

OPTIMIZATION OF PHYSICAL PARAMETERS OF α -AMYLASE PRODUCING *BREVIBACILLUS BOROSTELENIS* R1 IN SUBMERGED FERMENTATION

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Abstract

Bacteria have been regarded as treasure of many useful enzymes viz., amylases, proteases, lipases, hydrolases and reductases. Among them amylolytic enzymes have great biotechnological applications and economic exploitations. The production of α -amylases by fermentation had been thoroughly investigated and shown to be affected by a variety of physicochemical factors, such as the composition of the growth medium, the type of strain, cell growth, methods of cultivation, inoculum concentration, time of incubation, pH, temperature, salinity, carbon, nitrogen and mineral sources. The present study was carried out to optimize the α -amylase production of *Brevibacillus borstelensis* R1 using ten different media viz., Nutrient broth, Luria Bertain broth, Clarks & Lub medium, Pikovskaya's medium, Tendler's non-synthetic medium, Amylase production medium, Soluble starch beef extract medium, Soybean casein digest medium, Yeast extract peptone dextrose glucose medium and Tryptone glucose beef extract medium. Among these ten media, Pikovskaya's (PK) medium proved to be optimal for α -amylase production ($1861 \pm 17U/ml$). The optimized α -amylase production in PK medium by submerged fermentation (SmF) was subjected to varying physical parameters such as 24hrs incubation time, 2% inoculum size, $37^{\circ}C$, pH 7.0 and 1% 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: Pikovskaya's medium, incubation time, inoculum size, temperature, pH and salinity.

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1. INTRODUCTION

Screening is the detection and isolation of high-yielding species from the natural material such as soil, air and water containing heterogeneous microbial populations. Screening methods of bacteria include primary screening, secondary screening and strain improvement [1].

1.1 Primary Screening

Primary screening consists of basic elementary tests required to detect and to isolate new bacterial species exhibiting the desired property. Marine microorganisms are known to have a diverse range of enzymatic activity. Several reports of α -amylase producing bacteria include: *Streptomyces* spp. [2], *Lactobacillus* spp. [3], *Geobacillus stearothermophilus* spp. [4] and *Clostridium* spp. [5].

1.2 Measurements of Bacterial Growth

Growth is defined as an orderly increase of all cellular components, with multiplication as a consequence. Death is the irreversible loss of ability of organism to reproduce itself. The stages of growth curve are lag phase, log phase

(exponential phase), stationary phase and death phase (decline phase) [6].

There are four methods of growth measurements:

1.2.1 Direct Microscopic Count (Direct Cell Count Method)

It helps to count the total number of cells include both living and dead directly by direct microscopic counts using Petroff-hauser chamber (nine squares, each 0.1mm deep, volume of liquid over one square mm is 0.1 cubic mm).

1.2.2 Viable Cell Count Method

It is based on the principle that when material containing bacteria are cultured, every viable bacterium develops into a visible colony on a nutrient agar medium.

1.2.3 Turbidity Method

It helps to study the cloudiness of the suspension. Turbidity is directly proportional to the number of cells. It is quantified

with the spectrophotometer, which measures the amount of light transmitted directly through a sample. The cells suspended in the culture interrupt the passage of light. The amount of light absorbed through the suspension is measured as optical density.

1.2.4 Dry Weight Method

It helps to estimate the bacterial growth at regular time intervals during the incubation period. The growth of the bacteria is directly proportional to the dry weight harvested.

1.3 Physical Parameters

The physical parameters include pH, temperature and salinity which influence the production of amylase.

1.3.1 P^H

Sudden variations in cytoplasmic pH can harm bacteria by disrupting the plasma membrane, inhibiting the activity of enzymes and membrane transport proteins. pH is known to affect the synthesis, secretion and stability of α -amylase [7]. Selection of a suitable fermentation medium and initial pH is very important for the enhanced alpha-amylase production [8].

