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Optimization of Slight Halotolerant α-Amylase Producing Brevibacillus Borstelensis R1 in Various Media by Submerged Fermentation

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Abstracts

Bacteria in marine habitat have modified structure of enzymes, ribosomes, and transport proteins which require high levels of potassium for stability and activity. In the present study, α-amylase producing *Brevibacillus borstelensis* R1 was isolated from marine waters of Bay of Bengal, Rusikonda, Visakhapatnam by applying different primary screening techniques. The secondary screening of α-amylase production of *Brevibacillus borstelensis* R1 was carried out at varying % of NaCl using ten different media. The sodium chloride response towards 1.0%, 2.0%, 3.0% and 4.0% was positive and the response was negative at 5.0% NaCl. *B.borstelensis* R1 was categorized under slight halophile which shows optimum growth at 2–5% NaCl. Alpha-amylase produced by *B.borstelensis* R1 have many applications in starch processing, desizing of textiles, paper sizing, detergent additive, bread improvement, ethanol production, sewage treatment, effluent treatment and other fermentation processes.

Keywords: *Brevibacillus borstelensis* R1, alpha-amylase, media, slight halotolerant.

Introduction

Bacteria in marine habitat have modified structure of enzymes, ribosomes, and transport proteins which require high levels of potassium for stability and activity. In addition, the plasma membrane and cell wall of *Halobacterium* are stabilized by high concentrations of sodium ion and plasma membrane literally disintegrates if it is low. Halophilic bacteria can be classified according to their salt requirement and growth pattern. Slight halophiles show optimum growth at 2–5% NaCl, moderate halophiles at 5–20% NaCl and extreme halophiles show at 20–30% NaCl respectively [4, 6, 2, 9, and 12]. Many halophiles and halotolerant microorganisms can grow over a wide range of salt concentrations, occasionally depending on environmental and nutritional factors for the growth and tolerance [15].

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 spread 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) [1]. The zone of hydrolysis measuring more than 11mm were selected for further screening of amylase activity.

Optimization of salinity:

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 Glycerol Dextrose Medium (YPGD) and Tryptone Glucose Beef Extract (TGB) Medium were taken in 250ml Erlenmeyer flasks. All the 10 fermentation media were prepared with different NaCl (0.5, 1.0, 1.5, 2.0 and 2.5 %w/v).

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**

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(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), **YPGD** (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. All ingredients of the media were procured form Himedia, India.

Submerged fermentation (SmF):
Two percent of pure culture of *Brevibacillus borstelensis* R1 isolated from coastal waters of Bay of Bengal, Visakhapatnam from pre-incubated pure strain was inoculated to each of 250ml Erlenmeyer flask (Borosil). All the ten media with different NaCl (0.5, 1.0, 1.5, 2.0 and 2.5 %w/v) were incubated in orbital shaking incubator (Remi) at 120rpm, 37^OC for 24 hours. After incubation, the sample was subjected for centrifugation at 5,000 rpm for 15 minutes at room temperature. The supernatant was collected in sterile test tubes and the pellet was discarded.

Assay of α-amylase:
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. Estimation of α-amylase activity was carried out according to the dinitro salicylic acid (DNS) method [11].

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

Sodium chloride response:
Ten ml of peptone broth of different concentration (1.0%, 2.0%, 3.0%, 4.0% and 5.0%) of NaCl was inoculated with one loopful of culture ($30x10^9$ colony forming per ml). The broth was observed for pellicle formation after incubation at 37^0C for 24 hours.

