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Comparative Study of Alpha Amylase Production by Locally Isolated Bacillus and Aeromonas Species from Various Soil Habitats Around Ranchi District

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Abstract:

This study focuses on the isolation, production and comparative analysis of α-amylase from strains of Bacillus and Aeromonas using three different habitats (cow dung dump waste site, fruit and vegetable waste dump site and rice field waste dump) site. Alpha amylase (α-1,4-glucan-glucanhydrolase, EC 3.2.1.1) is an extracellular enzyme that breaks down α-1,4 glycosidic linkages in starch and related substrates, producing oligosaccharides such as maltose, glucose, and alpha limit dextrin. Results showed that the α-amylase production by *Bacillus subtilis* strain was highest with 8.57 IU/ml/min and *Aeromonas jandaei* produced minimum amylase which is 4.80 IU/ml/min among all the six strains. Upon optimization of various parameters maximum production was observed at 24 hours to 48 hours, and the crude enzyme characterization revealed optimal activity at pH 7 and 37°C.

Key words: soil bacteria; enzyme; amylase; molecular identification; optimization; starch.

1. Introduction:

To support life, there are approximately 3,000 known enzymes, but only a few are utilized in industrial applications (Shipra Das et al. 2011). These are primarily extracellular hydrolytic enzymes (Shivaji Bole et al. 2013) that break down naturally occurring polymers like starch, proteins, pectin, and cellulose (Bhaskar Rao et al. 2011). Amylase is an enzyme that breaks down starch into sugar (Shyam Sundar et al. 2013). Starch, a primary storage polysaccharide, is a crucial component of the human diet and is enzymatically processed into various products

for consumption (A Pandey et al. 2000). It is broken down by amylolytic enzymes. While amylases can originate from animals, plants, and microorganisms, microbial amylases are particularly suited to industrial demands due to their short growth period and high productivity (S. Mohamad et al.2014)(L. Yanhong et al.2017). Due to its extensive applications in the food, starch liquefaction, saccharification, detergent, brewing, paper, textile, and distilling industries, there is a growing demand for increased indigenous production of α-amylase (R. Gupta et al 2003). With advancements in biotechnology, the range of amylase applications has expanded into various other fields, including clinical, medical, and analytical chemistry. Many industrial enzymes are extracellular and derived from microorganisms (R. Vidyalakshmi et al. 2009). Among these, α-amylase is the most commercially exploited. Bacteria and fungi secrete amylases outside their cells to perform extracellular digestion. After breaking down soluble starch, the resulting products, such as glucose or maltose, are absorbed back into their cells. D. Monnet et al, 2010 mentioned that the demand for microbial amylases has increased due to their reaction specificity, the mild conditions required, and lower energy consumption compared to traditional chemical methods. The ability of microorganisms to produce amylase depends on factors such as the strain type, growth medium, cultivation technique, cell growth, nutritional requirements, incubation time, pH, temperature, presence of metal particles, and thermostability (V. Rivathsan et al. 2022). Notable Bacillus strains used extensively for alpha amylase production include B. amyloliquefaciens, B. licheniformis, B. stearothermophilus, B. subtilis, B. megaterium, and B. circulans. Mostly all Bacillus strains are Gram-positive, endospore-forming bacterium found in soils and plant materials worldwide. This organism is widely used in large-scale industrial production of exoenzymes due to its ability to secrete significant amounts of proteins, ranging from 20–25 g/l. Utilizing submerged culture is beneficial because it facilitates easy sterilization and process control.

This study aimed to examine the pattern and compare the production of α-amylase by three strains of Bacillus and three species of Aeromonas, using four different carbon sources

2. Materials and methods:

2.1 Sample collection:

Soil samples were collected from cow dung dump waste site, fruit and vegetable waste dump site and rice field waste dump an agricultural field in, all located in the Ranchi district.

