Trop J Nat Prod Res, November 2023; 7(11):5172-5177 **ISSN 2616-0684 (Print)**
**ISSN 2616-0692 (Electronic)**

# Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

*Original Research Article*

# Cold Active Amylase Production from *Bacillus cereus* RGUJS2023

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ARTICLE INFO

*Article history:*
Received 31 August 2023
Revised 21 October 2023
Accepted 02 November 2023
Published online 01 December 2023



ABSTRACT

Cold-active enzymes have significant biotechnological prospects and provide a number of ecological and economic advantages by reducing the heating cost. The present investigation describes the production and characterization of a novel cold-active enzyme from the RGUJS2023 strain of *Bacillus cereus*. This enzyme would be used for the benefit of the food, starch, and pharmaceutical industries. The growth kinetics of this strain showed that starch utilization with reducing sugar production was within 4 hours of the exponential growth of the strain with the initial production of enzyme. The starch was fully utilized in the media within 6 hours of culture growth; however, the produced reducing sugar remained same up to 20 hours of the cell's growth. The highest production of the enzyme was in the late exponential to early stationary phase. This strain produced the highest enzyme after incubating for 16 hours at 37°C and at pH 6.9. This enzyme was characterized as cold-active alpha-amylase. This enzyme showed maximum activity with 2% starch solution at 28°C and at pH 6.5 after 30 min of incubation and was stable at room temperature (30°C) for a long time. Cold active enzyme activity was stimulated in the presence of $Mn^{2+}$, $Mg^{2+}$ and $Sn^{2+}$ and inhibited in the presence of $Pb^{2+}$ and $Cu^{2+}$. After a careful consideration of the above finding, it may be considered that this cold-active alpha-amylase from *Bacillus cereus* RGUJS2023 is an outstanding, affordable, and reliable choice for industrial applications.

***Keywords***: *Bacillus cereus*, Characterization, Cold-active alpha amylase, Growth kinetics, Starch hydrolysis.

## Introduction

Amylases are extracellular enzymes (endo-1,4-D-glucanohydrolases, E.C. 3.2.1.1) that hydrolyze starch polymer to glucose.[1] World-wide, amylases account for 30% of the market for industrial enzymes.[2] Amylases has been shown to have a wide range of importance in industry.[3] Cold-active amylases are suitable for a range of industrial processes, including detergents for cold washing, leather softening, bioremediation, biofuel production, textiles, alcohol production, paper industry, the food industry, pharmaceuticals, and molecular biology applications.[4,5] From ancient times to present, amylase has been discovered in animal, plant, and microbial sources; however, microbial sources are more preferred for commercial usage.[6] The primary benefits of using microorganisms in the synthesis of amylase include their ability to perform a variety of modifications to produce enzymes with desired properties quickly.[7] In particular, bacterial amylases from *Bacillus spp*. are sought-after industrial organisms because of their ability to produce the extracellular enzymes and have rapid growth rates that result in fast fermentation times, which are crucial for the starch processing industries.[8] Bacillus species make up the vast majority of the bacteria that create cold-active amylases.[9] In order to produce cold active amylase for commercial use, *Bacillus spp*. (*Bacillus cereus*, *Bacillus amyloliquefaciens*, *Bacillus marisflavi* and *Bacillus subtilis*)[10,11,12] have been widely employed. Several of the species were isolated, characterized, and optimized for amylase synthesis.

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**Citation:** Samanta A, Pradhan S, Jana SC. Cold Active Amylase Production from *Bacillus cereus* RGUJS2023. Trop J Nat Prod Res. 2023; 7(11):5172-5177. http://www.doi.org/10.26538/tjnpr/v7i11.20

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

But industries still import this pricey enzyme from other nations today. In this situation, industries require highly active enzymes that will function at standard temperatures and have a broad pH tolerance for the manufacture of sugar.[13] Each biocatalyst can only react within a specific temperature range which increases its advantages and cost effectiveness.[14] In order to address the never-ending need for industrial enzymes, research is always being conducted to isolate novel microbial strains. According to Hamid *et al.*[15] a range of pharmaceuticals, fine chemicals, foods, and beverages must be manufactured at low temperatures in order to prevent high temperature chemical side reactions and contamination problems. The cold active enzyme used in this study doesn't require high temperature to activate which saves the company money and energy.[7]

In this paper, the production and characterization of a cold-active acidic α-amylase by *Bacillus cereus* RGUJS2023 obtained from soil was described.