1.3.2 Temperature

High temperatures damage bacteria by denaturing enzymes, transport carriers, and other proteins. Thermostability is a desired characteristic of most of the industrial enzymes. Thermostable α -amylases were produced by mesophilic species of *Bacillus* [9]. Therefore, a high value is placed on extreme thermostability and thermoactivity of the enzymes.

1.3.3 Salinity

Bacteria in marine habitat have modified the structure of enzymes, ribosomes, and transport proteins which require high levels of potassium for stability and activity. Halophilic bacteria are 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 at 20–30% NaCl [10].

2 MATERIALS AND METHODS

2.1 Collection of the Marine Water Samples

Marine water samples were collected from Rushikonda coastal area of Visakhapatnam, Andhra Pradesh, India. The water samples were collected in sterile BOD bottles (Borosil) and brought to the lab, stored in the refrigerator until it was used.

2.2 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°C for 24 hours, the plates were flooded with Lugol solution (1% iodine in 2% potassium iodide w/v) [11]. The average cfu/ml, number of colonies forming clear halo zone of hydrolysis and zone of starch hydrolysis measured in mm.

2.3 Estimation of Amylase by DNS Method

Maltose produced by the hydrolytic activity of α -amylase on α -1, 4 linkages present in polysaccharides, reduce 3, 5 dinitro salicylate to an orange red colored 5-nitro 3-amino salicylate which can be measured at 520nm. 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°C 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°C 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 H₂O. The color developed was read at 520nm. One unit of enzyme activity was defined as the amount of enzyme that release 1.0 mmol of reducing sugar (maltose) per minute under the assay conditions [12].

2.4 Fermentation Media

Optimization of α -amylase production by *B. borostelensis* R1 was carried out by using ten submerged fermentation media; Nutrient Broth (NB), Luria Bertain Broth (LB), Clarks and Lub Medium (CL), Pikovskaya's Medium (PK), Tendler's Non-synthetic (TNS) Medium, Amylase Production Media (APM), Soluble Starch Beef Extract Medium (SB), Soybean Casein Digest Medium (SCD), Yeast Extract Peptone Dextrose Glucose Medium (YPDG) and Tryptone Glucose Beef Extract Medium (TGB) procured from Himedia, India.

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 K₂HPO₄ 5.0), **PK** (Glucose 10.00, Ca₃(PO₄)₂ 5.0, (NH₄)₂SO₄ 0.50, MgSO₄·7H₂O 0.10, MnSO₄·7H₂O 0.01, FeSO₄ 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, K₂HPO₄ 0.3, MgSO₄ 0.5 and Starch 2.0), **APM** (Starch 2.0, Na₂HPO₄ 3.0, KH₂PO₄ 6.0, NH₄Cl 1.0, CaCl₂ 0.15, MgSO₄·7H₂O 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 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 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 3.00 and Beef extract 1.00). The final pH was adjusted to 7.0 with 0.1N HCl and 0.1N NaOH before autoclaving.

2.5 Incubation Period

The growth curve of *Brevibacillus borstelensis* R1 was constructed by using direct microscopic count method, viable cell count method, turbidity method and dry weight method in Pikovskaya's Medium. The α -amylase production during growth phase (0-30hours) with an interval of 2hrs of *B. borstelensis* R1 was assayed by DNS method. The incubation period with maximum amylase production was determined during growth cycle.

2.6 Inoculum Size

Two loopful of culture was aseptically inoculated in Pikovskaya's Medium. The flask was incubated (37°C) in rotary shaking incubator at 120rpm, for 24hrs. After incubation the inoculum optical density was adjusted to 0.9 with sterile distilled water at 600nm in colorimeter. Each ml contains 10^{12} cells which were in stationary phase (α -amylase production phase) as calibrated by growth curve measurement methods.

The effect of different sizes (1% to 5%) of inoculum on the α -amylase production of *Brevibacillus borstelensis* R1 in Submerged fermentation (SmF) was investigated. The Erlenmeyer flask was incubated (37°C) in rotary shaking incubator at 120rpm, for 24hrs.

