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Optimization of cold active amylase production by mesophilic *Bacillus cereus* RGUJS2023 under submerged fermentation

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Abstract

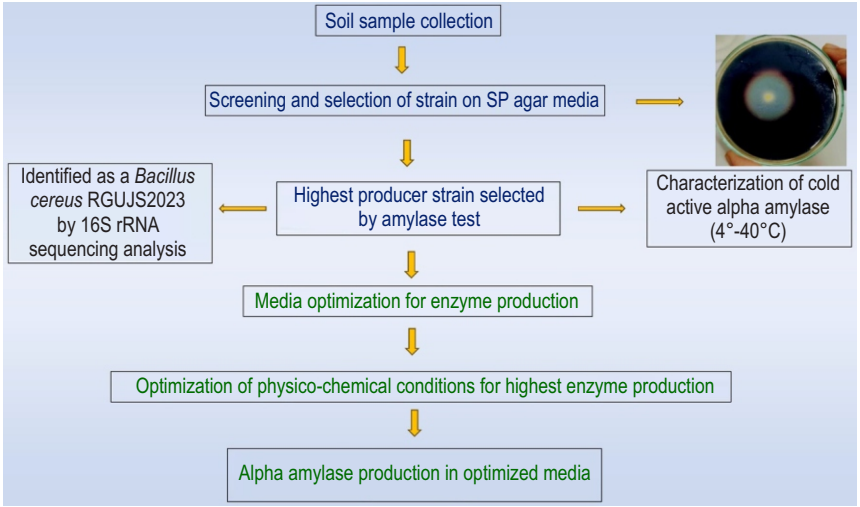
Aim: To produce the highest amount of cold-active alpha-amylase within a short time using mesophilic bacteria with optimized media to save the energy consumption cost and obtain higher enzyme production.

Methodology: Amylase producing twenty-three strains were isolated on starch peptone agar plates. Among them, one strain, A5 was selected on the basis of highest clear (12 mm) zone on starch peptone agar plates. It was characterized and identified following Bergey's Manual of Systematic Bacteriology. Enzyme was characterized as Cold alpha amylase. All physico-chemical parameters (temperature, pH, Inoculum size) including carbon, nitrogen, metal ion and amino acid sources were optimized for maximum production of the enzyme. The optimized media was used for enhancing the cold active amylase production.

Results: The strain, A5 was identified as *Bacillus cereus* RGUJS2023 by 16S rRNA sequencing analysis for further experiments. This strain showed the highest activity (9.922± 0.143 U ml^{-1}) on the basal starch peptone media. Though, crude enzyme showed its activity at 4°C to 48°C temperature, but the temperature was 28°C. The highest cold active enzyme was produced (18.87±0.06 U ml^{-1}) at 16 hr of bacterial growth at 35 °C with a pH 6.5 in the optimized media containing 0.5% starch, 0.1% peptone and 0.03% $MgSO_4.7H_2O$ as a carbon, nitrogen and metal ion sources, respectively, with addition of 0.03% arginine.

Interpretation: The cold active alpha amylase could be used commercially for the benefit of pharmaceutical and starch processing industries.

Key words: *Bacillus cereus* RGUJS2023, Cold-active amylase, Starch hydrolysis



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Introduction

Among the most essential industrial enzymes, Alpha-amylase is the most important in starch industry (Jujjavarapu and Dhagat, 2019). Due to their starch-degradation properties, amylase is used in the pharmaceutical, food, textile, paper, detergent and alcohol industries (Farooq *et al.,* 2021). Currently, amylase has occupied 30% of the global enzyme market andits demand has increasing continuously (Zaferanloo *et al.,* 2014). Amylase is found in animals, plants and microbial sources, but for commercial purpose, microbial sources are preferred because of their lower production cost, greater stability and easy extraction procedures (Gopinath *et al.,* 2017). Among the *Bacillus* species *B. subtilis, B. licheniformis, B. amyloliquifaciens, B. stearothermophilus* and *B. cereus* are known for high amylase production (Singh *et al.,* 2022). A perfect source for unidentified functional organisms is the soil which possesses about 99% of its microorganisms is yet to be studied in the laboratory (Ogunniran *et al.,* 2023). Many strains have been isolated from soil samples and optimized for amylase production (Abo-Kamer *et al.,* 2023; Kumar *et al.,* 2013; Samanta *et al.,* 2013; Halder *et al.,* 2014).