## Materials and Methods

*Strain*

*Bacillus cereus* RGUSJ2023 (GenBank accession number OQ984972) was used in this study. This strain was isolated from garbage soil in Midnapur district, India, with the GPS coordinates of 87° 46' 34.8132" E and 21° 56' 14.2368" N.

*Culture Media*

The strain was grown in sterile starch peptone broth with a pH of 6.9 using the subsequent composition: Sigma soluble starch (0.5%), peptone (0.09%), $MgSO_4.7H_2O$ (0.01%), KCl (0.01%), $NaH_2PO_4$, $2H_2O$ (0.05%) and $(NH_4)_2HPO_4$ (0.04%) in the Orbital shaking incubator (REMI, CIS-24 PLUS, India) with 130-rpm at 37°C.[16]

*Study of growth kinetics with enzyme production*

*Bacillus cereus* RGUSJ2023 culture (5%) from exponential phase was transferred to grow in sterile starch peptone (SP) broth for 30 hours up

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

to its stationary phase. The fermented medium (5 mL) was taken out at various intervals to assess the starch content, extracellular enzyme activity, released reducing sugar, and growth optical density (O.D.).[17]

*Characterization of enzyme*

*Starch degrading pattern of the enzyme*

The reaction mixture, which contained 0.2 mL of crude enzyme, 1 mL of sodium phosphate buffer with the pH of 6.9 and 0.5 mL of 2% gelatinized starch, was incubated at 37°C. Then, the reaction mixture was withdrawn in 10 min intervals and their starch content with corresponding reducing power were determined by iodine staining method.[18]

*Effects of temperature and pH on the activity of α-amylase*

The crude enzyme was incubated with substrate at different temperatures between 7°C and 42°C to determine the cold activity of the enzyme. The pH optima of the enzyme were determined between 5.0 and 8.0. Amylolytic activity of the enzyme was measured by the method of Bernfeld[7,19] as above.

*Enzyme stability*

Stability of crude enzyme was studied by incubating the enzyme at different pH (6.0, 6.5, 7.0 and 7.5) in different temperatures (10°C, 30°C and 40°C) and from each experimental set, the incubated enzyme was assayed in several time intervals to check the activity. Triplicate sets of each test were performed.[20,21]

*Effect of metal ions*

Different concentrations (0.5 mM and 1 mM) of metal ions in sodium phosphate buffer (pH 6.9) were used to determine the effects of various metal ions (NaCl, KCl, $SnCl_2$, $MgCl_2$, $BaCl_2$, $CdCl_2$, $CaCl_2$, $MnCl_2$, $SrCl_2$, $CoCl_2$, $PbCl_2$, and $CuCl_2$) on amylase activity. The control set of experiment was prepared without metal ions.[20]

*Effect of substrate-enzyme reaction time*

The amylolytic activity was determined following the incubations of the amylase and substrate at 5 mins intervals (5 to 60 min) under laboratory conditions.[21]

*Substrate concentration*

The crude enzyme was added to varied starch concentrations (0.2 – 3%) and the activity was measured using method of Bernfeld[7] in order to observe the effect of different substrate concentration on amylase activity.[22,23]

*Statistical analysis*

The data were presented as the mean with standard deviation (mean±SD) and each experiment was performed in a set of three separate replicates (n = 3). Microsoft Excel on Windows 10 was used to analyze the data.

*Analytical methods*

*Growth measurement*

Bacterial growth was determined by measuring the absorbance of culture at 660 nm in the double-beam UV-vis spectrophotometer (BI-2700, BR Biochem, India).[18]

*Enzyme extraction*

Culture was centrifuged at 10,000 rpm for 10 min in a Refrigerated centrifuge (CPR-24 Plus, REMI, India) at 4°C and the supernatant was used as a crude enzyme for further experiments.[17]