2.2 Isolation:

Isolation of bacterial strains from soil samples involved diluting the samples using a serial dilution technique, where approximately 0.1 ml from dilutions of 10^{-5}, 10^{-6}, and 10^{-7} were used. Colonies producing amylase were screened by aseptically transferring dilutions of each soil sample onto starch agar plates using the spread plate technique. These plates were labelled as "C", "F", and "R" respectively, and then incubated at 37°C for 24 hours (Bharat Pokhrel et al. 2013).

2.3 Screening:

The colonies producing amylase were screened using the starch iodine test (Kunal Madhav et al. 2011). Isolates from each sample demonstrating the largest zone of clearance and similar morphology were chosen for further study (Arvinder Kaur et al. 2012). Pure cultures of each isolate were obtained through repeated sub-culturing and designated as "C", "F", and "R" respectively.

2.4 Biochemical analysis

The isolates were identified through a series of biochemical tests, including Gram staining, Methyl red test, VP test, Indole test, Urease test, Catalase test and oxidase test. The results were analyzed according to Bergey's manual of microbiology (Musliu et al. 2012, Sasmita Mishra and Niranjan Behera, 2008).

2.5 Amylase production

The isolates were induced to produce amylase by transferring 1 ml of culture aseptically into a complex production medium containing starch (1 g/100 ml), yeast extract (0.04 g/100 ml), diammonium hydrogen phosphate (0.4 g/100 ml), potassium chloride (0.1 g/100 ml), and magnesium sulphate hepta hydrate (0.05 g/100 ml). The cultures were then labelled accordingly and incubated at 37°C in a shaking incubator.

2.6 Extraction of crude amylase and quantification (enzyme assay)

The crude enzyme from each isolate was obtained after various incubation periods (12 hrs, 24 hrs, and 36 hrs) by centrifuging 10 ml of culture at 5000 rpm for 20 minutes at 4°C . Enzyme

activity was quantified using the colorimetric method described by Fisher and Stein. In this procedure, 1.0 ml of crude enzyme was placed in appropriately labelled test tubes and 1.0 ml of substrate (soluble starch) was added to each tube. The tubes were covered and incubated at 35°C for 15 minutes in a water bath. After incubation, 2.0 ml of DNS reagent was added to each tube to halt the reaction, followed by boiling in a water bath for 5 minutes. The absorbance was then measured at 590 nm using a colorimeter against a blank that had the reaction stopped without prior incubation (0 time blank).

The rate of amylase activity was quantified by comparing against a standard graph of maltose, and the results were expressed in terms of maltose liberated per ml of crude enzyme after 15 minutes of incubation with soluble starch substrate.

2.7 Extraction of genomic DNA: Colonies from a single streak on the agar plate were scraped and suspended in PBS, then centrifuged. The resulting pellet was dispersed, and 600 µl of cell lysis buffer (guanidine isothiocyanate, SDS, Tris-EDTA) was added. The mixture was inverted for 5 minutes and incubated for 10 minutes with gentle mixing until the suspension appeared nearly transparent. Next, 600 µl of isopropanol was layered on top of the solution. The layers were mixed gently until white strands of DNA became visible and the solution was homogeneous. The DNA strands were spooled using a pipette tip and transferred into a new vial. Then, 500 µl of 70% ethanol was added to the spooled DNA, which was spun at 10,000 rpm for 10 minutes to precipitate the DNA. The supernatant was discarded, and the pellet was air-dried without completely drying it. Finally, 50 µl of 1X TE was added to the pellet, which was then suspended and incubated for 5 minutes at 55–60°C to increase the solubility of the genomic DNA (H. G. Murray and W. F. Thompson , 1980).

2.8 Amplification of the 16srRNA gene: Amplification of the 16S rRNA gene was performed using the primer sets 16S rRNA fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rP2 (5′-ACGGCTACCTTGTTACGACTT-3′) [13]. PCR was conducted in a total volume of 50 µl, starting with an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1.5 minutes, with a final extension at 72°C for 5 minutes. The PCR products were then subjected to electrophoresis in 1% agarose gel, stained with ethidium bromide, and visualized under ultraviolet (UV) light. The length of the amplicon was approximately 1.5 kb (W. G. Weisburg et al. 1991).