Industries require active enzymes that will act at ambient temperature with a wide pH tolerance for sugar production (Shinde and Vamkudoth, 2022). Biocatalyst can react in a particular temperature range which renders its applicability and cost effectivity. Cold active enzymes have immense potential and are widely used in industrial processes. (Ottoni *et al.,* 2020). Low temperatures are required for many industrial processes where high temperature chemical side reactions and contamination issues occur during the manufacturing of a variety of pharmaceuticals, fine chemicals, foods and beverages (Hamid *et al.,* 2022). Most of the cold active enzymes, in particular amylases, are produced by psychrotolerant and psychrophiles, (Liu *et al.,* 2023). As per the present report, cold amylase from mesophilic bacteria was very scanty and it was not much studied. (Santiago *et al.,* 2016). Cold active enzyme production from extremophiles is costly due to energy consumption to maintain their growth and enzyme production (Mangiagalli *et al.,* 2020). So, it is evident that the isolation of cold active enzyme producing mesophilic bacteria are necessary to reduce the enzyme production cost.

The research is going on continuously for the isolation of new microbial strains to meet the never-ending demand for industrial enzymes. Additionally, the production of enzymes can be optimized either by modifying the process of physical and nutritional variables or making genetic changes in the microorganisms (Gois *et al.,* 2020; Dou *et al.,* 2018). The cold active enzyme needs no high temperature for its activation thereby, saving energy and money for the industry (Arabacı and Arıkan, 2018). The objective of the present study was to produce the maximum amount of cold active alpha-amylase in a short time from mesophilic bacteria with an optimized media to save energy consumption cost.

Materials and Methods

Sample collection: Different soil samples (5 to 10 cm deep) were collected from different locations, East Medinipur (87° 46' 34.8132" E and 21° 56' 14.2368" N), Howrah (88° 10' 49.2816" E and 22° 34' 58.0728" N), Hooghly (88°23'48.26" E and 22°54'31.57" N), Darjeeling (88°15'29.3"E and 27°02'14.3"N), Uttar Dinajpur (88° 7' 32.1132" E and 25° 37' 6.7080" N) and Bankura (87°03'58.2"E and 23°14'12.4"N) District in West Bengal, India for the isolation of bacterial strain. The upper part, 5 to 10 cm of the soil, was removed manually with a knife and then the soils were collected.The strain was cultivated in sterile starch peptone medium (pH 6.9) in a BOD shaker incubator at 37°C and 130 rpm .

Isolation and selection of amylase producing strain: One gram of sample was transferred to 20 ml of starch peptone broth and incubated at 37°C for 12 hr. The samples (0.1 ml) from incubated broth were poured on the starch peptone agar plate and incubated at 37°C for 24 hr. Twenty-three different colonies (A1 to A23) were selected and purified by streaking repeatedly on starch peptone agar plates. High productive strains were screened and selected on the basis of formation of clear zone around the colonies after plates were flooded with Lugol's iodine. The selected strains were grown in starch peptone broth at 37°C for 12 hr for further test of amylase assay following the method of Bernfeld (1955). Cultures were centrifuged at 10,000 rpm at 4°C for 10 min to get cell free extract as crude amylase. The highest amylase producing isolate was picked for further experimental use.

Identification of bacterial strain: The best selected isolate was identified by morphological, physiological and biochemical methods following Bergey's Manual of Systematic Bacteriology (Garrity *et al.,* 2005) and molecular identification was carried out by analyzing its 16S rRNA gene sequencing. Briefly, DNA was extracted using lysis method and 16S rRNA gene was amplified. A single discrete 1500 bp PCR amplicon band was identified when separated on agarose gel. To eliminate contaminants, this PCR amplicon was purified. The forward and reverse DNA sequencing test of PCR amplicon was performed by using BDT v3.1 Cycle sequencing kit on an ABI 3730xl Genetic analytical apparatus. The 16S rRNA gene sequence was used to carry out BLAST with the 'nucleotides' database of NCBI GenBank database. The sequencing was performed by Syngenx India, Bangalore, India. Based on maximum identity score sequences and other strains of *Bacillus,* sequences were aligned using neighbor joining method. Phylogenetic tree was generated with MEGA 11 software (Kimura, 1980).

Effect of physico-chemical factors for α-amylase production from the strain: A number of factors affecting cell growth and the production of cold active α -amylase were studied using one factor at a time approach (Elmansy *et al.,* 2018). Optimized parameters such as temperature (10, 15, 20, 25, 30, 35, 40 and 45°C), initial pH of the medium (4, 4.5, 5, 5.5, 6.0, 6.5, 7.0, 7.5 and

8.0), inoculum size (1%, 3%, 5%, 7% and 9%), nitrogen, carbon, amino acid and divalent salt sources in various concentrations were studies.

