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Optimization of Mineral sources on α-amylase Production by *Brevibacillus borstelensis* R1 in Submerged Fermentation

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Abstract

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 minerals separately to Pikovskaya's fermentation medium by maintaining constant physical parameters (24hrs incubation time, 2% inoculum size, 37^0C, pH 7.0 and 1% NaCl). The optimum production was found with 0.8% calcium chloride (2962 ± 2.0U/ml). The α-amylase produced by *Brevibacillus borstelensis* R1 has a number of applications in many fields such as bakery industry, food preparations, automation dishwashing, ethyl alcohol dual fermentation, fodder production, laundry and textile industries.

Keywords: *Brevibacillus borstelensis* R1, Mineral sources, Submerged Fermentation.

Introduction

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 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 many workers [1&2]. The supplementation of essential nutrients greatly affects the growth of bacteria and α-amylase production [3]. Natural mineral sources are with undefined mineral composition. They contain little amounts of carbohydrates, proteins, lipids and vitamins. The metal stimulators present in the natural sources influence the α-amylase activity. Synthetic mineral sources have well defined concentration. Most of the enzymes are metalloenzymes. Metals are used to stabilize the transitional state of enzyme substrate complex. Metalloenzymes are usually thermostable. Alpha-amylases are metalloenzymes.

The effects of metal ions on the activity of α-amylase were studied in *Bacillus sp.* strain KSM-1378 [4]. Higher metal ion concentrations often inhibit microbial growth and enzyme production. Adequate concentrations of specific metal ions are very essential for microbial growth in *Bacillus subtilis* [5]. Chakraborty *et al.* [6] reported inhibition of amylase by divalent metal ions. He also reported the effect of some mono and trivalent cations on the enzyme activity of *Bacillus stearothermophilus.*

The stimulatory effect of calcium chloride was reported in *Bacillus subtilis* [7] and *Bacills thermoloeovorans* Np 54 [8]. The stimulatory effect of Magnesium ions was reported in *Bacillus spp.*[9] and *Bacillus licheniformis* A-4041 [10]. Stimulatory effect of ferrous ions was reported in *Bacillus spp.* [11] and *Bacillus halodurans* LBK 34 [12]. The strong inhibitory effect of mercuric ions in α-amylase production was reported in *Bacillus spp.* [13 & 14].The inhibitory effect of zinc sulphate in *Gluconacetobacter diazotrophicus* [15], *Bradyrhizobium sp.* strain INPA R- 991 [16] and *Bacillus ferdowsicous* [17] were reported.

Materials and methods

Optimization of the media:

Brevibacillus borstelensis R1 was cultured in Pikovskaya's medium with additional source of

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natural and synthetic carbon (1-5%w/v), nitrogen (1-5%w/v), and minerals (0.2-1.0%w/v) separately by keeping the physical parameters (Incubation period 24hrs, inoculum size 2%, pH 7.0, temperature 37^0C and salinity 1%) constant. Samples were incubated in orbital shaking incubator (120rpm) for 24hrs [18].

Optimization of the Natural and synthetic mineral sources:

Natural sources include small dried fish, green gram dal, black gram dal, onion, tea powder, salt, amaranth tender leaves, cheese, green dry peas and mint. Synthetic sources include copper sulphate (Qualigens fine chemicals), calcium chloride, magnesium sulphate (Qualigens fine chemicals), mercuric chloride, calcium carbonate, sodium sulfite, sodium chloride, manganese sulphate, zinc sulphate and ferrous sulphate (all the synthetic mineral sources are procured from Merck except labelled). Varying concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 % w/v) of ten natural and synthetic mineral 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, for natural sources of minerals, they were ground to powder with a mortar and pestle.

Submerged Fermentation:

Two ml of inoculum of *Brevibacillus borstelensis* R1 was inoculated to the 100ml of production medium (Pikovskaya's Medium) in Erlenmeyer flask and incubated in the orbital shaking incubator for 24hrs. After incubation, the medium was subjected to centrifugation at 5,000rpm for 15minutes at room temperature (25^0C). 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.