2.9 16srRNA gene analysis and identification of bacteria

The amplified DNA was visualized using gel electrophoresis, and the PCR products were purified with the Gel Extraction Kit (Omega Bio-tek). The purified products were then sequenced using the Sanger method. The most similar bacterial species were identified through a BLAST search on GenBank (http://www.ncbi.nlm.nih.gov/). Neighbor-joining phylogenetic trees were constructed based on the 16S rRNA sequences using ClustalW.

2.10 Optimization of different parameters for amylase production

2.10.1 Optimization of different pH

When the pH deviates from the optimum, amylase activity decreases. The effect of pH on amylase activity was assessed by preparing buffers at various pH levels (3, 5, 7, 9, and 12) and conducting an enzyme assay using the DNS method under standard conditions at 540 nm.

2.10.2 Optimization of different temperature

The stability of the enzyme is always influenced by temperature. The effect of temperature on amylase activity was determined by incubating the reaction mixture at various temperatures (4°C, 28°C, 37°C, 55°C, and 80°C) and then performing the assay under standard conditions.

2.10.3 Optimization of different incubation time

The enzyme activity produced in the media was monitored using an enzymatic assay method at regular intervals of 24, 48, 72, 96, and 120 hours. This was done to determine the time period and the substrate-containing medium that resulted in the highest enzyme production.

3. Results and discussion

3.1 Isolation

From all the three soil samples a total of 36 bacteria were isolated. Pure culture was maintained for further investigation.

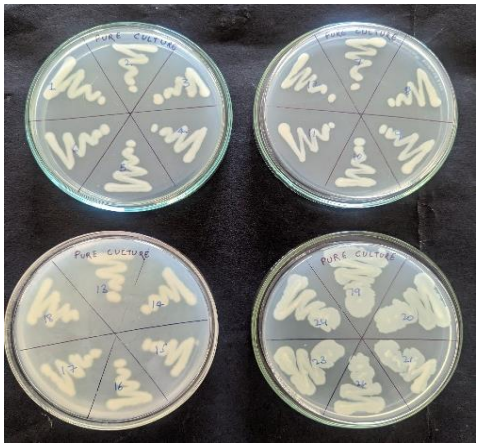


Fig 1: pure culture of 1-24 isolates

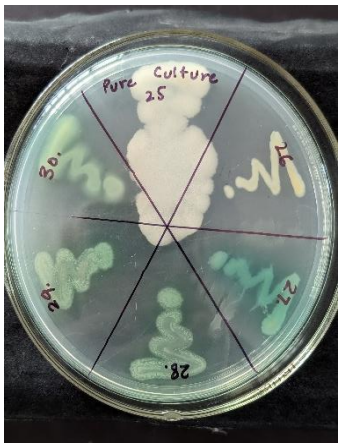


fig2: pure culture of 25-30 isolates

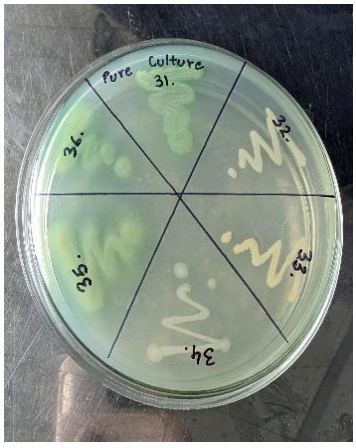


fig 3: pure culture of 31-36 isolates

3.2 Iodine test

For the confirmation of amylase production, starch hydrolysis test was performed and total of 19 bacteria showed positive result. Results are mentioned in table number 1.