Bacterial growth and amylase production under submerged fermentation process with optimized media were considered in the present study with a possible combination of different physico-chemical factors The factors were considered one at a time and measured the enzyme activity comparing with the basal media. The factors were considered as temperature, pH, inoculum size, nitrogen, carbon, metal ion and amino acid sources.

Enzyme assay: Enzyme activity was assayed by 3,5- dinitro-salicylic acid method by measuring the released reducing sugar (Priya and Renu 2018 and Sanchez *et al.,* 2019). Amount of released reducing sugar was determined from glucose standard curve. One unit of amylase activity (U ml^{-1}) is the amount of enzyme that releases the reducing sugar equivalent to 1 µmol of glucose per min per ml from substrate under the optimum assay conditions.

Cold-activity test: Crude enzyme with starch solution was incubated at different temperature for half an hour to check cold activity of the enzyme. Stability of crude enzyme was studied by incubating the enzyme at different temperatures and enzyme was assayed at ragular intervals to check the activity. Amylolytic activity of cold incubated enzyme was measured by the method of Bernfeld (1955).

Determination of amylolytic nature: The enzyme was treated with 10 mml^{-1} phytic acid for 2 hr in optimum enzyme assay conditions. Phytic acid treated enzyme activity was compared with a set of untreated enzymes, alpha amylase (9000-85-5-sigma) and beta amylase (9000-91-3-sigma), as a control to determine the amylolytic nature of the enzyme.

Statistical analysis: The data were expressed as mean with standard deviation and all experiments were run in triplicate set. Two-way analysis of variance (ANOVA) was used to statistically analyze the data. Version 7.0 Design-Expert software was used to evaluate the results.

Results and Discussion

Twenty-three strains that showed amylolytic activity and growth on starch peptone agar plates were selected for enzyme assay. The highest amylase producing strain, A5 was considered for further studies. The strain A5 was characterized and identified as genus *Bacillus,* based on the morphological and biochemical characteristics. 16S rRNA sequencing was analyzed to confirm the species level. The analyzed partial sequence was compared with the GenBank database using BLAST search in NCBI and sequence homology was 100% similar to *Bacillus cereus* (Fig. 1). Therefore, it was named as *Bacillus cereus* RGUJS2023. The sequence was registered to GenBank at NCBI with its accession number OQ984972. Enzyme was completely inactivated when it was treated with phytic acid. Phytic acid is one of the alpha amylase inhibitors (Ray *et al*., 1994). The untreated isolated enzyme showed complete activity under similar condition. The alpha-amylase from *Bacillus cereus* RGUJS2023 was confirmed by the above experiments.

The stability and activity of this enzyme were studied at temperatures ranging from 4° to 48°C (Fig. 2a). Though this amylase showed optimal activity at 28°C, it also showed low activity at 4°C. Ottoni *et al.* (2020) discovered cold-active amylase from the Antarctic psychrophilic bacterium *Carnobacterium iners* and *Arthrobacter* sp., which showed highest activity at 20°C, however, significantly dropped at 5°C. The enzyme obtained from RGUJS2023 strain showed a good range of activity between 16 and 40 °C. The activity was low at lower temperature, below 16 °C as compared to 28°C. Ranjan *et al.* (2016) also reported similar results. Previous research have also reported a variety of cold-

