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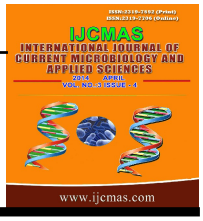



**Original Research Article**

# Optimization of various Nitrogen sources for the production of α-Amylase using *Brevibacillus borstelensis* R1 by Submerged fermentation

**K.Suribabu[1*], T.Lalitha Govardhan[1] and K.P.J Hemalatha[2]**

[1]PG Department of 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
***Corresponding author***

**Keywords**

Pikovskaya's fermentation medium; beef extract; *Brevibacillus borostelensis* R1

**A B S T R A C T**

Secondary screening provides information pertaining to the effect of different components of the medium. This is valuable in designing the medium that may be attractive as far as economic consideration is concerned. Optimization of α-amylase production was carried out by the addition of supplementary sources of nitrogen separately to Pikovskaya's fermentation medium by maintaining constant physical parameters (24hrs incubation time, 2% inoculum size, $37^0$C, pH 7.0 and 1% NaCl). The optimum production was found with 1% beef extract (3001 ± 1.0U/ml). The α-amylase production with optimized physical and chemical parameters was 3120±14U/ml. The α-amylase produced by *Brevibacillus borostelensis* R1 has a number of applications in many fields such as bakery industry, food preparations, automation dishwashing, ethyl alcohol dual fermentation, sago and rice industrial effluent treatment, sewage water treatment, fodder production, laundry and textile industries.

## Introduction

The production medium must have suitable chemical composition and contain a source of carbon, nitrogen and mineral salts. The production of α-amylase by *Bacillus spp.* in natural and synthetic culture medium has been reported earlier by Mei & chen, 1997a. The supplementation of essential nutrients greatly affects the growth of bacteria and α-amylase production (Fogarty *et al.*, 1999). Natural nitrogen sources have undefined composition with small amounts of carbohydrates, lipids and minerals. Sometimes the natural sources contain stimulators like vitamins and minerals which influence the α-amylase production. Nitrogen is needed for the synthesis of amino acids, purines, pyrimidines, carbohydrates, lipids, enzyme cofactors, and other substances. Synthetic nitrogen sources have well defined concentration of the nitrogen. The nitrogen helps in formation of amino acids which in turn forms proteins. Since all enzymes are

proteins it may stimulate the secretion of more amylase enzyme.

The addition of different nitrogen sources reported to be greatly affected the production of α-amylase by *Bacillus subtilis* (Birol *et al.,* 1997). Nitrogen sources that stimulated amylase production are - Yeast extract (Santos & Martins, 2003), beef extract (Thaddeus *et al.,* 2005), peptone (Aiyer, 2004), ammonium sulphate (Dercova *et al.,* 1992), ammonium chloride (Saxena *et al.,* 2007), casein (Goto *et al.,* 1998), cysteine (Mahmoud & Hani, 2012), urea (Ramesh & Lonsane, 1990), potassium nitrate (Haq *et al.,* 2002b) and ammonium nitrate (Okolo *et al.,* 1996).

## Materials and Methods

### Primary screening

Marine water samples collected from Rushikonda, the coastal area isolated from the city, Visakhapatnam, India were diluted by serial dilution technique and cultured on starch agar medium. After incubation at $37^0$C the colonies observed with zone of starch hydrolysis by iodine staining were chosen. Discrete colonies on plate exhibiting the zone of starch hydrolysis were identified.

### Optimized parameters

*Brevibacillus borostelensis* R1 was cultured in Pikovskaya's medium with additional source of natural and synthetic nitrogen (1-5%w/v) separately by keeping the physical parameters (Incubation period 24hrs, inoculum size 2%, pH 7.0, temperature $37^0$C and salinity 1%) constant (Suribabu *et.al.,* 2014). Samples were incubated in orbital shaking incubator (120rpm) for 24hrs.