Alpha Amylase Assay:

Estimation of α-amylase activity was according to 3, 5 dinitro salicylic acid (DNS) method [19]. One unit of enzyme activity is defined as the amount of enzyme that releases 1.0 mmol of reducing sugar (maltose) per minute under the assay conditions.

Each concentration was assayed in triplicate sets and recorded for statistical analysis.

Statistical analysis:

All the experiments were conducted in triplicate. The results were given as mean value ± standard deviation. The conditions were analysed to determine the singnificant 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.

Result

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

Natural and synthetic mineral supplements were-small dried fish, green gram dal, black gram dal, onion, tea powder, salt, amaranth tender leaves, cheese, green dry peas, mint (Figures 1A-J), copper sulphate, calcium chloride, magnesium sulphate, mercuric chloride, calcium carbonate, sodium sulfite, sodium chloride, manganese sulphate, zinc sulphate and ferrous sulphate (Figures 2 A-J). They were added to the PK Medium at varying concentrations ranging from 0.2 to 1.0 %.

The production of α-amylase was estimated at different concentrations of mineral supplements and the highest activity at optimum concentrations are shown in Table 1. The production with synthetic mineral sources was higher than the natural supplements. The highest production (2962 ± 2.00U/ml) of amylase was found in the PK medium with additional synthetic mineral source of 0.8% Calcium chloride.

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

Natural mineral source	% of mineral source	Amylase activity (U/ml)
Small dried fish	0.2	720 ± 0.25
Green gram dal	0.2	2041 ± 0.50
Black gram dal	0.4	1001 ± 0.50
Onion	0.6	1541 ± 0.50
Tea powder	1.0	2230±14.00
Salt	1.0	2050±71.00
Amaranth tender leaves	1.0	2761 ± 1.00
Cheese	1.0	2781 ± 1.00
Green dry peas	1.0	2062 ± 1.50
Mint	1.0	2221 ± 0.70
Synthetic mineral source	% of mineral source	Amylase activity (U/ml)
Copper sulphate	0.2	1141 ± 1.00
Calcium chloride	**0.8**	**2962 ± 2.00**
Magnesium sulphite	0.2	961 ± 1.00
Mercuric chloride	0.2	1041 ± 1.00
Calcium carbonate	0.2	1982 ± 2.00
Sodium sulfite	0.2	1501 ± 1.00
Sodium chloride	1.0	981 ± 1.00
Manganese sulphate	0.6	600 ± 0.3
Zinc sulphate	0.2	960 ± 0.3
Ferrous sulphate	1.0	761 ± 1.00