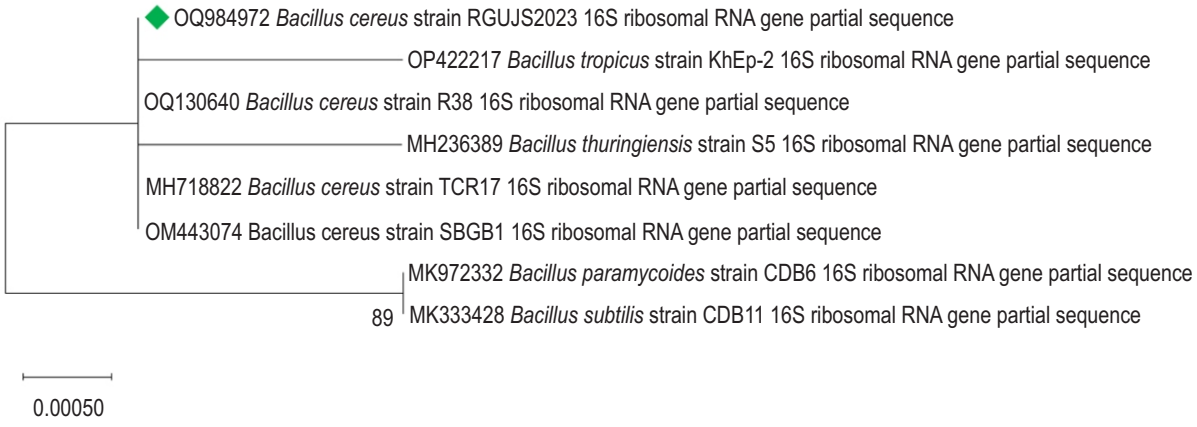

Fig. 1: Phylogenetic tree build based on 16S rRNA sequences of *Bacillus cereus* RGUJS2023 and its closest *Bacillus* species using Neighbor Joining Method. 1,000 replications were used to calculate the bootstrap probabilities.

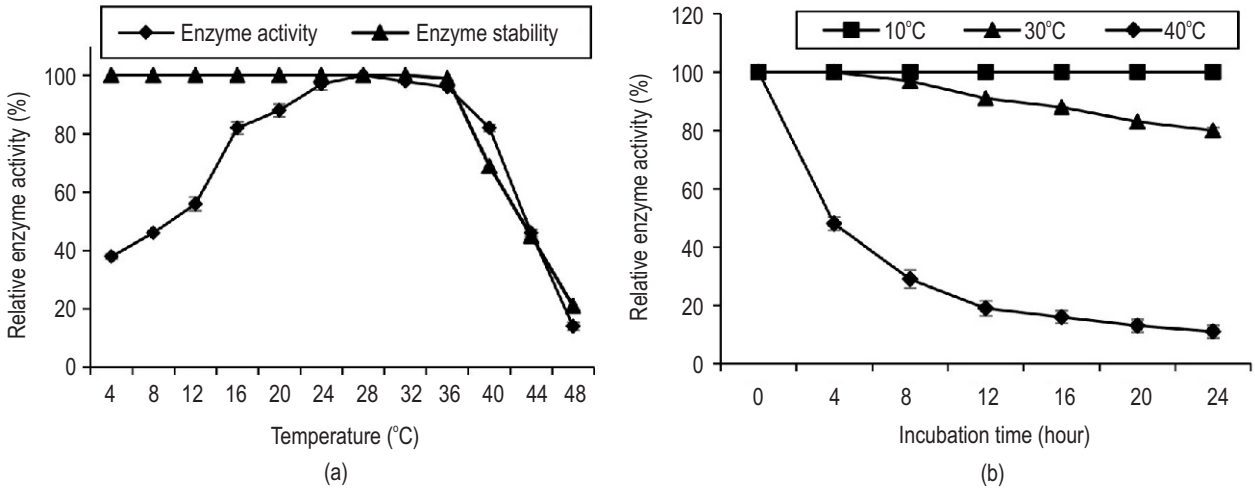

Fig. 2: Effect of temperature on enzyme activity and stability. (a) Enzyme activity at different temperatures (rhombus) and thermal stability was determined at each temperature by incubating the enzyme for 2 hr (triangles) and (b) Enzyme stability at 10°C (squares), 30°C (triangles) and 40°C (rhombus) measured at different time intervals.

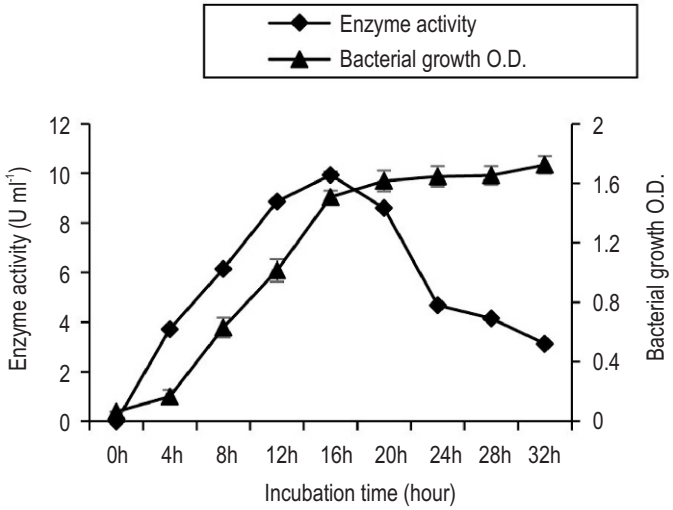

Fig. 3: Effect of incubation time on enzyme synthesis and subsequent growth of *Bacillus* RGUJS2023 strain.

active amylase producers from cold environments, specifically from the microorganisms of Antarctic region (Daskaya-Dikmen *et al.,* 2018; Sanchez *et al.,* 2019). According to Zhang and Zeng, (2007), this instant reduction in the catalytic activity value above 40°C is a unique character of cold active enzymes. These findings indicate that an enzyme is a cold-active enzyme with catalytic activity at lower temperatures. The stability of this enzyme was studied with a set of time intervals for each temperature at 10°C, 30°C and 40°C and its activity was then estimated under laboratory conditions (Fig. 2b).