2.7 Temperature

Hundred ml of the Pikovskaya's Medium was taken in sterile erlenmeyer flasks. Inoculum size (2%) of pure culture from pre-incubated pure strain was inoculated to each of flasks. Medium was incubated in orbital shaking incubator (120rpm) at different (4°C , 25°C , 37°C , 50°C and 60°C) temperatures for 24hrs.

2.8 pH

Fermentation medium (Pikovskaya's Medium) was prepared at different pH (2.0, 5.0, 7.0, 9.0 and 11.0) by adjusting the pH with 0.1N HCl and 0.1N NaOH. Hundred ml of different pH medium were taken in sterile erlenmeyer flasks. Inoculum size (2%) from preincubated pure strain was inoculated to each of Erlenmeyer flask. The flasks were incubated in orbital shaking incubator (120rpm) at 37°C for 24hrs.

2.9 Salinity

Hundred ml of the Pikovskaya's Medium was taken in different erlenmeyer flasks. Inoculum size (2%) of pure culture from preincubated pure strain was inoculated to each Erlenmeyer flask. Each flask with different concentrations of NaCl (0.5, 1.0, 1.5, 2.0, and 2.5 % w/v) was incubated in orbital shaking incubator (120rpm) at 37°C for 24hrs.

After incubation, the samples were subjected to centrifugation at 5,000 rpm for 15 minutes at 25°C . The supernatant was collected in sterile test tubes and the pellet was discarded. Supernatant (0.5 ml) was used for the amylase assay by DNS method.

3. RESULTS

3.1 Optimization of the Media

The optimization of media for α -amylase production was carried out by inoculating *B. borstelensis* R1 for 24hrs in ten different submerged fermentation 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. The highest production of α -amylase in all media is shown in Chart -1. The optimal production of amylase ($1861 \pm 17 \text{U/ml}$) was observed when R1 strain was subjected to SmF in Pikovskaya's (PK) production media.

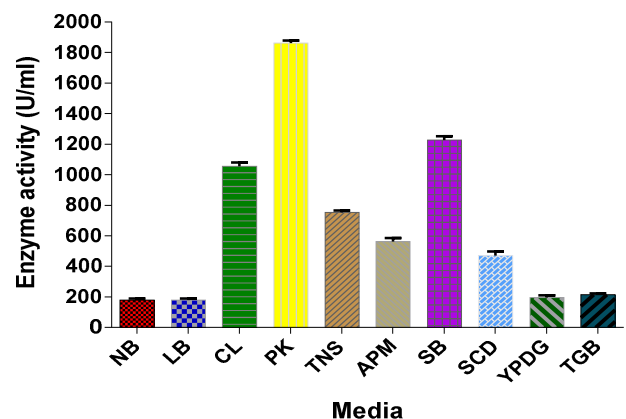


Chart -1: The α -amylase production (U/ml) in various submerged fermentation (SmF) media; Nutrient broth (NB), Luria bertain broth (LB), Clarks and lub medium (CL), Pikovskaya's medium (PK), Tendler's non-synthetic medium (TNS), Amylase production medium (APM), Soluble starch beef extract medium (SB), Soybean casein digest medium (SCD), Yeast extract peptone glycerol glucose medium (YPDG) and Tryptone glucose beef extract medium (TGB).

Y bars indicate the standard deviation of mean value.

**** P < 0.0001 Values differ significantly at p<0.05.

The optimum (1861±17U/ml) α-amylase production was observed with Pikovskaya’s Medium (PK) and the lowest production was observed (180±1U/ml) with two media- Nutrient broth and Luria bertain broth (Chart -1).