*Enzyme assay*

Enzyme activity was determined according to the method of Bernfeld[19] by measuring the produced reducing sugar. The reaction mixture, which contained 0.2 mL of crude enzyme, 1 mL of sodium phosphate buffer with a pH of 6.9 and 0.5 mL of 2% gelatinized starch, was incubated at 37°C. After determination of the optimum temperature and pH, all enzyme assays were conducted at 28°C with a pH of 6.5. The reaction between the enzyme and substrate was stopped by adding sodium hydroxide solution [0.1 mL of 2(N)]. Additionally, 0.5 mL of 3,5-dinitro-salicylic acid (DNS) solution was added to the mixture to boil it for 10 minutes. The optical density at 540 nm was measured with a spectrophotometer, when the reaction mixture was cooled. Without a substrate, the control was generated the same way. The glucose standard curve was used to calculate how much glucose was produced. One unit of amylolytic activity (U/mL) is the amount of enzyme that releases the reducing sugar equivalent to 1 µmol of glucose per min per mL from starch solution under the optimum assay conditions.[16]

*Determination of starch content*

Starch content was determined by measuring the blue colour value from the reaction of 5 mL samples and 100 µL Lugol's iodine (0.2% iodine and 0.2% potassium iodide) in a spectrophotometer at 660 nm against iodine water solution as a blank, where pure starch (S9765-sigma) used as a standard.[18]

## Results and Discussion

*Study of growth kinetics with enzyme production*

The kinetics of the growth of *Bacillus cereus* RGUJS2023 showed that the enzyme production 3.721± 0.21 U/mL was initiated in early log phage (within 4 hours of incubation) with the cell growth at $O.D._{660\ n.m.,}$ 0.167±0.01 at pH 6.8 and maximum reducing sugar, 15.98±0.667 mg/mL was detected in 4 hours grown culture and starch utilization was almost completed within 6 hours of growth (Figure 1).

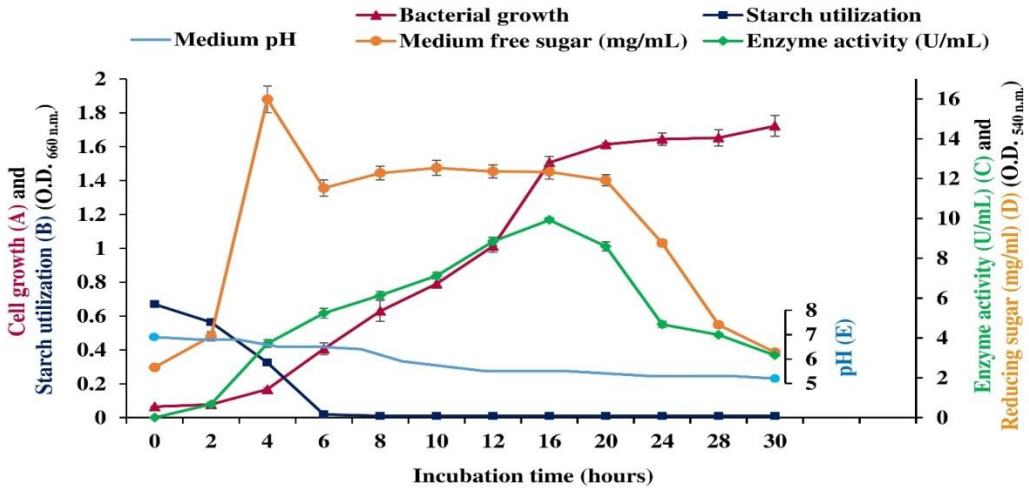


**Figure 1:** Growth kinetics study of *Bacillus cereus* RGUJS2023 with subsequent alpha amylase synthesis. Growth (A), starch utilization (B), Enzyme activity (U/mL) (C), free reducing sugar (mg/mL) (D) and pH (E).

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 1: Stability of alpha amylase at different temperature (°C) with pH 6.0, 6.5, 7.0 and 7.5.