This experiment showed that the enzyme remained stable between 4 and 36 °C. The enzyme stability decreased slowly above 36°C. When the enzyme was incubated for 24 hr at 40°C, only 11% of the relative activity was retained. Dou *et al.* (2018) showed that the cold activity of amylase (Amy D-1) from marine isolated *Bacillus* sp. was approximately 35% at 4°C. According to Cotarlet *et al.* (2009), only 80% activity of amylase retained after incubation at 30°C for 60 min, which was studied in case of *Streptomyces* 4 Alga. In the present study, cold-active alpha amylase from *Bacillus cereus* RGUJS2023 remained 100%

stable after 4 hr at 30°C. The relation between the growth and amylase production is shown in Fig. 3. The cold active alpha amylase was produced simultaneously with the initiation of bacterial growth. The maximum enzyme production was noted at 16 hr of incubation, 37°C and pH 6.9 (9.922± 0.143 U ml^{-1}) at the exponential phase of bacterial growth.

The enzyme production decreased at stationary phase. As the highest production of enzymes was found at 16 hr, the 16 hr growth culture was used as the enzyme source for all the experiments. According to Abo Kamer *et al.* (2023), *Bacillus cereus* produced maximum alpha amylase after 48 hr from MK1 strain. Cold active amylase production was also reported from *Bacillus* species after 24 and 96 hr of incubation (Kuddus and Ahmad, 2012; Arabacı *et al.,* 2018). Although *Bacillus cereus* is reported to grow slowly for enzyme production in comparison to other *Bacillus* species, *Bacillus cereus* RGUJS2023 showed enzyme production with the shortest production time, (16 hr only). *Bacillus cereus* RGUJS2023 was grown at 10 to 45°C in starch peptone medium at pH 6.9 for 16 hr (Fig. 4a) and the enzyme production was measured. The highest enzyme production from *Bacillus cereus* RGUJS2023 was found at 35°C (10.95 ±0.302 U ml^{-1}) when growth O.D.660 nm was 1.571± 0.018.

Similar results were recorded in the mesophilic bacterial strain studied by Poddar *et al.* (2012).The findings of the present study indicated that the enzyme produced was cold active but producer strain was mesophilic. According to Putri and Nakagawa (2020), several mesophilic bacterial strains, mainly *Bacillus* species were known that produce thermophilic alpha-amylases. The growth and enzyme production decreased significantly above 40°C (Samanta *et al.,* 2013), which indicated the mesophilic character of the strain. The present investigation with the *Bacillus cereus* RGUJS2023 corroborates with the reports of Elechi *et al.* (2022). Abou-Elela *et al.* (2009) observed that the synthesis of cold-active amylase from *Nocardiopsis aegyptia* occurred between 25 and 30°C. However, Kuddus and Ahmad (2012) reported that cold active amylase production from *Bacillus cereus* was maximum at 20°C. Fig. 4b shows the effect of initial fermentation pH (4.0- 8.0) on the synthesis of cold active amylase incubated for 16 hr under 130 rpm 35°C.

The growth of *Bacillus cereus* RGUJS2023 and amylase production was maximum at pH 6.5 (11.372± 0.392 U ml^{-1}) with growth O.D.660 nm 1.583± 0.047. However, Kuddus and Ahmad (2012) reported that production of amylase from *Bacillus cereus* was maximum at pH 10. Though, the production of most cold active amylase have been reported at alkaline pH range (Arabacı and Arıkan, 2018 and Ottoni *et al.,* 2020), however, in this study, *Bacillus cereus* RGUJS2023 produced maximum cold active amylase at acidic pH (6.5). The maximum enzyme was produced (11.372 U ml^{-1}) with 5% inoculum (growth O.D.660 nm 1.583), at 35°C maintaining the pH of 6.5 in the production medium. The effect of inoculum size (2%, 3%, 4%, 5%, 6% and 7%) on the growth and extracellular amylase synthesis is shown in Fig. 4c. However, Saha and Mazumdar (2019) and Elechi *et al.* (2022)