3.2 Incubation Period

Measurements of growth curve of *B. borstelensis* R1 to determine incubation period: The growth curve of *B. borstelensis* R1 at stationary phase was constant from 18-

24hours. It was measured by adopting direct microscopic count by using Neubauer chamber, 12 log cfu/ml (Chart -2a), viable cell count method, 12 cfu/ml (Chart -2b), turbidity method, 0.923±0.012 O.D at 600nm (Chart -2c) and dry weight method, 1.526667±0.003gm/10ml (Chart -2d). Distinct phases of the growth curve were observed in each method.

The Rate of Growth (R) measured by direct microscopic count, viable standard plate count method and turbidity method was 1.63. The specific growth rate (μ) was 1.65 and the generation time (G) was 0.61hrs or 36.60minutes.

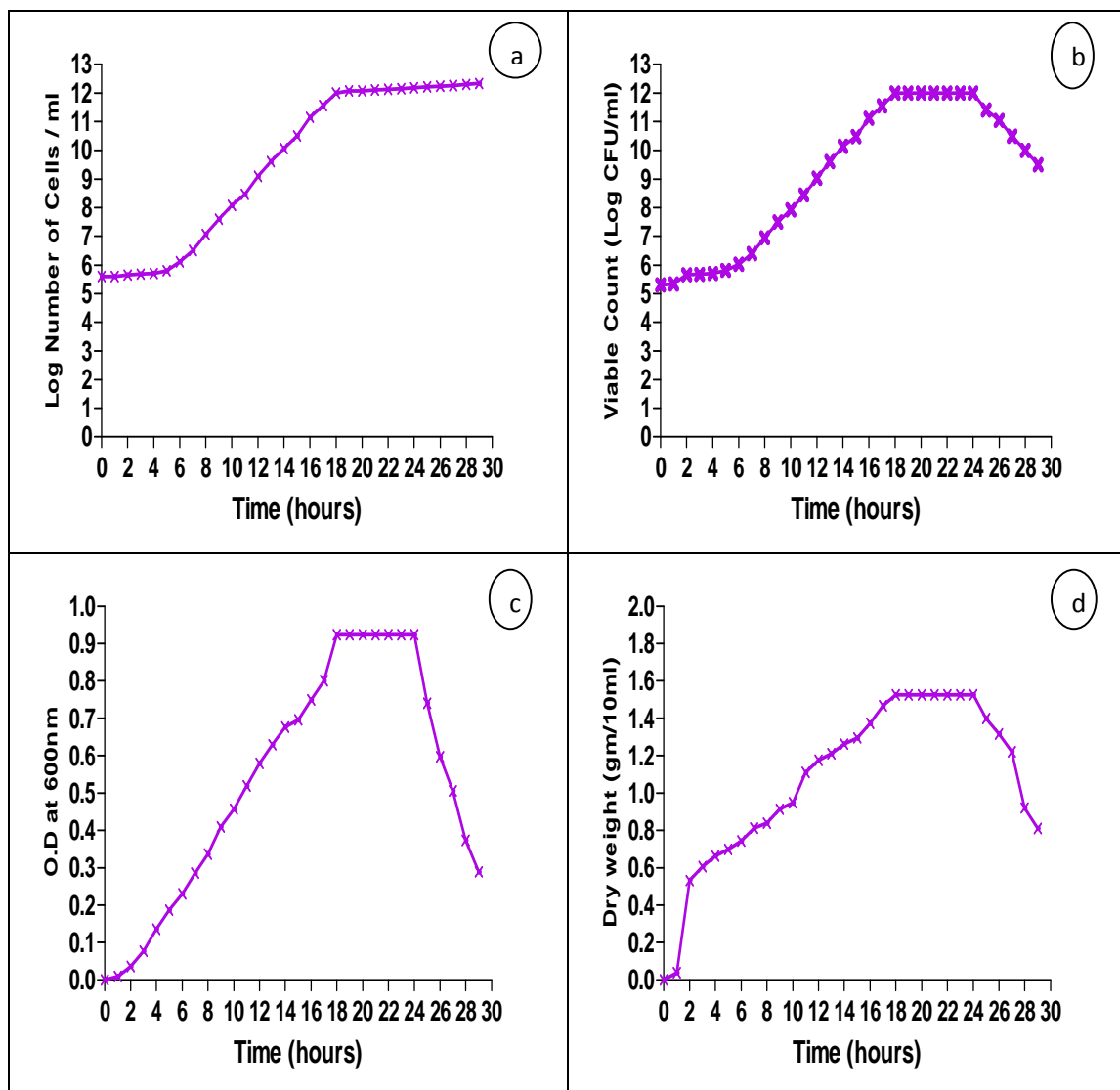


Chart -2: Growth curve of *Brevibacillus borstelensis* R1: a, Direct microscopic count method; b, Viable cell count method; c, Turbidity method and d, Dry weight method.

Y bars indicate the standard deviation of mean value.

**** P < 0.0001 Values differ significantly at p<0.05 with moderate degree of positive correlation.

The Pikovskaya's (PK) Medium was found to be the best among ten fermentation media used. The lag phase (0-2hrs),