Result

The potent α-amylase producing *Brevibacillus borstelensis* R1 was isolated during primary screening. The *B.borstelensis* R1 was identified by colony, morphological, biochemical and 16S gene sequencing. *B.borstelensis* 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, *Bacilli* measuring 1.838 ± 0.123µm x 0.774 ± 0.048µm in size. The scanning electron micrograph of *B. borstelensis* R1 was shown in figure1.
The optimization of α-amylase was carried out by culturing *B. borstelensis* R1 for 24hours in ten different media at varying salinity (0.5, 1.0, 1.5, 2.0 & 2.5). The highest α-amylase activity was observed in various media at different percent of salinity; at 1.0 % in PK (2132 ±1 U/ml), SB (975 ± 0.13 U/ml) and TGB (231 ±1 U/ml), 1.5% in CL (1250 ± 0.25 U/ml) and YPGD (526 ± 1 U/ml), 2.0% in NB (240 ± 0.13 U/ml), LB (570 ± 0.125 U/ml), APM (571 ± 0.50 U/ml) and SCD (611±1 U/ml) and 2.5% in TNS (461 ± 1 U/ml). The lowest amylase activity was observed in different media at different percent of salinity: 0.5% in SCD (400 U/ml) and YPGD (105 U/ml), 1.0% in NB (125 U/ml), CL (680 U/ml) and TNS (340 U/ml), 1.5% in APM (450 U/ml), 2.0% in PK (1170 U/ml), 2.5 % in LB (310 U/ml), SB (450 U/ml) and TGB (100 U/ml). The range of α-amylase activity observed in different media at different percent of salinity: NB (125-245 U/ml) was shown in figure 2, LB (310-575 U/ml) was shown in figure 3, CL (680-1255 U/ml) was shown in figure 4, PK (1170-2135 U/ml) was shown in figure 5,TNS (340-465 U/ml) was shown in figure 6, APM (450-575 U/ml) was shown in figure 7, SB (450-976 U/ml) was shown in figure 8, SCD (400-615 U/ml) was shown in figure 9, YPGD (105-530 U/ml) was shown in figure 10 and TGB (100-235 U/ml) was shown in figure 11.

The pellicle formation in peptone broth represents the tolerance of the *Brevibacillus borstelensis* R1 to NaCl concentration. The sodium chloride response towards 1%, 2%, 3% and 4% was positive and the response was

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negative at 5% NaCl. *B.borstelensis* R1 was categorized under slight halophile which shows optimum growth at 2–5% NaCl.

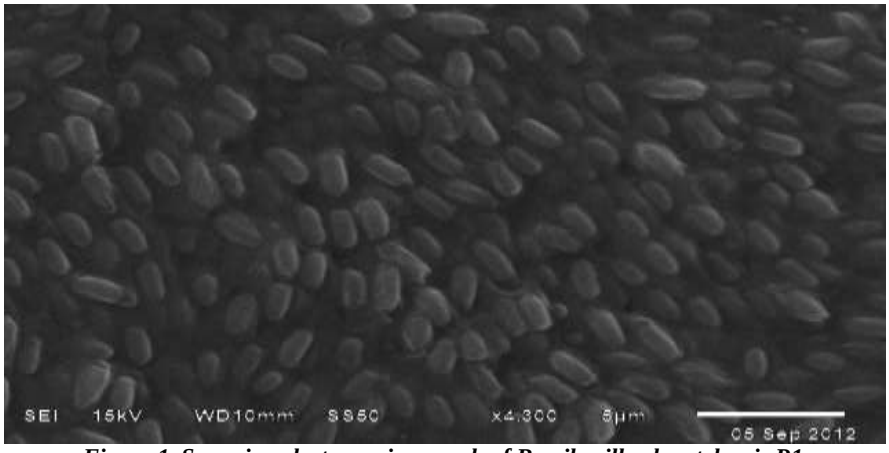

Figure 1. Scanning electron micrograph of Brevibacillus borstelensis R1

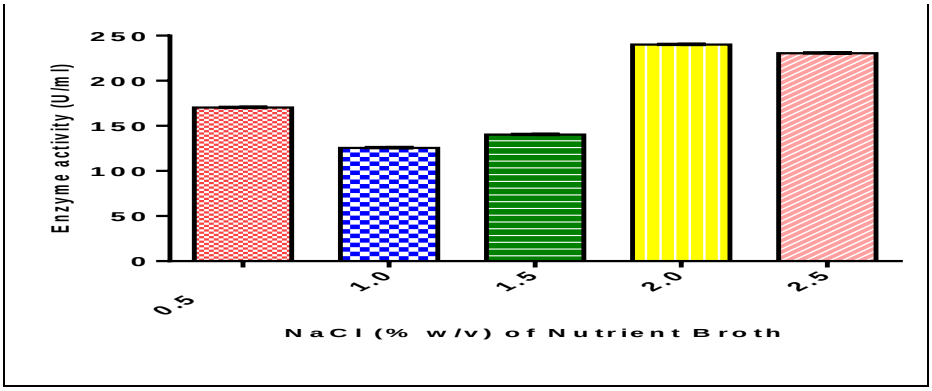

Figure 2. Effect of salinity on the production of α-amylase by B. borstelensis R1 in Nutrient Broth (NB).