| Incubated temperature (°C) | pH | Relative enzyme activity (%) 8 h | 16 h | 24h |
|---|---|---|---|---|
| 10°C | 6 | 95 ± 0.001 | 91 ± 0.001 | 83 ± 0.004 |
| | 6.5 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| | 7 | 100 ± 0.00 | 100 ± 0.00 | 100 ± 0.00 |
| | 7.5 | 88 ± 0.001 | 73 ± 0.001 | 65 ± 0.025 |
| 30°C | 6 | 87 ± 0.001 | 71 ± 0.002 | 59 ± 0.018 |
| | 6.5 | 100 ± 0.00 | 98 ± 0.001 | 96 ± 0.001 |
| | 7 | 100 ± 0.00 | 95 ± 0.001 | 91 ± 0.001 |
| | 7.5 | 74 ± 0.0012 | 56 ± 0.024 | 48 ± 0.030 |
| 40°C | 6 | 47 ± 0.002 | 38 ± 0.011 | 17 ± 0.048 |
| | 6.5 | 89 ± 0.0013 | 53 ± 0.018 | 41 ± 0.026 |
| | 7 | 82 ± 0.001 | 45 ± 0.026 | 28 ± 0.061 |
| | 7.5 | 74 ± 0.002 | 19 ± 0.042 | 9 ± 0.04 |

The values are represented as Mean ± SD here 100% relative enzyme activity is 13.172 U/mL.

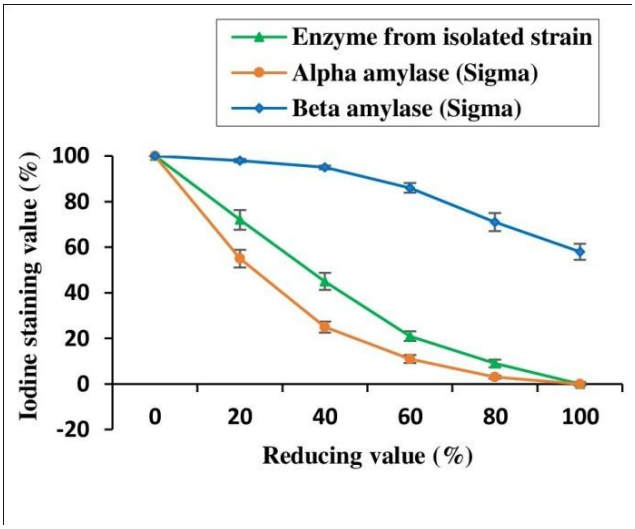


**Figure 2:** Iodine staining value and reducing value curves of isolated enzyme from *Bacillus cereus* RGUJS2023, Beta amylase and alpha amylase.

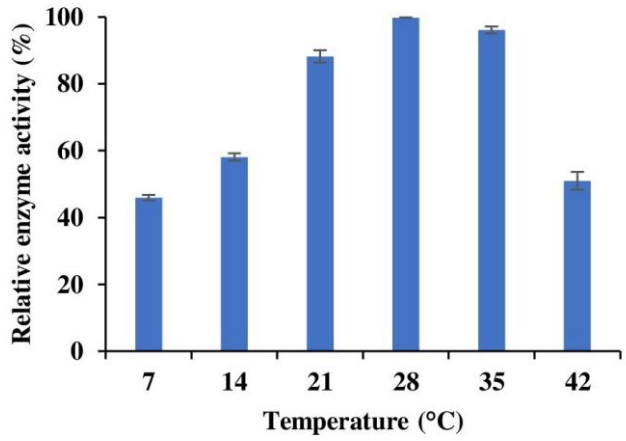


**Figure 3:** Effect of temperature (°C) on alpha amylase activity.

Instead of ceasing enzyme production, enzyme production was steadily increased with the low level of starch which was depleted abruptly in the fermentation medium within the 4 hours of growth. Starch was not detected in the culture media after 6 hours of growth ($O.D._{660\ n.m.}$ 0.406± 0.035) and the reducing sugar was decreased from 15.98± 0.667 mg/mL to 11.52± 0.41 mg/mL and remained same 12.16±0.152 mg/mL throughout the remaining exponential phase to early stationary phase (6 to 20 hours). Enzyme production increased 3.721± 0.21 U/mL to 9.922± 0.24 U/mL within 16 hours of exponential phage where the medium pH was lowered from 6.9 to 5.5. Maximum enzyme, above 7.122±0.176 U/mL was produced at late exponential phage to early stationary phase (10 to 20 hours) though highest production, 9.922±0.24 U/mL were observed after 16-hour incubation. Since the 16-hour growing culture produced highest enzymes, it served as the source of enzymes for all of the experiments. Amylase production from *Bacillus cereus* was reported maximum at 48 hours of incubation.[23] However, cold active amylase production from *Bacillus cereus* GA6 was maximum at 120 hours of incubation during exponential phage as per the report of Kuddus and Ahmad[11]. Suganthi *et al.*[24] reported highest amylase from *Bacillus vallismortis* was produced in 26 hours of incubation in late stationary phage. Mishra and Behera[22] also observed amylase were maximum produced from *Bacillus* sp. in 72 hours of incubation in the stationary phage. Again, the *Bacillus cereus* produced the highest amount of alpha-amylase after 72 hours from the A26MB[34], PW4 strains[35] and 48 hours from MK1 strain.[36] According to Kuddus and Ahmad[11] and Arabacı *et al.*[7], cold-active amylase production was also observed in *Bacillus* species after 120 hours and 24 hours of incubation, respectively. As a comparison of the cold active enzyme production time with the available literature source, it could be stated that this cold active enzyme production from the bacterial strain RGUJS2023 is much faster than the other reported results. So, the producer, *Bacillus cereus* RGUJS2023 and the cold active alpha-amylase of this study will be a great importance in the starch industry.