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

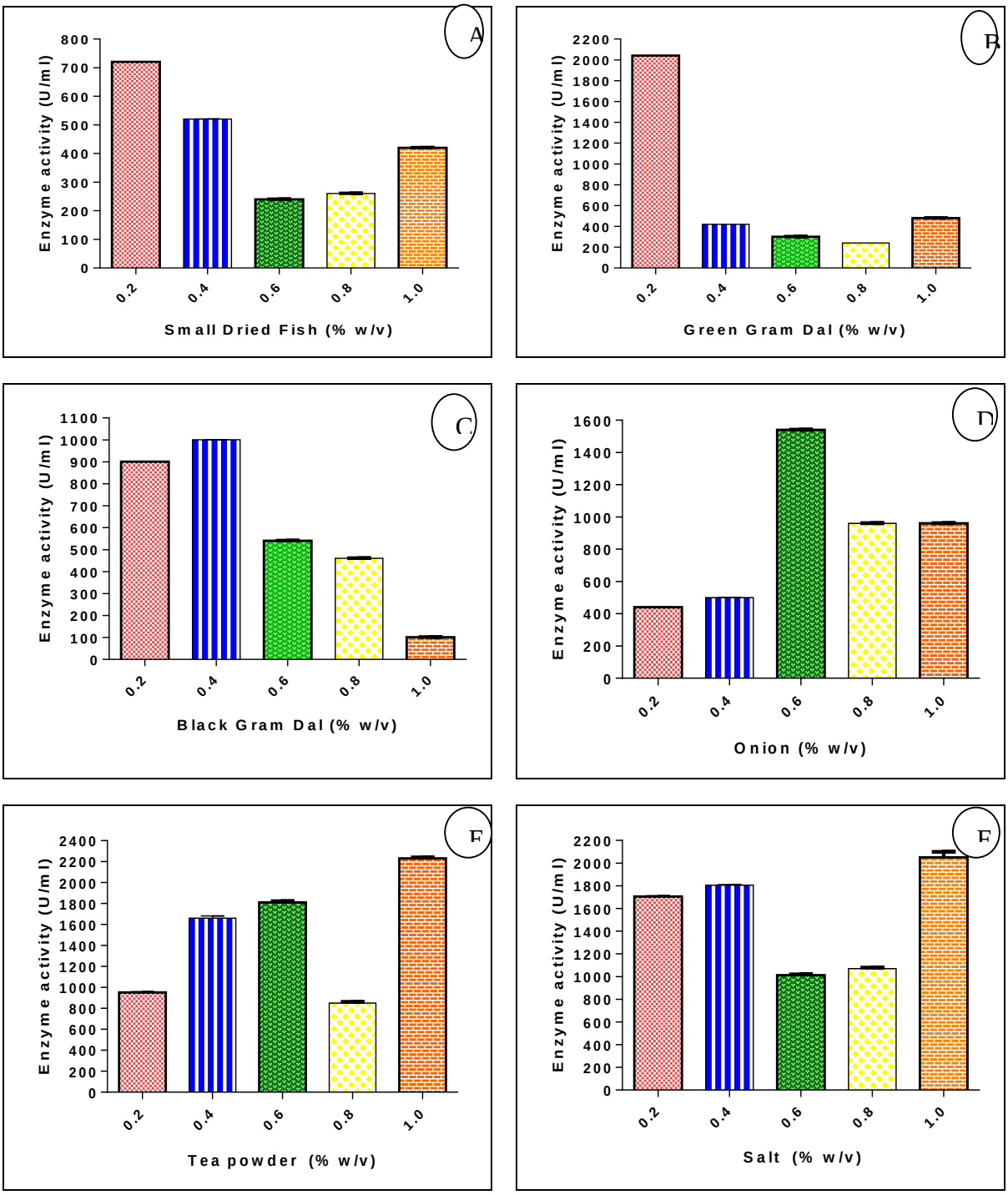

Figure 1. Effect of different concentrations of natural mineral sources on the production of α-amylase by Brevibacillus borstelensis R1: A, Small dried fish; B, Green gram dal; C, Black gram dal; D, Onion; E, Tea powder and F, Salt.

Y bars indicate the standard deviation of mean value.