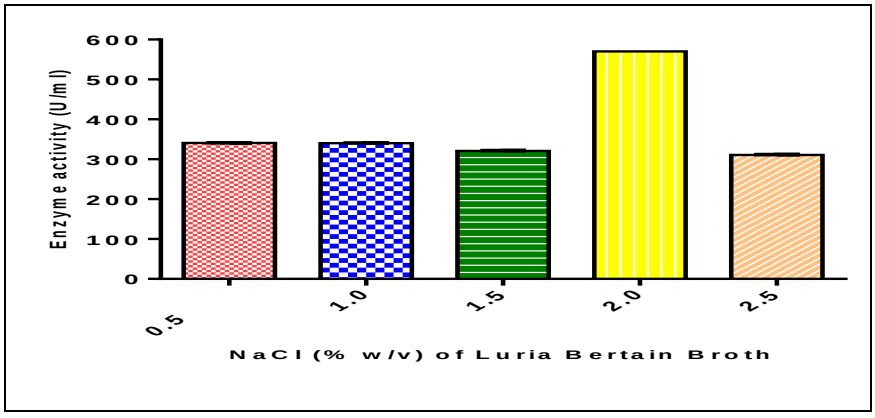

Figure 3. Effect of salinity on the production of α-amylase by B. borstelensis R1 in Luria Bertain Broth (LB).

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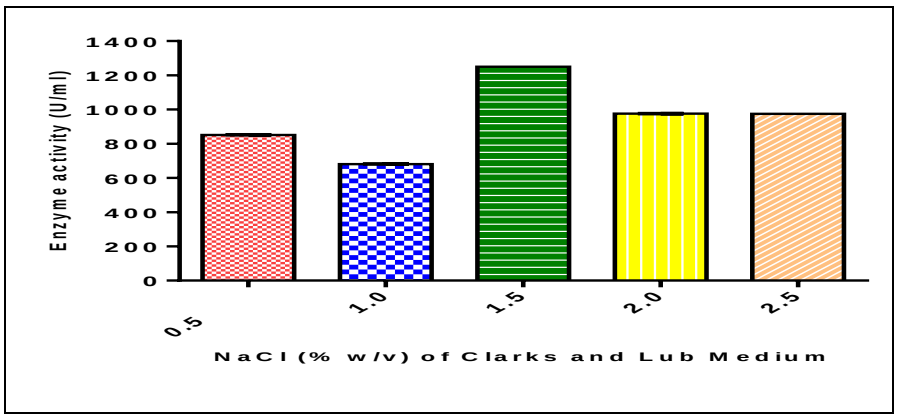

Figure 4. Effect of salinity on the production of α-amylase by B. borstelensis R1 in Clarks and Lub Medium (CL).

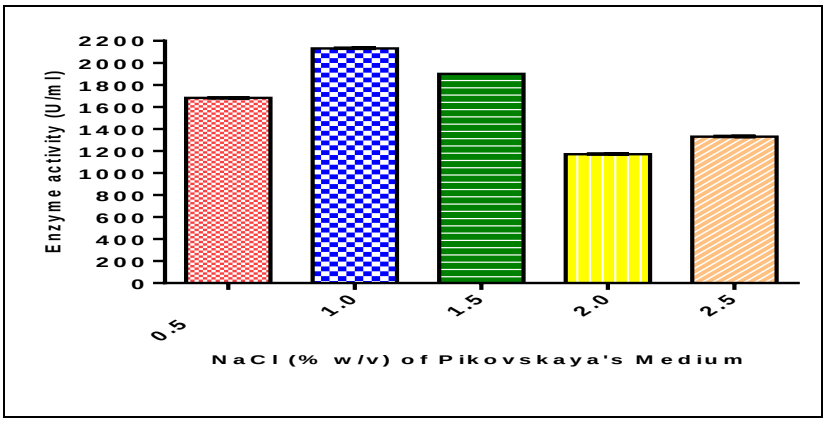

Figure 5. Effect of salinity on the production of α-amylase by B. borstelensis R1 in Pikovskaya's (PK) Medium.