*Characterization of enzyme*

*Amylolytic nature*

The pattern of the starch degradation was determined in iodine staining value which showed the complete similarity with commercially available purified alpha amylase (9000-85-5-sigma) (Figure 2). This result disagreed with the findings of Ray *et al*. where they showed the beta amylase pattern of starch degradation.[17]

*Effect of temperature on the activity of alpha amylase*

The enzyme activity was observed at temperatures between 7 °C to 42 °C (Figure 3). Though this amylase showed optimal temperature at 28°C, it also showed activity below 10°C. In comparison to 28°C, activity was decreased below 14°C. This result is in agreement with

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

the observation of the Arabacı and Arıkan.[7] According to Zhang and Zeng,[25] this rapid decrease in enzyme activity beyond 40°C is a distinctive character of cold active enzymes. Cold-active amylase from the RGUJS2023 strain showed the highest activity of 99.8±0.02% (13.146 U/mL) at 28°C. As per the report by Ottoni *et al.*[33], cold-active amylase from Antarctica isolate showed the highest activity, approximately 90%, at 20°C and activity decreased at 0°C. Dou *et al.*[12] demonstrated that at 4°C, the amylase (AmyD-1) from marine-isolated *Bacillus sp.* exhibited about 35% of its cold activity. Cold active amylase from *B. cereus* RGUJS2023 showed 45.9±0.83% activity at 7°C. Cold-active amylase from *B. cereus* RGUJS2023 was better than other reported works.

*Effect of pH on the activity of alpha amylase*

The cold-active enzyme from *B. cereus* RGUJS2023 displayed good activity at wide pH levels, where an optimum pH was observed at 6.5 (100% active) (Figure 4). Amylase activity from *Bacillus cereus* showed above 90% activity at the pH 6 to 7. The activity of this enzyme was also observed 64.04±0.76% and 61±1.68% at 28°C with pH 5 and 8, respectively. According to Abo-Kamer *et al.*[28] report, highest amylase activity from *Bacillus cereus* was reported in acidic pH range at 5.5. Amylases activity was maximum at 6.0 pH from *Bacillus* spp. reported by Msarah *et al.*[20] and Evurani *et al.*[29] Arabacı and Arıkan[7] found that amylase activity was at optimum pH 8.0. The majority of cold active amylase production has been documented in alkaline pH ranges.[7,11,25,33] However, the current study employing *Bacillus cereus* RGUJS2023 produced the highest amount of cold active amylase at an acidic pH of 6.5.

*Stability of cold active alpha amylase*

This enzyme stability was investigated at a pH of 6–7.5 with a set of time intervals for each temperature of 10°C, 30°C and 40°C, and its activity was then assessed under laboratory conditions (Table 1). This study showed the enzyme was stable between the pH of 6.5 and 7.0 at refrigerator temperature (10°C). The stability of this enzyme was decreased at 40°C with 6.5 pH. Just 41% of the residual activity was finally recorded after 24 hours of incubation at 40°C. As per the report, Zhang and Zeng,[26] cold adapted amylase was less than 30% activity at the temperature 45°C after 1 hour incubation. This cold active enzyme was stable up to 96% and 91% at 30°C with the respective pH 6.5 and 7 after 24 hours of incubation. Cotarlet *et al.*[27] found that only 80% activity of amylase was present after 60 minutes of incubation at 30°C in the case of *Streptomyces 4 Alga*. Cold active N8 α-amylase was stable at 25°C in the pH range 8.0 to 12.0 with its 53% activity as per the report Arabacı and Arıkan.[7] From the comparison with the above discussed work, it was found that this cold active amylase was better than the reported results.