**** P < 0.0001 Values differ significantly at p<0.5.

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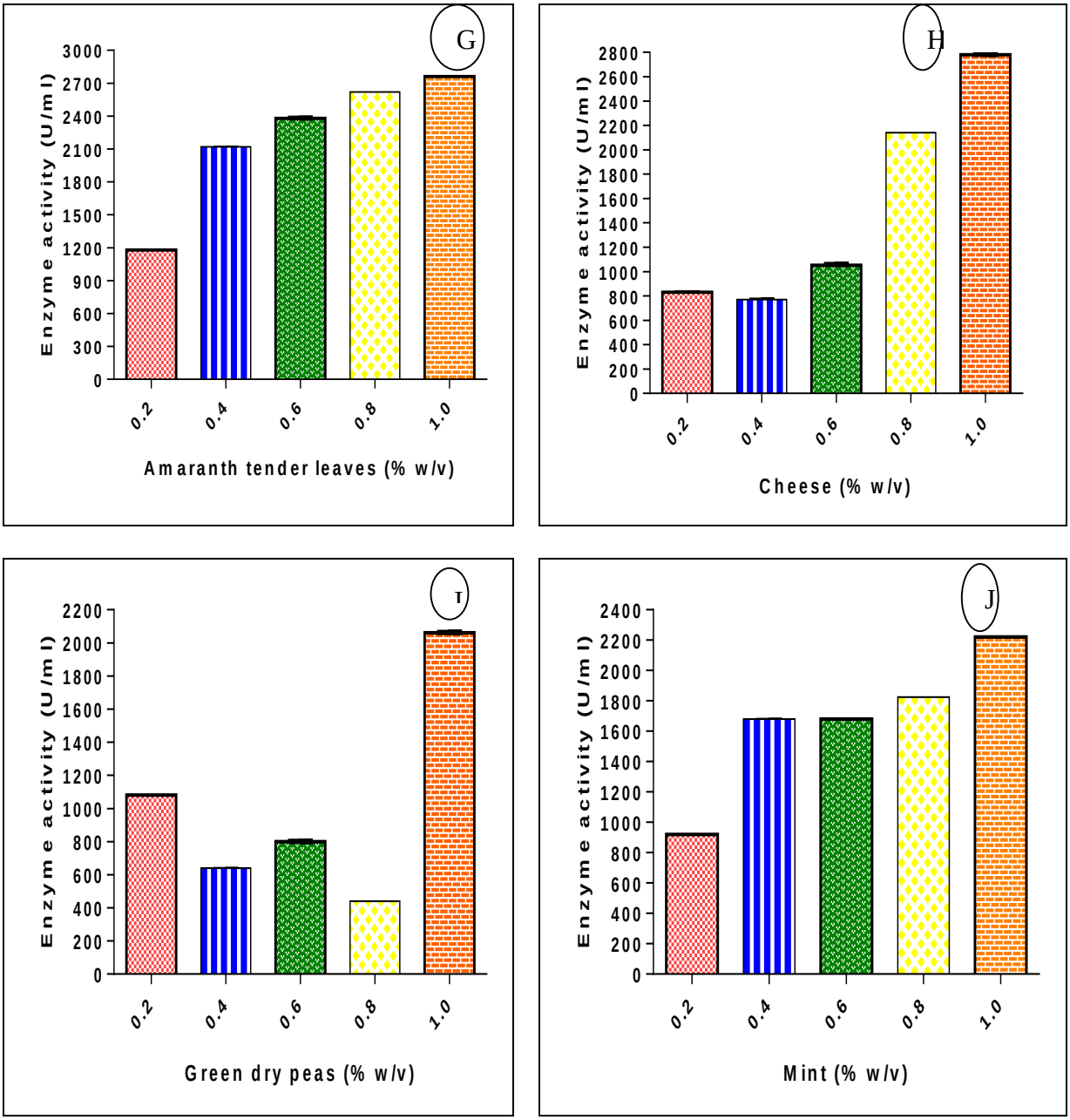

Figure 1. Effect of different concentrations of natural mineral sources on the production of α-amylase by Brevibacillus borstelensis R1: G, Amaranth tender leaves; H, Cheese;I, Green dry peas and J, Mint.

Y bars indicate the standard deviation of mean value.