log phase (2-18hrs), stationary phase (18-24hrs) and decline phase followed. The highest α -amylase production (1,800U/ml) was found during the stationary phase (18-24hrs) (Chart -3). Amylase production at 24hours was taken as standard time in all the following experiments.

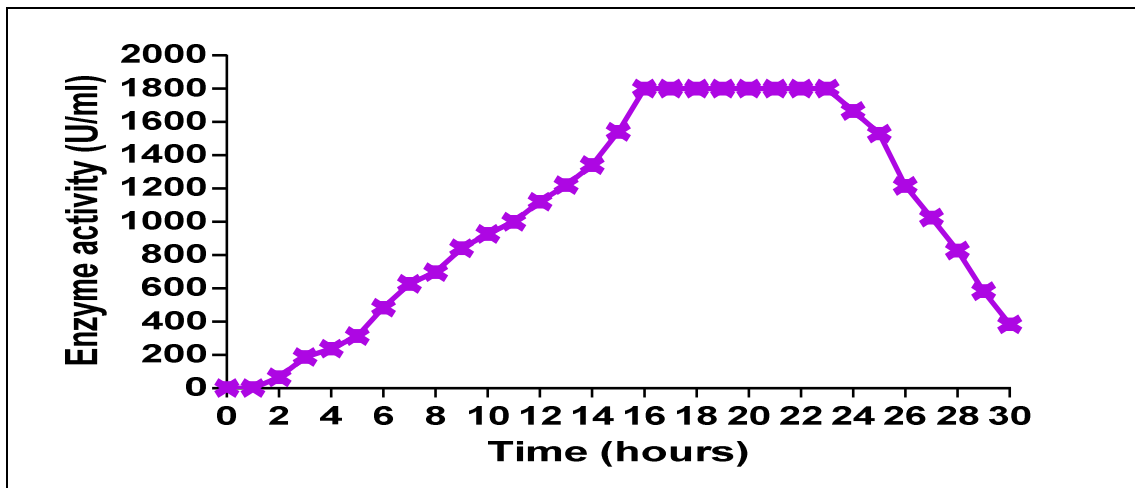


Chart -3: Effect of incubation period on α -amylase production of Brevibacillus borstelensis R1

Y bars indicate the standard deviation of mean value.

**** P < 0.0001 Values differ significantly at p<0.05 with moderate degree of positive correlation.

3.3 Inoculum size

The Pikovskaya's (PK) Medium and incubation period (24hrs) were optimized. The optimum inoculum size was optimized in PK medium. The effect of different sizes (1% to 5%) of

inoculum on the production of α -amylase by Brevibacillus borstelensis R1 using submerged fermentation (SmF) was investigated. The production of amylase was increased with the increase in the level of inoculum upto 2% (1813±23U/ml). As the level of inoculum was further increased, the productivity of amylase was decreased (Chart -4). Thus, the inoculum level of 2%v/w was found to be optimum for α -amylase synthesis.