*Effect of metal ions on the activity of alpha amylase*

Table 2 showed the effect of metal ions on cold active amylase activity from *Bacillus cereus* RGUJS2023. Amylase activity remarkably increased in the presence of $Mn^{2+}$, $Mg^{2+}$ and $Sn^{2+}$ with 1mM concentration. But Enzyme activity was decreased in the presence of heavy metals such as $Pb^{2+}$ and $Cu^{2+}$. Enzyme activity in this investigation was mostly increased up to 132±0.046%, 122±0.004% and 1120±0.002% with the addition of (0.5 mM) $Mn^{2+}$, (1 mM) $Mg^{2+}$ and (1 mM) $Sn^{2+}$ respectively, as compared to the control. Evurani *et al.*[20] showed that activity of amylase from *Bacillus cereus* SM22 was increased in presence of NaCl, $Mg^{2+}$ and $Ca^{2+}$ and decreased by $Al^{3+}$. Qin *et al.*[30] also found that the cold active amylase activity was stimulated in the presence of $Sr^{2+}$, $Mg^{2+}$, $Fe^{3+}$ and $NH^{4+}$ and inhibited by $Cu^{2+}$. But as per the Arabacı and Arıkan[7] report, cold active N8 α-amylase was inhibited by $MnCl_2$ $CaCl_2$, $BaCl_2$ and $ZnCl_2$. Liu *et al.* found the best stimulator as $Co^{2+}$ and $Ca^{2+}$ and inhibitor as $Mn^{2+}$, $Hg^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Al^{3+}$ and $Fe^{3+}$ for cold active amylase activity.[31]

*Effect of incubation time on the activity of alpha amylase*

This alpha amylase showed above 97% relative activity in 30 min of enzyme-substrate incubation with 0.05M phosphate buffer (pH 6.5) at 28°C (Figure 5). Sethi *et al.*[21] reported the optimum enzyme-substrate reaction time at 60 min. According to Alva et al., highest yield was found during the enzyme -substrate reaction time at 5 min.[31]

**Table 2:** Effect of metal ions on alpha amylase activity

| Metal ions | Relative enzyme activity (%) at that concentration | |
|---|---|---|
| | 0.5 mM | 1 mM |
| Control | 100 ± 0.00 | |
| $SnCl_2$ | 112 ± 0.002 | 109 ± 0.001 |
| NaCl | 98 ± 0.001 | 97 ± 0.001 |
| KCl | 97 ± 0.001 | 95 ± 0.001 |
| $MgCl_2$ | 120 ± 0.031 | 122 ± 0.004 |
| $BaCl_2$ | 104 ± 0.00 | 103 ± 0.00 |
| $CdCl_2$ | 87 ± 0.003 | 38 ± 0.050 |
| $Mncl_2$ | 132 ± 0.046 | 127 ± 0.047 |
| $SrCl_2$ | 89 ± 0.04 | 78 ± 0.008 |
| $CoCl_2$ | 96 ± 0.00 | 82 ± 0.062 |
| $PdCl_2$ | 46 ± 0.002 | - |
| $CuCl_2$ | 37 ± 0.004 | - |

The data is shown as Mean ± SD; in this case, 100% relative enzyme activity is 13.172 U/mL.