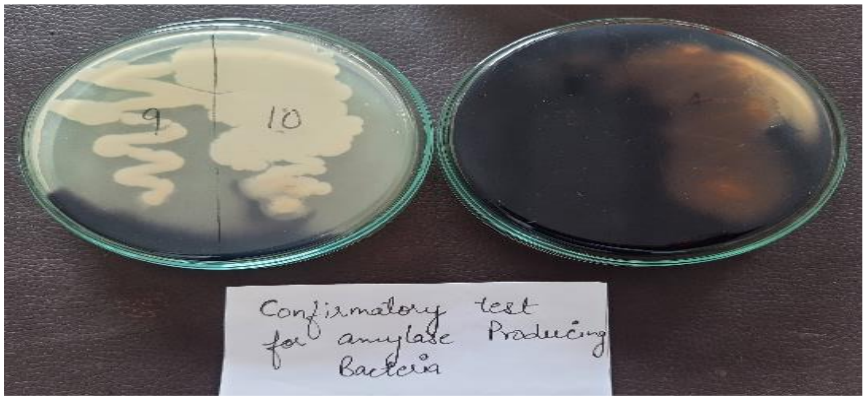


Fig 4: starch hydrolysis test for amylase

Table 1: Number of isolates showing zone of clearance

Sr. no	Area	No of Isolates	No of isolates showing zone of clearance
1	Fruit and vegetable waste dump site	16	9
2	Dairy waste dump site	9	4

3	Rice field waste dump site	13	6

3.3 Biochemical analysis

Biochemical analysis of all the strains is mentioned in the table number 2.

Table 2: Biochemical characterization of all the isolated bacteria

Sr. no	Bacteria	Gram staing	catalase	oxidase	MR	VP	urease	Indole	Colony colour
1	*A.jandaei*	-ve short rod	+ve	+ve	-ve	+ve	-ve	+ve	green
2	*A.hydrophila*	-ve	+ve	+ve	-ve	+ve	-ve	+ve	green
3	*A.veronii*	-ve	+ve	+ve	-ve	+ve	-ve	+ve	green
4	*B.subtilis*	+ve rod	+ve	-ve	-ve	+ve	-ve	-ve	creamy
5	*B.cereus*	+ve rod	+ve	-ve	-ve	+ve	-ve	-ve	white

3.4 Molecular identification

Molecular identification reveals three species of Bacillus and three species of Aeromonas. The sequence of all the strains were submitted in the NCBI and accession number was obtained. Details of all the identified strains are mentioned in table number 3.