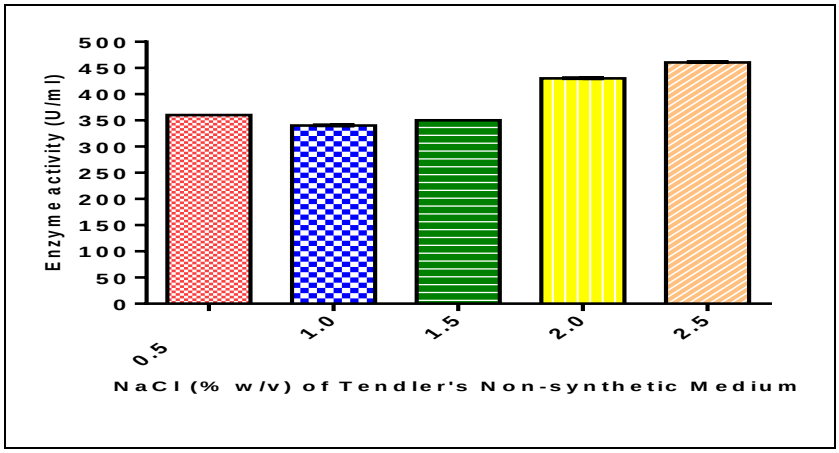

Figure 6. Effect of salinity on the production of α-amylase by B. borstelensis R1 in Tendler's Non-synthetic Medium (TNS).

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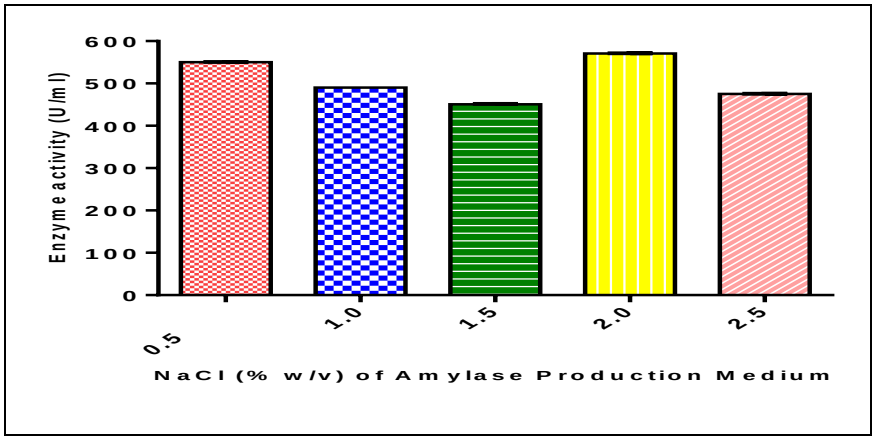

Figure 7. Effect of salinity on the production of α-amylase by B. borstelensis R1 in Amylase Production Medium (APM).

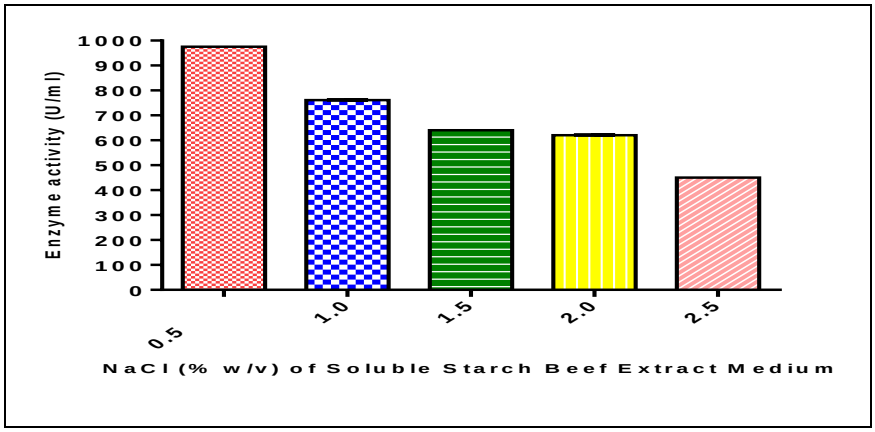

Figure 8. Effect of salinity on the production of α-amylase by B. borstelensis R1 in Soluble Starch Beef Extract Medium (SB).

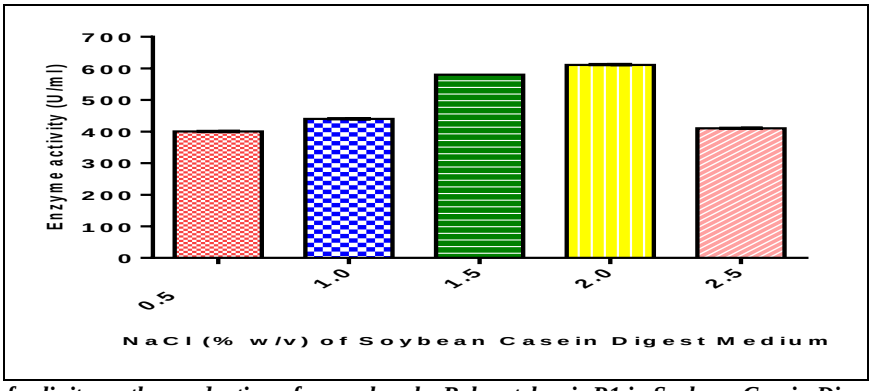

Figure 9. Effect of salinity on the production of α-amylase by B. borstelensis R1 in Soybean Casein Digest Medium (SCD).