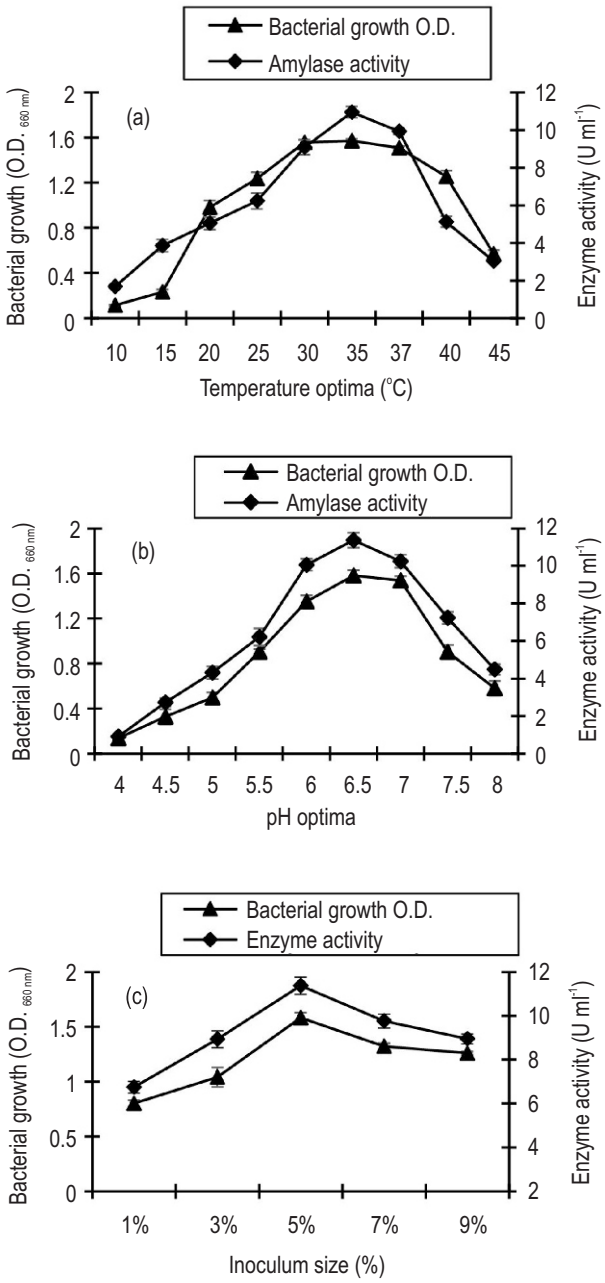

Fig. 4: Effect of temperature (a), pH (b) and inoculum size (c) on the growth and enzyme production by RGUJS2023 strain.

reported that the highest amylase production was obtained with 3% and 2% inoculum, respectively. Among several organic nitrogen sources studied, 0.10% peptone supplemented in the basal media produced maximum enzyme (11.398 U ml^{-1}) as compared to the supplied nitrogen sources, 0.10% beef extract (9.07 U ml^{-1}) and 0.05% tryptone (6.75 U ml^{-1}) at optimum temperature, pH and incubation time for *Bacillus cereus* RGUJS2023 (Fig. 5a). However, with the increased concentration