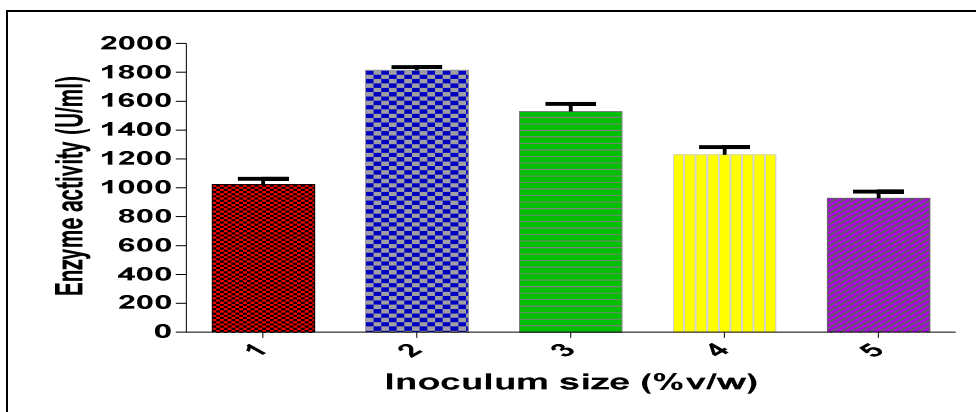


Chart -4: Effect of inoculum size on α -amylase production of Brevibacillus borstelensis R1

Y bars indicate the standard deviation of mean value.

**** P < 0.0001 Values differ significantly at p<0.05.

3.4 Temperature

The Pikovskaya's (PK) Medium, incubation period (24hrs) and 2% inoculum size were optimized. The optimization of

temperature was carried at different temperatures (4°C, 25°C, 37°C, 50°C & 60°C). The highest amylase activity was recorded at 37°C (2086±71U/ml) and the lowest at 4°C (1131±57U/ml) (Chart -5).

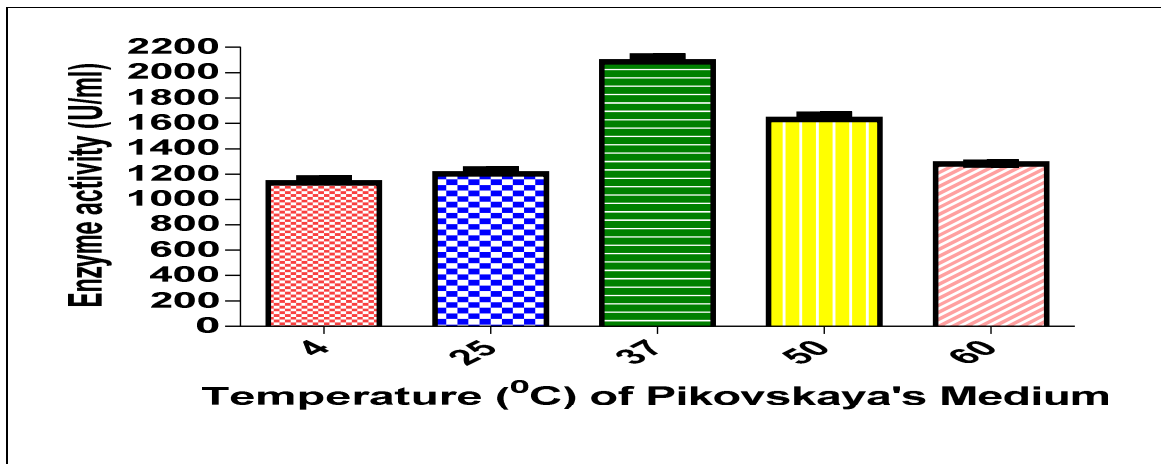


Chart -5: Effect of Temperature on α-amylase production of *Brevibacillus borstelensis* R1

Y bars indicate the standard deviation of mean value.

**** P < 0.0001 Values differ significantly at p<0.05.

3.5 pH

The Pikovskaya's (PK) Medium, incubation period (24hrs), 2% inoculum size and temperature (37°C) were optimized.