**** P < 0.0001 Values differ significantly at p<0.5.

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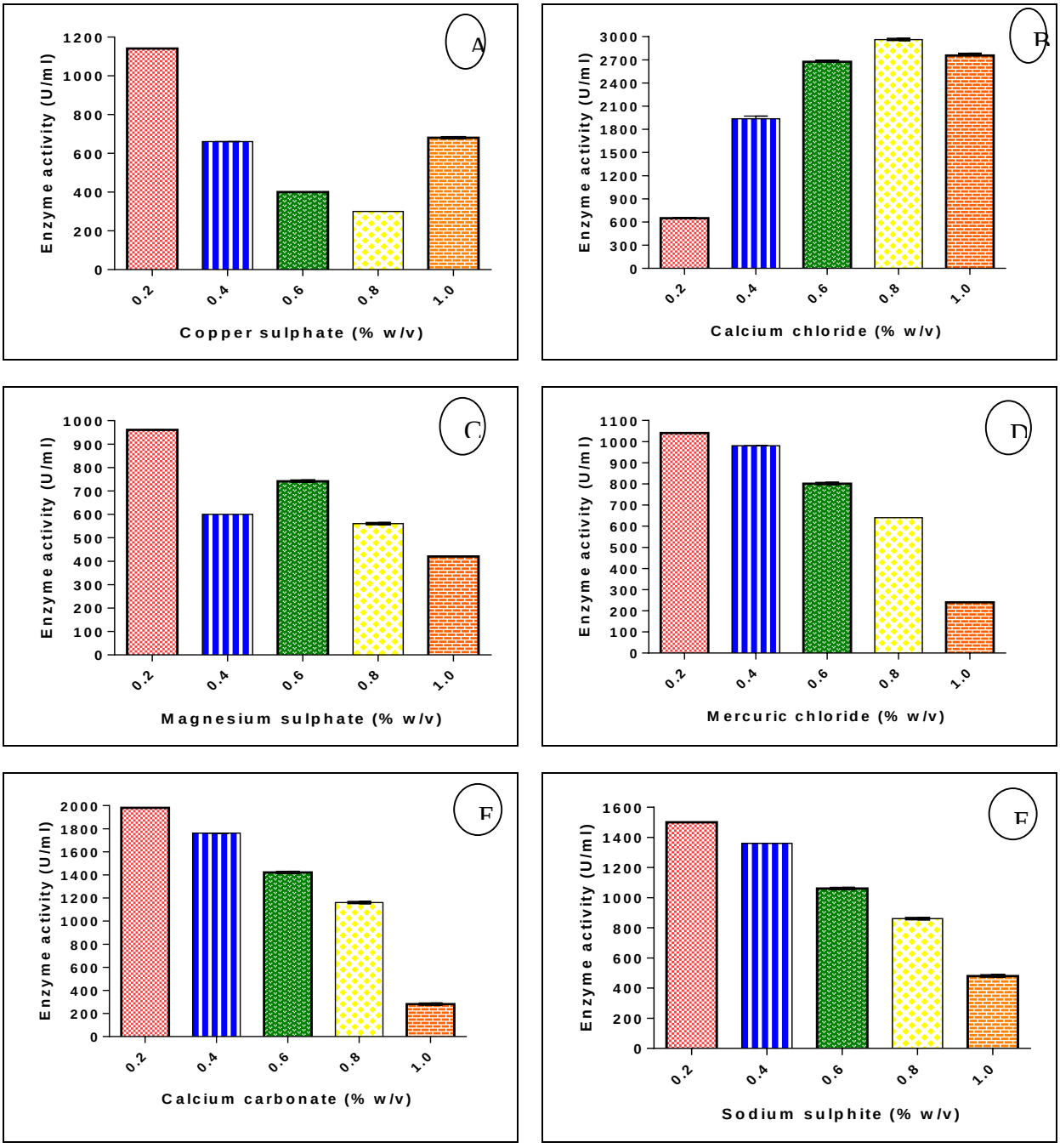

Figure 2. Effect of different concentrations of synthetic mineral sources on the production of α-amylase by Brevibacillus borstelensis R1: A, Copper sulphate; B, Calcium chloride; C, Magnesium sulphate; D, Mercuric chloride; E, Calcium carbonate and F, Sodium sulphite.