### Nitrogen sources

Soybean meal, channa dal, milk powder, sesame seed, ground nut (roasted), baker's yeast, badam, cashew nut, dry minced fish and sprouted green gram are naturally occurring nitrogen sources. Synthetic sources: yeast extract, beef extract, peptone, ammonium sulphate, ammonium chloride, tryptone, casein, L-cysteine, urea and potassium nitrate (all the synthetic nitrogen sources are procured from Merck).Varying concentrations (1, 2, 3, 4 and 5 % w/v) of ten natural and synthetic nitrogen sources were added to the 100 ml of Pikovskaya's fermentation medium separately. As synthetic sources need no pretreatment they were added directly into the culture at varying concentrations. However, natural sources of nitrogen were ground to powder with a mortar and pestle.

### Submerged Fermentation

Two ml inoculum of *Brevibacillus borostelensis* R1 was inoculated to the 100ml of production medium (Pikovskaya's Medium) and incubated in the orbital shaking incubator for 24hrs.

Pikovskay's Medium : (g/l ) ( pH 7.0)

| | |
|---|---|
| Glucose | : 10.05gm |
| $(NH_4)_2SO_4$ | : 0.5gm |
| $Ca_3(PO_4)_2$ | : 5.0gm |
| $MgSO_4.7H_2O$ | : 0.1gm |
| $MnSO_4.7H_2O$ | : Trace |
| $FeSO_4$ | : Trace |
| KCl | : 0.2gm |
| Yeast extract | : 0.5gm |

After incubation, the medium was subjected to centrifugation at 5,000rpm for 15minutes at room temperature ($25^0$C). The supernatant was collected in sterile

test tube and the pellet was discarded. Supernatant (0.5 ml) was used for the amylase assay by DNS method (Miller, 1959).

**Estimation of Maltose by Dinitro salicylic acid (DNS method)**

The enzyme extract (0.5 ml) was transferred to a test tube containing 0.5 ml of 1.0% soluble starch solution. The mixture was incubated at 37ºC for 10 min. Then 1.0 ml of dinitrosalicylic acid reagent (DNS) was added to each test tube. The tubes were placed in boiling water for 5 min and cooled at room temperature. The contents of tubes were diluted up to 10 ml with distilled water. The absorbance was read at 546 nm using a spectrophotometer and converted to mg of maltose from the standard. One unit of enzyme activity was defined as the amount of enzyme that releases 1.0 mmol of reducing sugar (maltose) per minute under the assay conditions.

**Statistical analysis**

All the experiments were conducted in triplicate. The results were given as mean value ± standard deviation. The conditions were analyzed to determine the significant difference between the variables by one way ANOVA, two way ANOVA and correlation analysis by using the scientific graph pad (Prism 6.1version software).
Analysis of variance (ANOVA) refers to the examination of differences among the sample means. It is used to examine the significance of the difference amongst more than two sample means at the same time.

## Results and Discussion

The Pikovskaya's (PK) Medium, incubation period (24hrs), 2% inoculum size, temperature ($37^0$C), pH (7.0) and salinity (1%) were optimized in submerged fermentation (SmF). The physical parameters optimized were maintained in chemical parameters optimization.

Optimization of α-amylase production was carried out by the addition of supplementary chemical sources of carbon, nitrogen and minerals to Pikovskaya's fermentation medium by maintaining constant physical parameters (24hrs incubation time, 2% inoculum size, $37^0$C, pH 7.0 and 1% NaCl).

Soybean meal, channa dal, milk powder, sesame seed, ground nut (roasted), baker's yeast, badam, cashew nut, dry minced fish and sprouted green gram were added separately as natural nitrogen supplements to the SmF PK medium at varying concentrations ranging from 1 to 5% (Figures 1 A-J). Ten synthetic nitrogen sources were utilized; yeast extract, beef extract, peptone, ammonium sulphate, ammonium chloride, tryptone, casein, L-cysteine, urea and potassium nitrate (Figures 2 A-J).

The production of α-amylase was estimated for all samples. The highest production of the enzyme at optimum concentrations of nitrogen supplements are tabulated in table 1. The synthetic sources resulted in higher production than the natural sources of nitrogen supplements. The addition of synthetic nitrogen (1% beef extract) to the PK medium yielded optimum enzyme activity of 2826 ±37.0U/ml.