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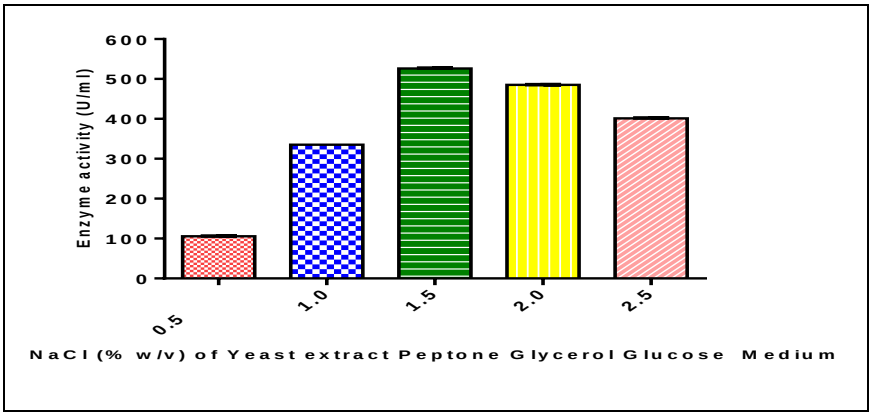

Figure 10. Effect of salinity on the production of α-amylase by B. borstelensis R1 in Yeast Extract peptone Glycerol Dextrose Medium (YPGD).

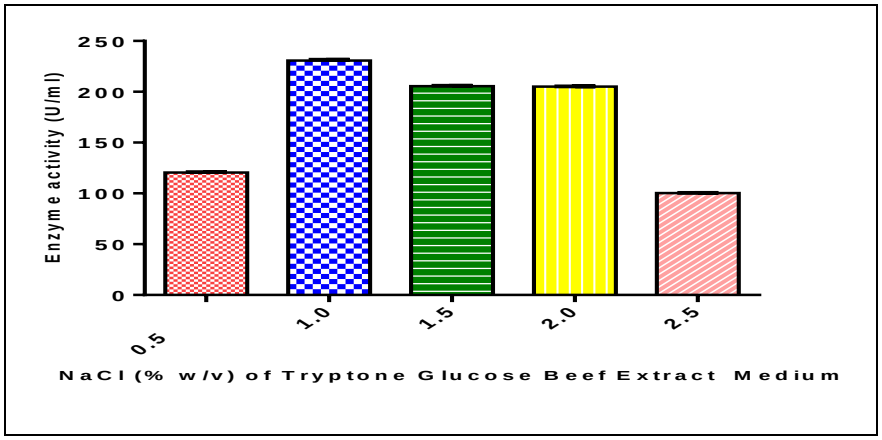

Figure 11. Effect of salinity on the production of α-amylase by B. borstelensis R1 in Tryptone Glucose Beef Extract (TGB) Medium.

Discussion

The amylase production of *Brevibacillus borstelensis* R1 was most favorable in Pikovskaya's medium with augment of 1.0% NaCl. Top production in CL and YPGD Medium with 1.5% NaCl in NB, LB, APM and SCD Media with 2.0% NaCl and TNS Medium with 2.5%NaCl but minimum production at 0.5% NaCl in all the media. The NaCl % source of metal ion was found to be a stirring affect on the production of amylase was disclosed in *Bacillus species* by Fukumoto [7], Proom & Knight [14], Jana & Patri, [10], Chandra *et al*., [5], Bernharsdotter [5], Wu *et al.*, [17], Haq *et al.*, [8] and Ashabil Aygan [2]. Parallel work with different NaCl concentration was carried out in *Bacillus sps.* at 0.1% [14, 9], 10.0% [16] and 3-15% [3]. But subduced effect was reported in *Bacillus species* [13].

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 physical parameters of pH 7.0, at 37^0C and 1.0% NaCl concentration. The minimum production was at 2.5 % in Tryptone Glucose Beef Extract Medium (100 U/ml). The sodium chloride response towards 1.0%, 2.0%, 3.0% and 4.0% was positive and the response was negative at 5.0% NaCl. *B.borostelensis* R1 was categorized under slight halophile which shows optimum growth at 2.0–5.0% NaCl.

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