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

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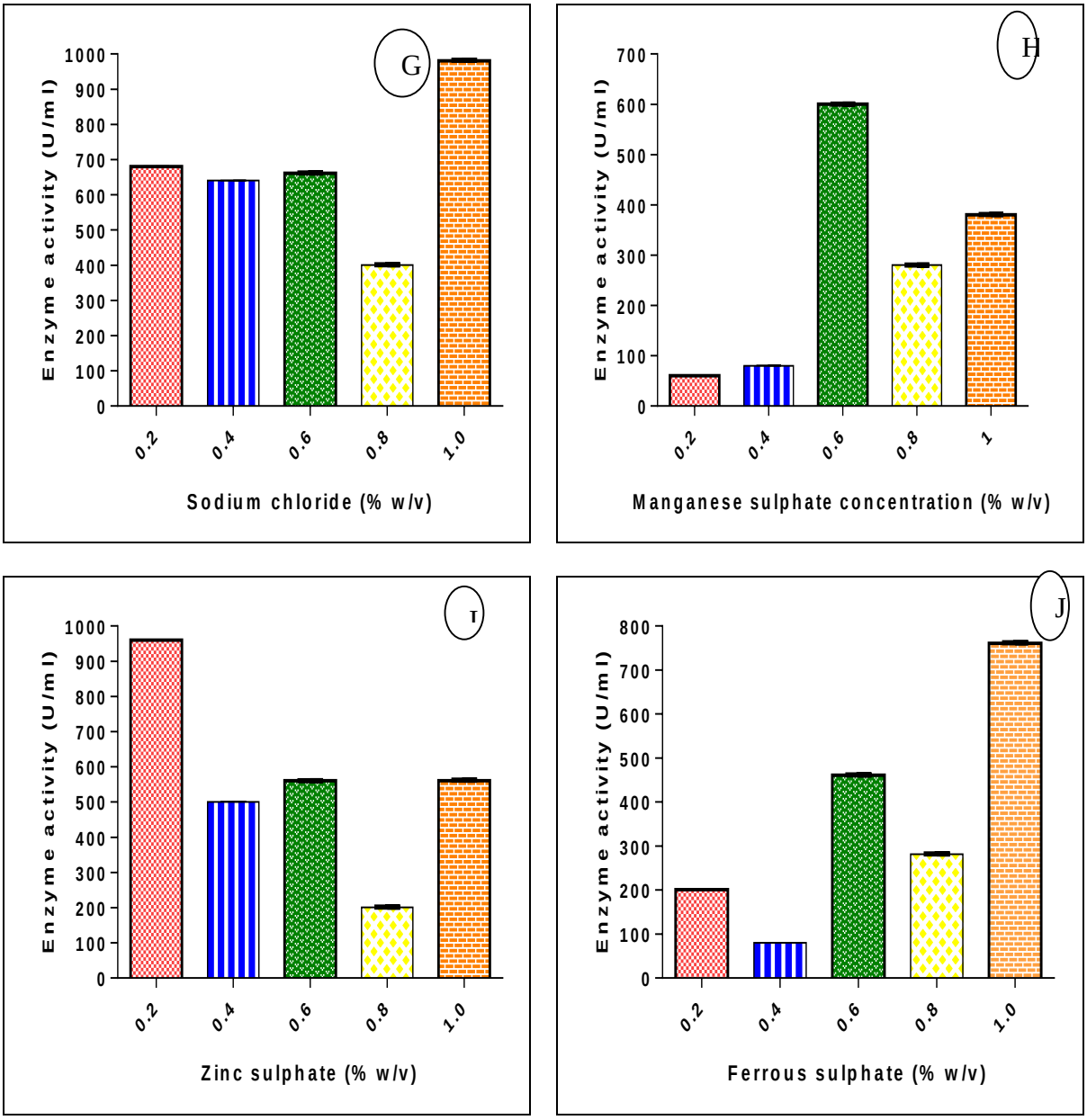

Figure 2 g-j. Effect of different concentrations of synthetic mineral sources on the production of α-amylase by *Brevibacillus borstelensis* R1: G, Sodium chloride; H, Manganese sulphate; I, Zinc sulphate and J, ferrous sulphate.

Y bars indicate the standard deviation of mean value.

**** P < 0.0001 Values differ significantly at p<0.5.

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Calcium chloride (0.8%) was found to produce maximum amylase activity (2962 ± 2U/ml) among 10 natural and 10 synthetic mineral sources used. Burhan *et al.* [20] have reported that *Bacillus sp.* in the presence of calcium increased enzyme production. Effect of different concentrations of calcium was reported by Ramesh & Lonsane [21] but the inhibitory effect of calcium on amylase production was studied by Muhammad Hamad Ashraf [22]. Asgher *et al.* [23] studied the effect of manganese ions on amylase production by *Bacillus species* but Zlem kiran *et al.* [24] reported the inhibitory effect of the Mn on amylase production.

Conclusion

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, 37^0C, pH 7.0 and 1% NaCl). The optimum production was found with with 0.8% calcium chloride (2962 ± 2.0U/ml).

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