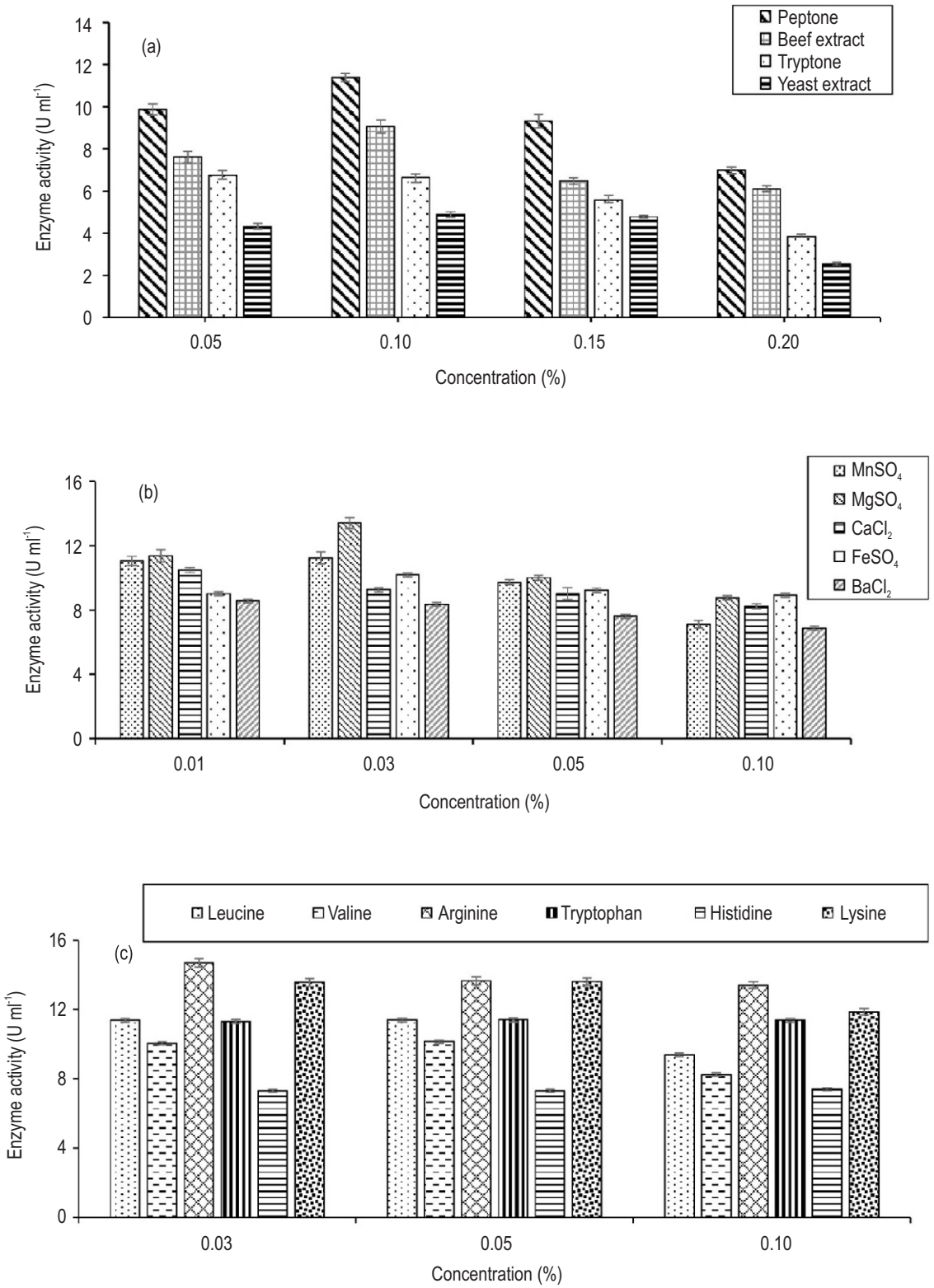

Fig. 5: Effect of different nitrogen sources (a), metal salts (b) and amino acid sources (c) on enzyme synthesis by *Bacillus cereus* RGUJS2023 strain at optimum pH and temperature.

Table 1: Effect of starch concentration on the growth and amylase production by *Bacillus cereus* RGUJS2023

	0.1%	0.5%	1%	1.5%	2%
Cell growth O.D.	1.031± 0.22	1.58± 0.047	1.546± 0.36	1.569± 0.18	1.654±0.027
Enzyme activity (U ml^{-1})	8.115± 0.16	11.372± 0.39	10.876± 0.42	10.435± 0.6	9.97± 0.20
Reducing sugar (mg ml^{-1})	9.673± 0.35	12.34±0.03	18.68± 0.40	26.54± 0.22	34.76±0.32
pH	5.5	5.5	5.4	5.4	5.4

Table 2: Effect of carbon sources on the growth and amylase production by *Bacillus cereus* RGUJS2023

Carbon sources (0.5%)	Growth (16 hr) O.D. at 660 n.m.	pH after fermentation	Enzyme activity (U ml^{-1})	Reducing sugar (mg ml^{-1})
Glucose	1.674± 0.22	5.1	0.14±0.02	17.121± 0.36
Maltose	1.598± 0.38	5.1	0.27±0.06	18.438± 0.14
Malto-Triose	1.687± 0.16	5	0.57±0.28	9.543± 0.26
Malto-tetraose	1.532± 0.26	5.5	9.337±0.047	0.924± 0.10
Pullulan	1.504± 0.12	5.4	9.85±0.028	1.006± 0.34
Dextrin	1.73± 0.28	5.2	2.08±0.03	4.363± 0.42
Starch	1.58± 0.047	6	11.372±0.397	12.34± 0.03

of nitrogen in the basal media, the enzyme production decreased. Saha and Mazumdar (2019) reported the highest production of amylase from *Bacillus cereus* on using sodium nitrate as a nitrogen source under one factor at a time method. Amylase production from *Bacillus* sp. was maximum with yeast extracts (Khusro *et al.,* 2017). Peptone is used as the best nitrogen source for amylase production, as reported by Tanyildizi *et al.* (2005).