Table 3: Identified Bacteria with accession number and its source of isolation

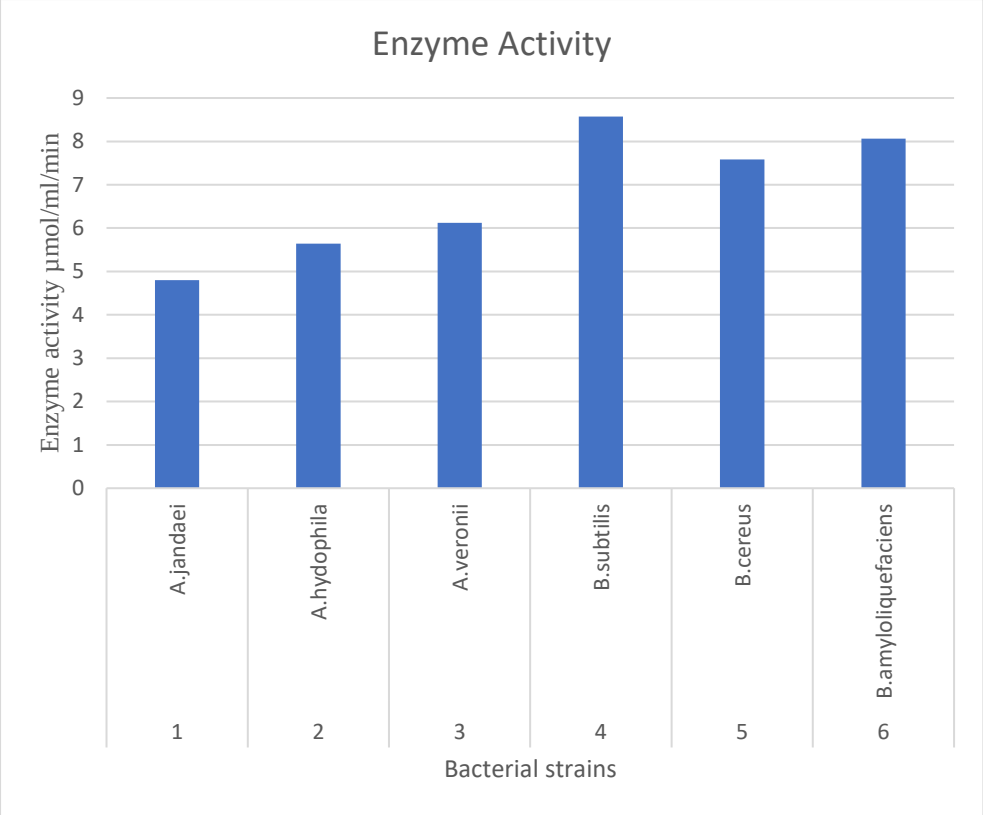
Sr. No.	source	Bacteria	Accession no
1	Fruit and vegetable	*Aeromonas hydrophila*	OR136168
2	Fruit and vegetable	*Aeromonas jandaei*	OR136166
3	cow dung	*Aeromonas veronii*	OR136284
4	Rice field	*Bacillus subtilis*	OR357659
5	Cow dung	*Bacillus cereus*	OR136189
6	Rice field	*Bacillus amyloliquifacens*	OR244382

3.5 Enzyme activity

The result of enzyme assay showed that among all the strains, *Bacillus subtilis* performed highest which is 8.57 µmol/ml/min and *Aeromonas jandaei* showed minimum enzyme activity which is 4.80 µmol/ml/min. the activity of all the strains is mentioned in table number 4.

Table 4: Enzyme activity of all isolated bacteria

Sr no.	Bacterial isolates	Enzyme Activity(µmol/ml/min)
1	*A.jandaei*	4.80
2	*A.hydophila*	5.64
3	*A.veronii*	6.12
4	*B.subtilis*	8.57
5	*B.cereus*	7.58
6	*B.amyloliquefaciens*	8.06



Graph 1: Graph showing enzyme activity of all the isolated straina.