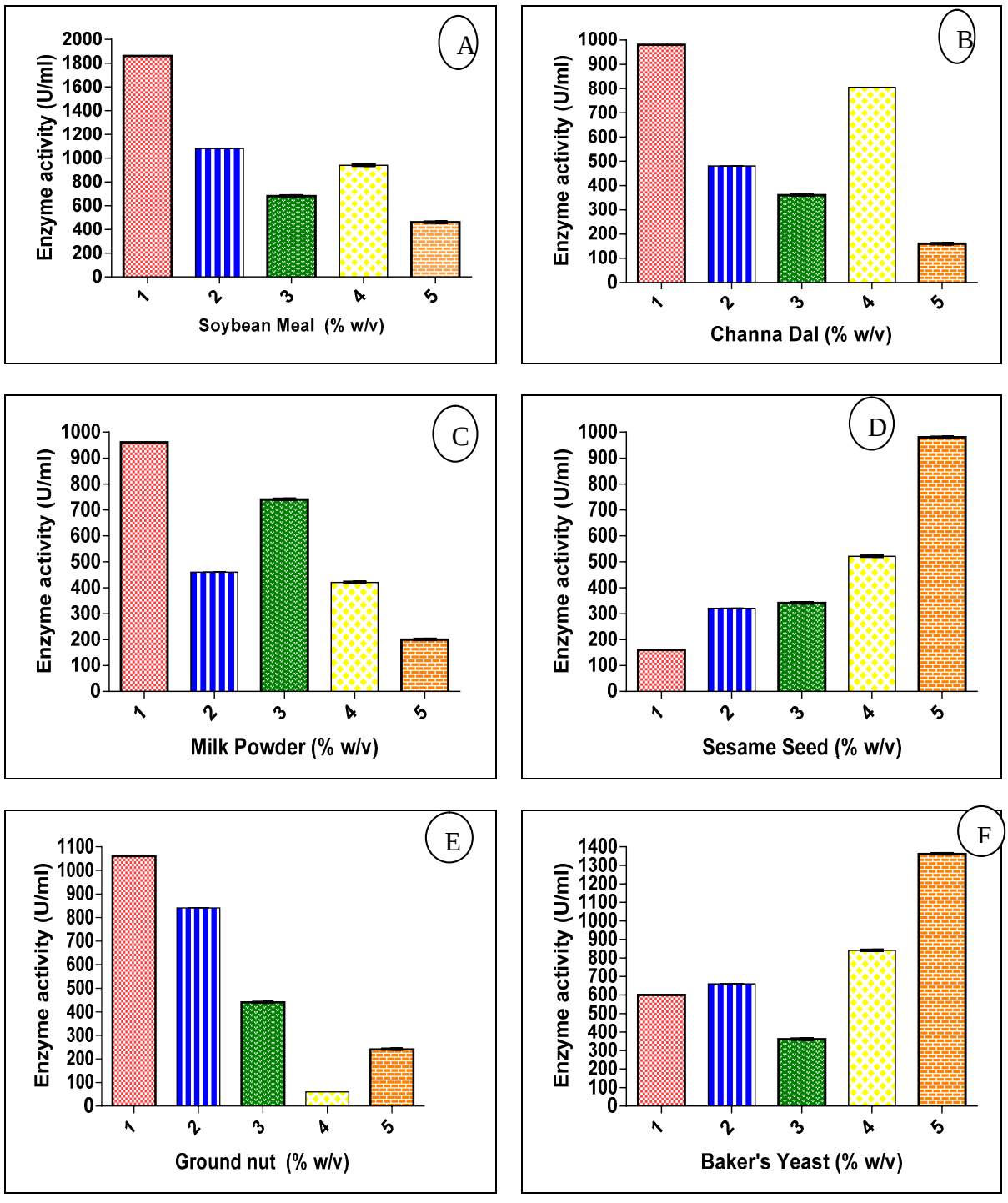
The production of α-amylase (2826±37U/ml) was found to be highest with 1% beef extract. The enhancing effect of beef extract on amylase production by