The optimization of pH was carried with different pH (2, 5, 7, 9 & 11). The pH was adjusted by using 0.1N HCl and 0.1N NaOH. The highest amylase activity was recorded at pH 7.0 (2083±56U/ml) and the lowest at pH 11.0 (565±77U/ml) (Chart -6).

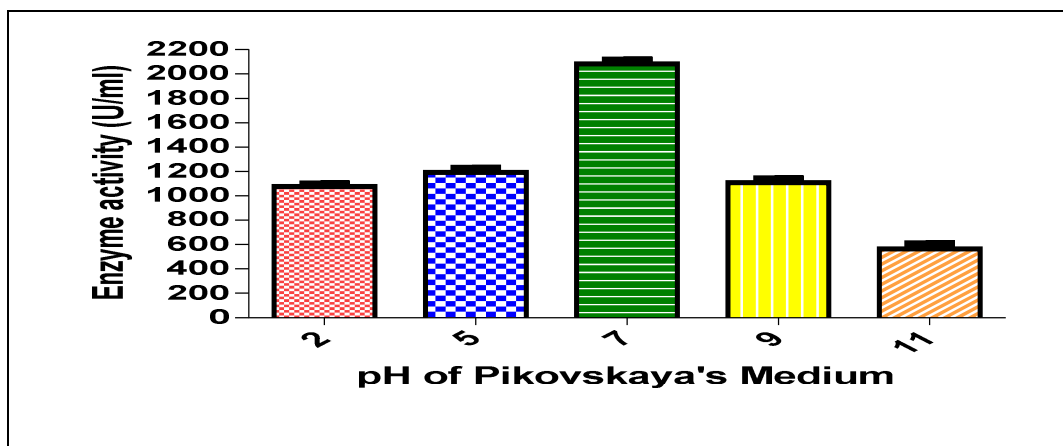


Chart -6: Effect of pH on α-amylase production of *Brevibacillus borstelensis* R1

Y bars indicate the standard deviation of mean value.

**** P < 0.0001 Values differ significantly at p<0.05.

3.6 Salinity

The Pikovskaya's (PK) Medium, incubation period (24hrs), 2% inoculum size, temperature (37⁰C) and pH (7.0) were

optimized. The optimization of salinity at different concentrations (0.5%, 1.0%, 1.5%, 2.0% & 2.5%) is depicted in Chart -7. The highest amylase activity was recorded at salinity of 1.0% (2082±58U/ml) and the lowest at 2.0% (1121±59 U/ml).

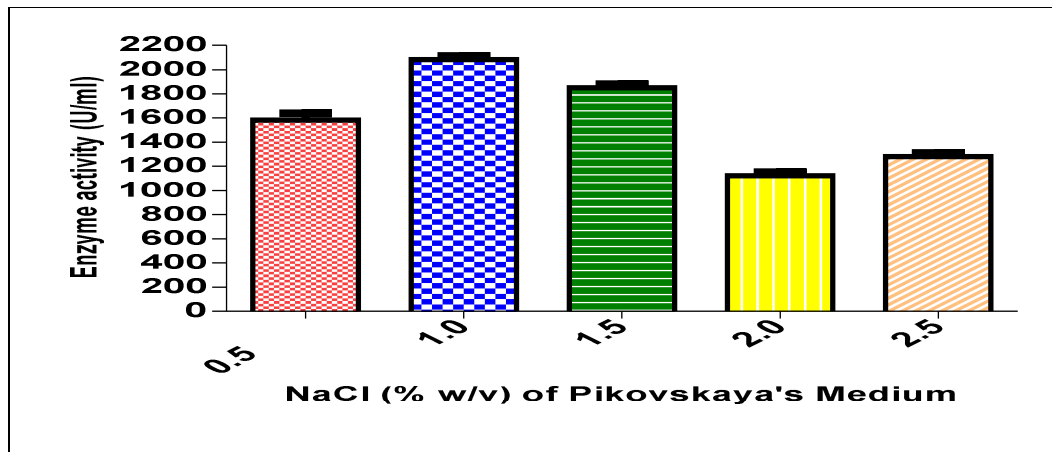


Chart -7: Effect of salinity on α -amylase production of *Brevibacillus borstelensis* R1

Y bars indicate the standard deviation of mean value.

