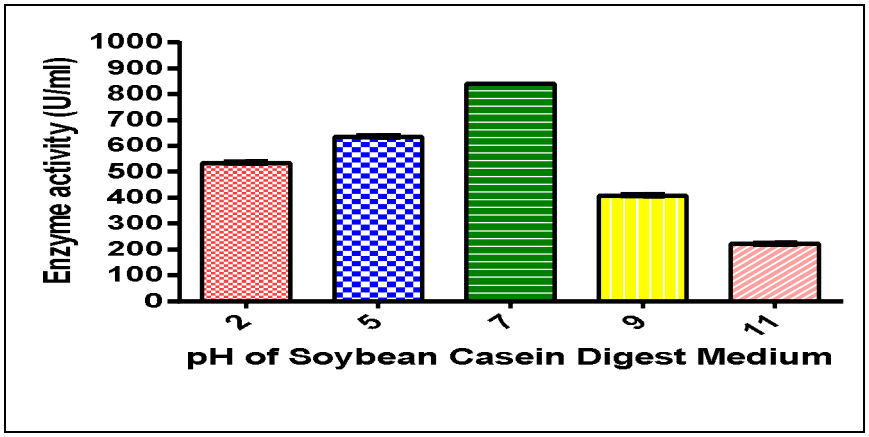

Figure 9: Effect of pH on the Production of α-Amylase by of *B. borstelensis* R1 in Soybean Casein Digest Medium (SCD)

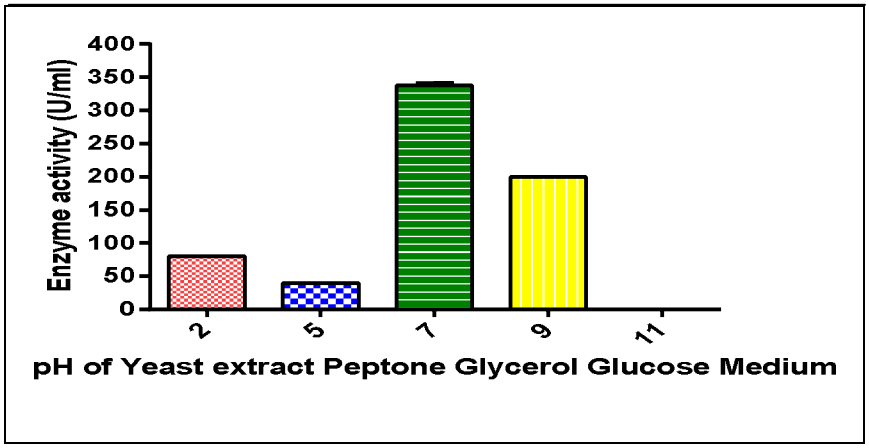

Figure 10: Effect of pH on the Production of α-Amylase by of *B. borstelensis* R1 in Yeast Extract Peptone Dextrose Glucose Medium (YPDG)

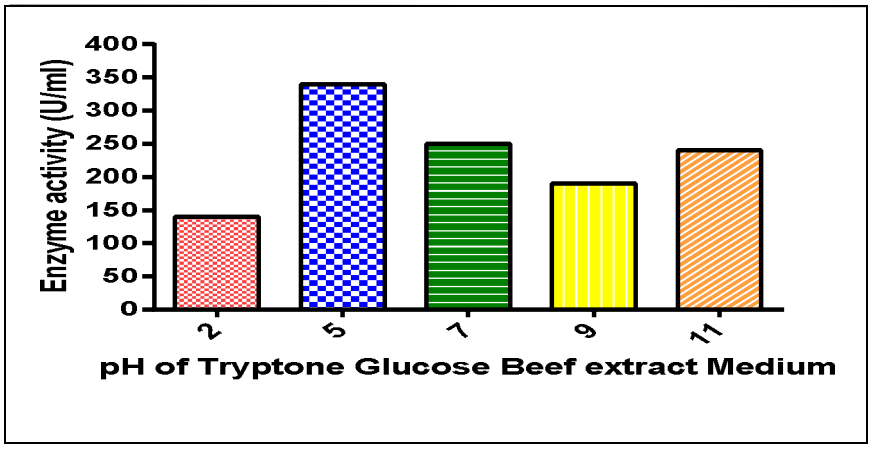

Figure 11: Effect of pH on the Production of α-Amylase by of *B. borstelensis* R1 in Tryptone Glucose Beef Extract (TGB) Medium