**Table.1** The highest production of α-amylase at optimal concentrations of nitrogen sources: Natural and Synthetic

| Natural nitrogen source | % of nitrogen source | Amylase activity (U/ml) |
|---|---|---|
| Soybean meal | 1 | 1861 ± 0.5 |
| Channa dal | 1 | 980 ± 0.3 |
| Milk powder | 1 | 961 ± 1 |
| Sesame Seed | 5 | 981 ± 0.5 |
| Ground nut (roasted) | 1 | 1061 ± 0.7 |
| Baker's yeast | 5 | 1361 ± 1.0 |
| Badam | 1 | 581 ± 0.5 |
| Cashew nut | 5 | 1141 ± 0.5 |
| Dry minced fish | 3 | 1662 ± 1.5 |
| Sprouted green gram | 3 | 1700 ± 0.0 |
| Synthetic nitrogen source | % of nitrogen source | Amylase activity (U/ml) |
| Yeast extract | 2 | 1741 ± 1.0 |
| **Beef extract** | **1** | **2826 ±37.0** |
| Peptone | 1 | 1801 ± 0.75 |
| Ammonium sulphate | 1 | 1982 ± 1.50 |
| Ammonium chloride | 1 | 1161 ± 1.00 |
| Tryptone | 1 | 681 ± 0.50 |
| Casein | 1 | 2621 ± 1.00 |
| L-Cysteine | 2 | 2410 ±14.00 |
| Urea | 3 | 1301 ± 0.500 |
| Potassium nitrate | 1 | 1121 ± 0.75 |

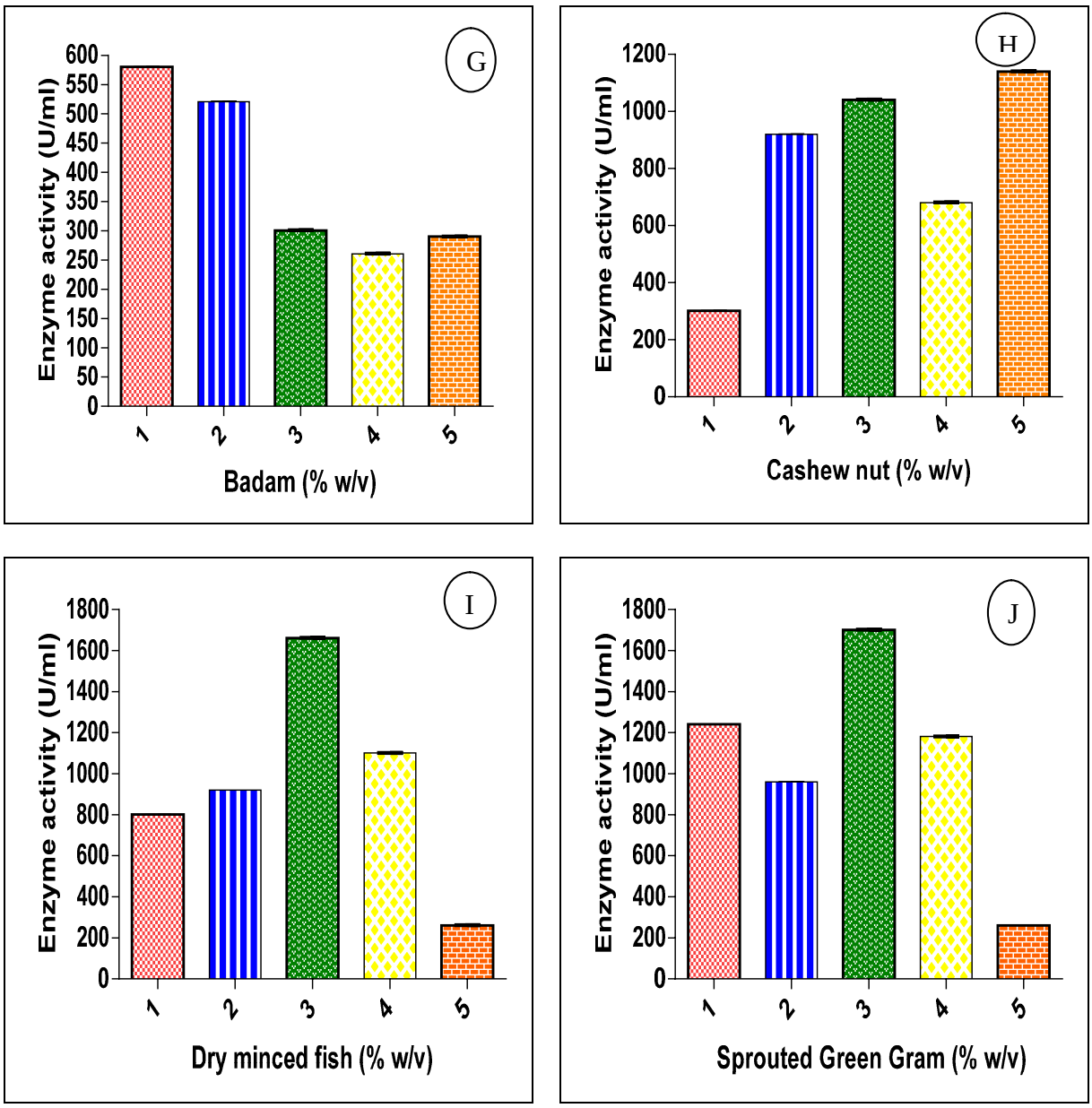
Values represented in the table are means of triplicates±SD.