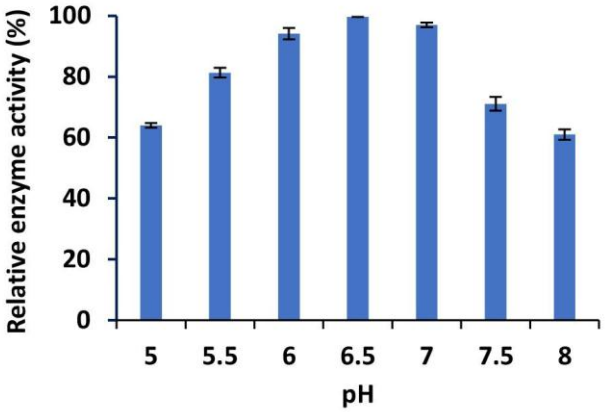


**Figure 4:** Effect of pH on alpha amylase activity.

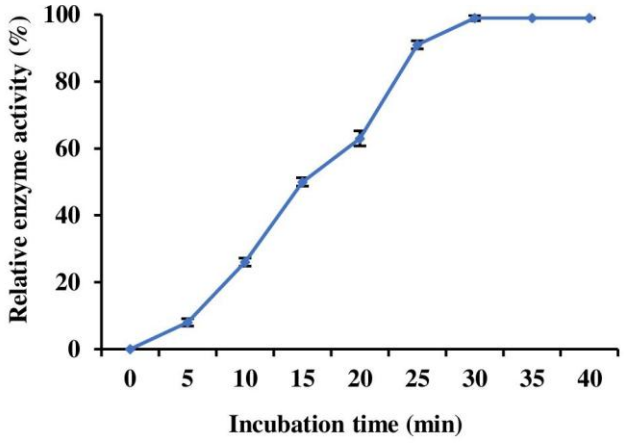


**Figure 5:** Effect of incubation time on alpha amylase activity at 28°C and pH 6.5.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

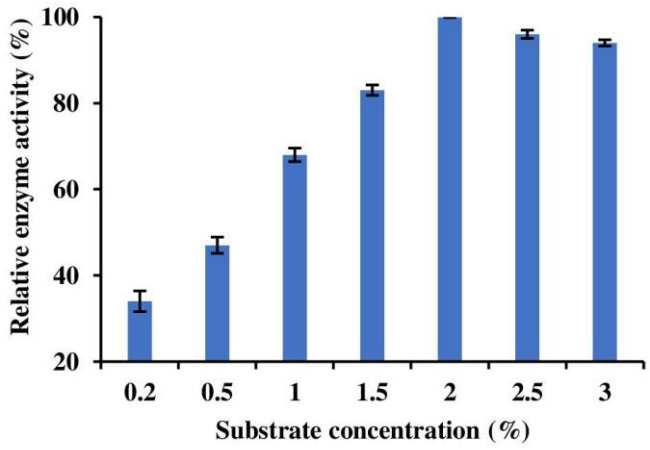


**Figure 6:** Effect of substrate concentration on alpha amylase activity at 28°C and pH 6.5.

*Effect of substrate concentration on the activity of alpha amylase*

The optimal substrate concentration for enzymatic reaction was estimated as 2.0% sigma starch as recorded in this work. Up to 2.0% of substrate, the amylase activity was increased, then activity was decreased (Figure 6). In their investigation, Mishra and Behera[22] reported a positive influence of 2% starch on amylase activity by *Bacillus sp.* Sethi *et al*.[21] and Doss and Anand[23] also showed that purified amylase activity from *Aspergillus* species were maximum in 1.5% to 2% substrate.

## Conclusion

The above findings demonstrate that *Bacillus cereus* RGUJS2023's alpha-amylase had outstanding activity and stability at low temperatures with acidic pH. The enzyme also showed higher activities in the presence of some metal ions. This enzyme may be designated as a novel cold active, acidic alpha amylase for starch processing industries in cold environments without maintaining the temperature, saving the energy and production cost. The novelty of this investigation with *Bacillus cereus* RGUJS2023 is the first report of the shorted time in cold amylase production that is within 16 hours of incubation. Additionally, this organism can be used in cold environment for bioremediation for cleaning starchy waste. More research is necessary not only for the commercialization of the enzyme but also for the proper utilization of this new bacterial strain *Bacillus cereus* RGUJS2023.

## Conflict of Interest

The authors declare no conflict of interest.

## Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

## Acknowledgements

Acknowledgments: The authors are thankful to Raiganj University, Raiganj, 733134, W. B., India. Authors are also thankful to professor Nirjhar Sarkar, Department of English, Raiganj University, Raiganj, West Bengal, India and L. K. Sahoo, Associate professor, Department of CIS, Raiganj University, Raiganj, West Bengal, India for their English editing of the text and also for the checking statistical analysis of the results. Amrita Samanta is grateful to Government of West Bengal, India for Swami Vivekananda Merit Cum Means Ph. D. Scholarship.

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