**** P < 0.0001 Values differ significantly at p<0.05.

4. DISCUSSION

The present work was carried out in one medium selected from ten media to optimize the physical and chemical parameters. The highest α -amylase production was found in Pikovskaya's Medium (PK) and the lowest in Nutrient broth and Luria bertain broth. The highest α -amylase production (1,800U/ml) was found during the stationary phase (18-24hrs). The production of amylase was increased with the increase in the level of inoculum at 2% (1813±23U/ml). The highest amylase activity was recorded at 37⁰C (2086±71U/ml), pH 7.0 (2083±56U/ml) and at salinity 1.0% (2082±58U/ml) and the lowest at 4⁰C, pH 11.0 and at salinity 2.0%.

The amylase production after 72hours of growth (*Bacillus subtilis*) was maximum (370U/mg). The effects of incubation period, pH of the medium and incubation temperature were optimized. The maximum production of enzyme was obtained at 30⁰C and pH 7.0 [13]. The production of the enzyme by *Bacillus sp.* was maximum at 10hrs after inoculation [14]. The enzyme production largely dependent on the type of strain, composition of medium, cell growth, initial pH and thermo stability [15, 16]. Increased incubation period decreased the α -amylase production. It might be due the depletion of nutrients, accumulation of toxic byproducts in the medium, proteolysis

of α -amylase and microorganisms [17]. To obtain maximum enzyme yield, development of a suitable medium and culture conditions were obligatory [18].

Raj Devi & Yogevaran [19] reported that the maximum enzyme production obtained after 24hrs of incubation in case of *Bacillus amyloliquefaciens* and 48hrs of incubation with *Micrococcus halobius*. The production of the enzyme was maximum at 10hrs after inoculation with *Bacillus sp.* [20]. Maximum growth was shown at 48hrs and amylase activity at 24hrs [21].

In the literature survey the optimum amylase production was delineated by several authors in different media. In acidic conditions (pH 4.0 - 6.5) the amylase production was reported in *Bacillus spp.* [22]. In neutral conditions (pH 6.5 - 7.5) the amylase production was reported in *Bacillus spp.* [23]. In alkaline conditions (pH 7.5 - 11) the α -amylase production was reported in *Bacillus spp.* [24].

The production was highest in Pikovskaya's Medium at 37⁰C. The amylase production in different media was reported in psychrophilic conditions (Temp. 4⁰C) in *Nocardiopsis sp.* 7326 [25] and *Arthrobacter psychrolactophilus* [26]. The zenith amylase production of mesophilic *Bacillus spp.* (Temperature 30⁰-45⁰C) was communicated by Nagarajan *et al.* [27].

The amylase production of *Brevibacillus borostelensis* R1 was most favorable in Pikovskaya's medium with augment of 1% NaCl. The NaCl % source of metal ion was reported to have a stirring effect on the production of amylase was disclosed in *Bacillus spp.* by Ashabil Aygan [28]. Parallel work with different NaCl concentrations (0.5-1.5%) was carried out in *Bacillus spp.* [29]. But subdued effect was reported in *Bacillus sp.* [30].

CONCLUSIONS

The present study was carried out to optimize the α -amylase production of *Brevibacillus borstelensis* R1 using ten different media. Among these ten media, Pikovskaya's (PK) medium proved to be optimal for α -amylase production (1861±17U/ml).

The optimized α -amylase production in PK medium by submerged fermentation (SmF) was subjected to varying physical parameters such as 24hrs incubation time, 2% inoculum size, 37°C, pH 7.0 and 1% NaCl).

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