**Figure.1** Effect of different concentrations of natural nitrogen sources on the production of α-amylase by *Brevibacillus borstelensis* R1 (A) Soybean meal (B) Channa dal (C) Milk powder (D) Sesame seed (E) Ground nut (roasted) and (F) Baker's yeast



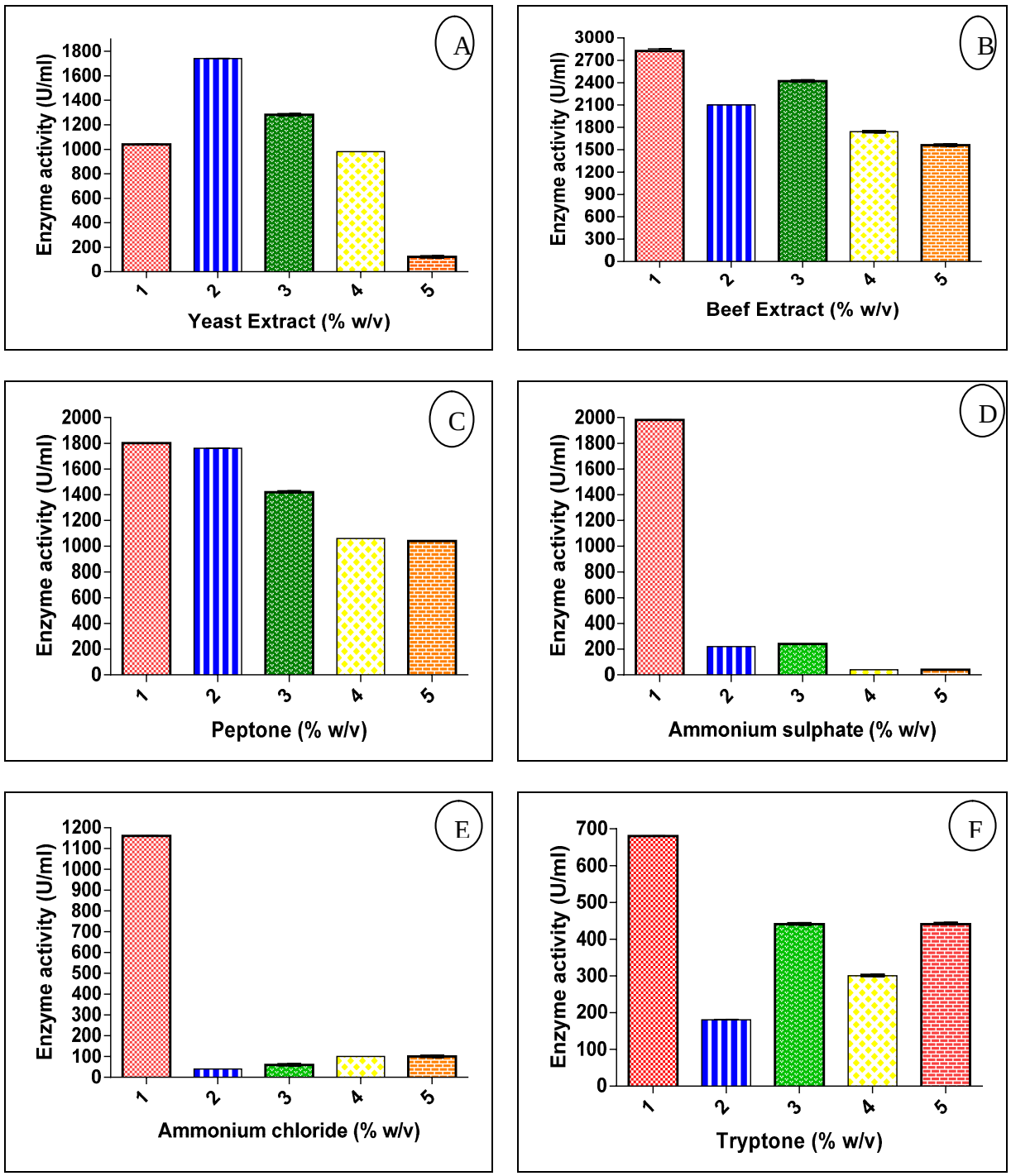

Y bars indicate the standard deviation of mean value.
**** P < 0.0001 Values differ significantly at p<0.5.

**Figure.1** Effect of different concentrations of natural nitrogen sources on the production of α-amylase by *Brevibacillus borstelensis* R1 (G) Badam (H) Cashew nut (I) Dry minced fish and (J) Sprouted green gram