In the study of different divalent salts with different concentrations, 0.3% $MgSO_4$ (13.411± 0.345 U ml^{-1}) salt acted as a better cofactor for highest amylase production instead of 0.1% $MgSO_4$ (Fig. 5b). In this study, no remarkable enzyme production was recorded with divalent cations, ($CaCl_2$, $MnSO_4$, $FeSO_4$ and $BaCl_2$) at any concentration in the basal media. However, Vishwanathan and Surlikar (2001) found that the production of amylase increased in the presence of calcium ions. Elechi *et al.* (2022) showed that 1% ferric chloride was the best metal ion source for amylase production from *Bacillus cereus*.

Supplementation of free amino acids to the growth medium of *Bacillus cereus* RGUJS2023, increased enzyme synthesis significantly, though the basal medium did not contain any external source of amino acids. Enzyme synthesis increased on adding arginine and/ or lysine in the medium. In the presence of histidine, enzyme production reduced in comparison to the enzyme production in the basal medium. Enzyme production in the presence of 0.03% and 0.05% arginine showed 14.711 U ml^{-1} and 13.67 U ml^{-1} enzyme activity, respectively, however in case of 0.05% lysine, it showed 13.62 U ml^{-1} enzyme activity (Fig. 5c). Amylase production was increased with 0.09% tyrosine and 0.01% phenyl alanine as reported by Saha and Mazumdar (2019) and Suganthi *et al.* (2015). As per the report by Suganthi *et al.* (2015), amylase production increased in the media containing 0.01% phenyl alanine as an amino acid source. In this study, 0.5% starch was reported to be the most effective carbon source for amylase synthesis by *Bacillus cereus* RGUJS2023 (11.372 U ml^{-1}) (Table 1). On increasing the starch concentration in the medium upto, 2%, the enzyme activity (9.97± U ml^{-1}) was noted to decrease with the increased cell growth (1.654) and the total free sugar in the medium increased from 12.34 to 34.76 mg ml^{-1}. Different carbon sources were tested separately at 0.5% concentration in the basal media, including some polysaccharides (starch, dextrin), di- and oligo-saccharides (maltose, triose, tetraose, pullulan) and monosaccharides (glucose) (Table 2). Khusro *et al.* (2017) reported that 1% starch was the best inducer as a carbon source for amylase production from *Bacillus* sp. by submerged fermentation. Amylase production was higher in the media containing polysaccharide such as pullulan (9.85 U ml^{-1}), starch (11.372 U ml^{-1}) and tetraose (9.337 U ml^{-1}) compared to glucose (0.14 U ml^{-1}), maltose (0.27 U ml^{-1}), triose (0.57 U ml^{-1}) and dextrin (2.08 U ml^{-1}) containing media. In the presence of glucose and maltose, the enzyme production mostly declined whereas the growth of *Bacillus cereus* RGUJS2023 was better in similar conditions. Though starch as substrate was the best inducer in this study, however, maltose and dextrose were also good inducers based on the reports of Elmansy *et al.* (2018) for amylase production by *Bacillus* sp.

For maximum enzyme production, *Bacillus cereus* RGUJS2023 was grown in optimized media maintaining all-optimal conditions in one at a time. The optimized media contain (g l^{-1}): peptone, 1.0; $(NH_4)_2HPO_4$, 0.4; KCl, 0.1; $MgSO_4.7H_2O$, 0.3; NaH_2PO_4, $2H_2O$, 0.5; soluble starch (S9765-Sigma), 5and arginine, 0.3. Optimized media was prepared by changing the

ingredient's concentration except starch in basal media where enzyme was increased compared to the previous results. In this study, 5% inoculum was incubated in the optimized medium for 16 hours of fermentation in a rotary shaker incubator (130 rpm) at 35°C for enzyme production and compared the enzyme production with basal media. Maximum enzyme production was observed in optimized media (18.87±0.06 U ml^{-1}) which was higher than basal media (9.922± 0.143 U ml^{-1}) under submerged fermentation. The novelty of the present investigation with *Bacillus cereus* RGUJS2023 for the highest cold alpha amylase production within 16 hours of incubation. This enzyme would be a great benefit not only for the pharmaceutical and starch industry but also save the import cost of the country.

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