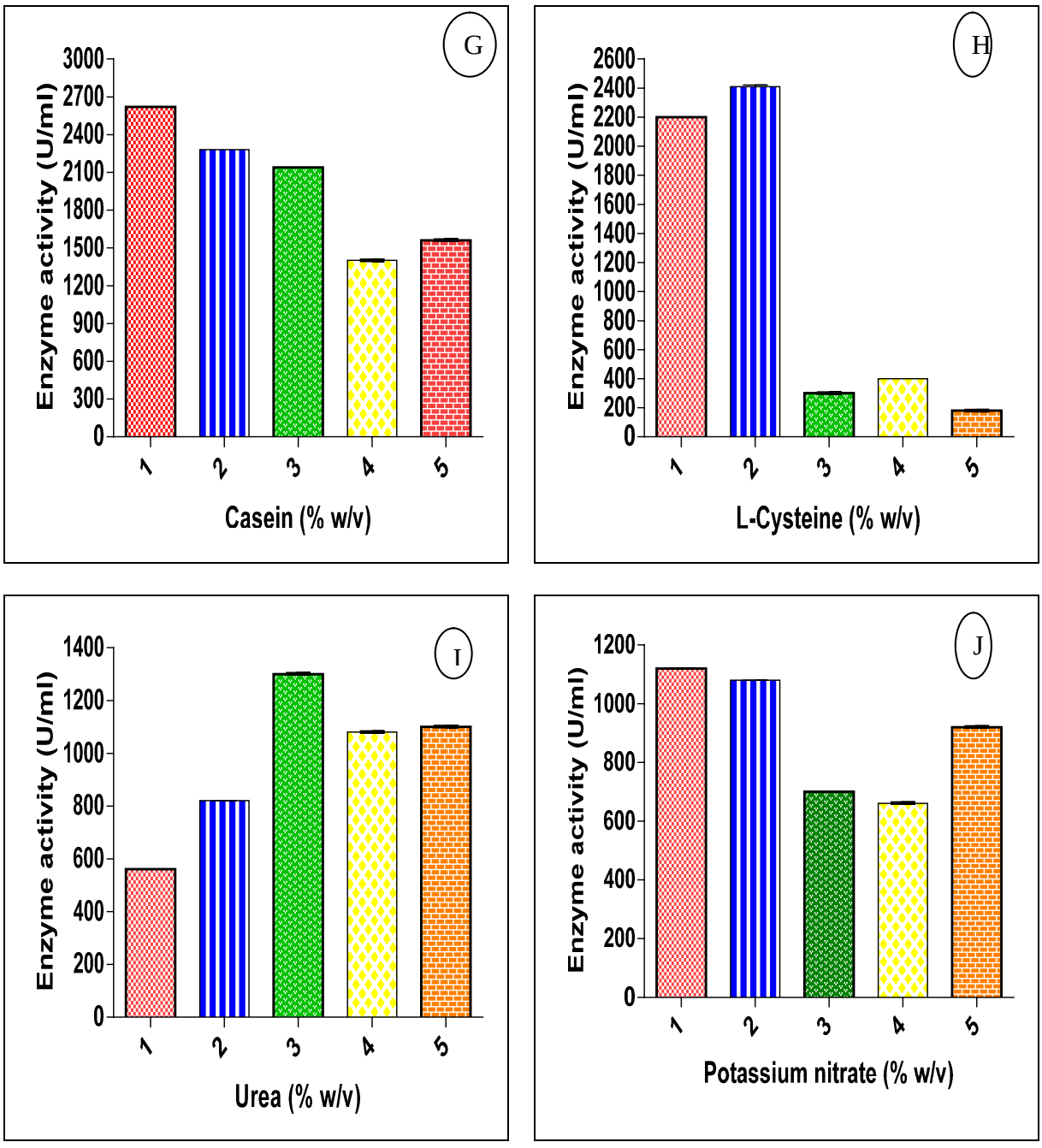

Y bars indicate the standard deviation of mean value.
**** P < 0.0001 Values differ significantly at p<0.5.

**Figure.2** Effect of different concentrations of synthetic nitrogen sources on the production of α-amylase by *Brevibacillus borstelensis* R1 (A) Yeast extract (B) Beef extract (C) Peptone (D) Ammonium sulphate (E) Ammonium chloride and (F) Tryptone




Y bars indicate the standard deviation of mean value.
**** P < 0.0001 Values differ significantly at p<0.5.

**Figure.3** Effect of different concentrations of synthetic nitrogen sources on the production of α-amylase by *Brevibacillus borstelensis* R1 (G) Casein (H) L-Cysteine (I) Urea and (J) Potassium nitrate




Y bars indicate the standard deviation of mean value.
**** P < 0.0001 Values differ significantly at p<0.5.

bacterial strains was circulated by Pederson & Nielsion (2000). Kammoun *et al*. (2008) have shown the positive effect of casein on amylase production by *Aspergillus oryzae*. The summit production was found in natural addition of soybean meal (1%). Corresponding studies in enhancing amylase production in *Bacillus sp.* were reported by Sodhi *et al.* (2005).

Elif Demirkan & Demirkan (2011) reported the stimulating effect of tryptone in *Bacillus sp.*. Malhotra *et al.* (2000) observed that tryptone (0.3%) showed optimum amylase production. Tryptone was reported to be as nitrogen source for the production of amylase in *B. thermooleovorans* (Sailas Benjamin *et al.*, 2013).

Optimization of α-amylase production was carried out by the addition of supplementary sources of carbon, nitrogen and minerals separately to Pikovskaya's fermentation medium by maintaining constant physical parameters (24hrs incubation time, 2% inoculum size, $37^0$C, pH 7.0 and 1% NaCl). The optimum production was found with 1% beef extract (2826±37U/ml). The α-amylase production with optimized physical and chemical parameters was 3120±14U/ml.

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