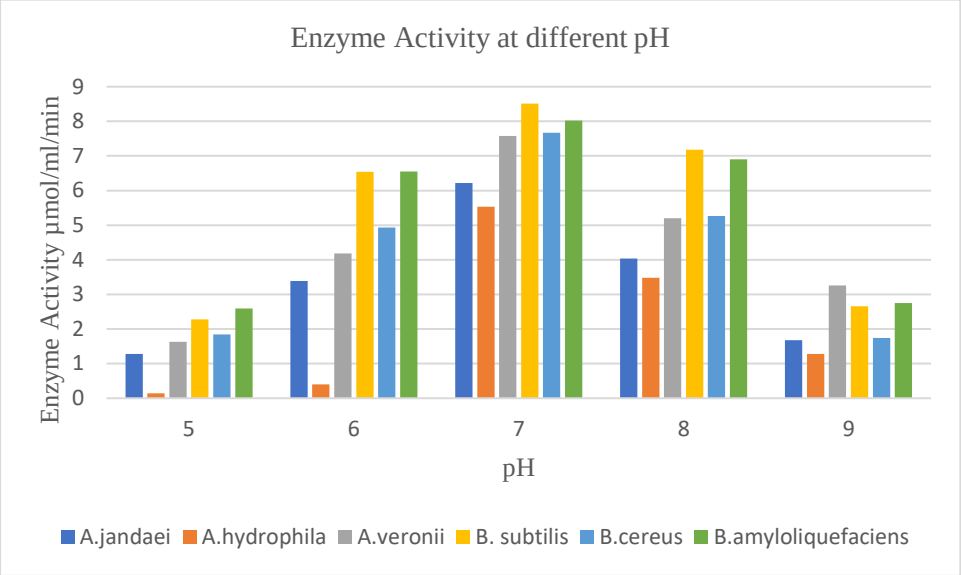
3.6 Optimization of parameters

3.6.1 Enzyme activity at different pH

Enzyme activity was calculated at different pH for maximum enzyme production. The result of which is mentioned in table number 5.

Table 5: Enzyme activity of all isolated strains at different pH

pH	*A.jandaei*	*A.hydrophila*	*A.veronii*	*B. subtilis*	*B.cereus*	*B.amyloliquefaciens*
5	1.28	0.14	1.63	2.28	1.84	2.59
6	3.39	0.4	4.18	6.54	4.93	6.55
7	6.22	5.53	7.58	8.51	7.67	8.02
8	4.04	3.48	5.2	7.18	5.27	6.9
9	1.68	1.28	3.26	2.66	1.74	2.75



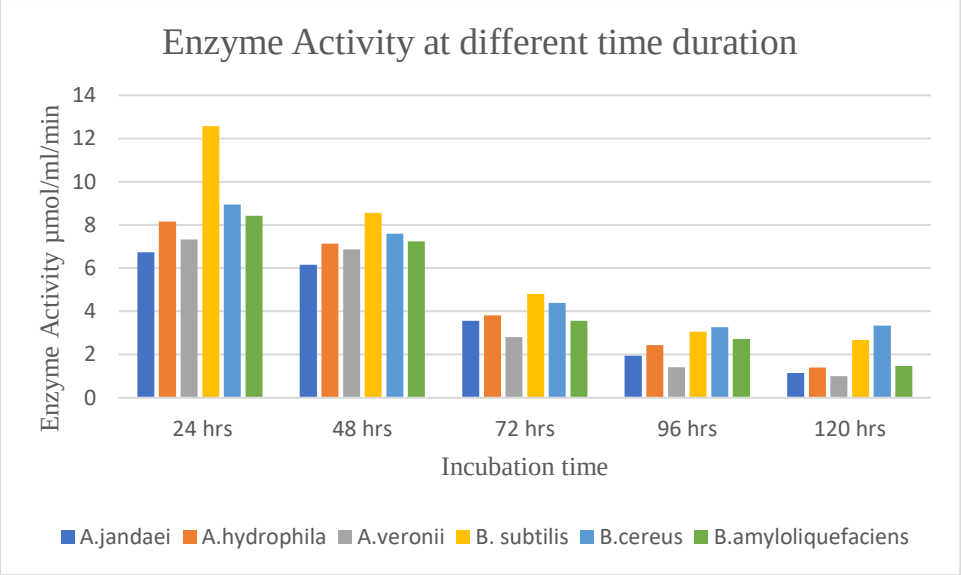
Graph 2: **Enzyme activity of all isolated strains at different pH**

3.6.2 Enzyme activity at different incubation time

To check the maximum production of enzyme all the strains were incubated for different time duration. The enzyme activity was calculated after every 24hours and the results are mentioned in the table number 6.

Table 6: Enzyme activity of all isolated strains at different incubation time

	A.jandaei	*A.hydrophila*	*A.veronii*	*B. subtilis*	*B.cereus*	*B.amyloliquefaciens*
24 hrs	6.73	8.15	7.32	12.58	8.94	8.43
48 hrs	6.15	7.13	6.87	8.55	7.6	7.24
72 hrs	3.56	3.81	2.81	4.8	4.39	3.56
96 hrs	1.94	2.43	1.41	3.06	3.27	2.72
120 hrs	1.15	1.39	0.99	2.67	3.34	1.47



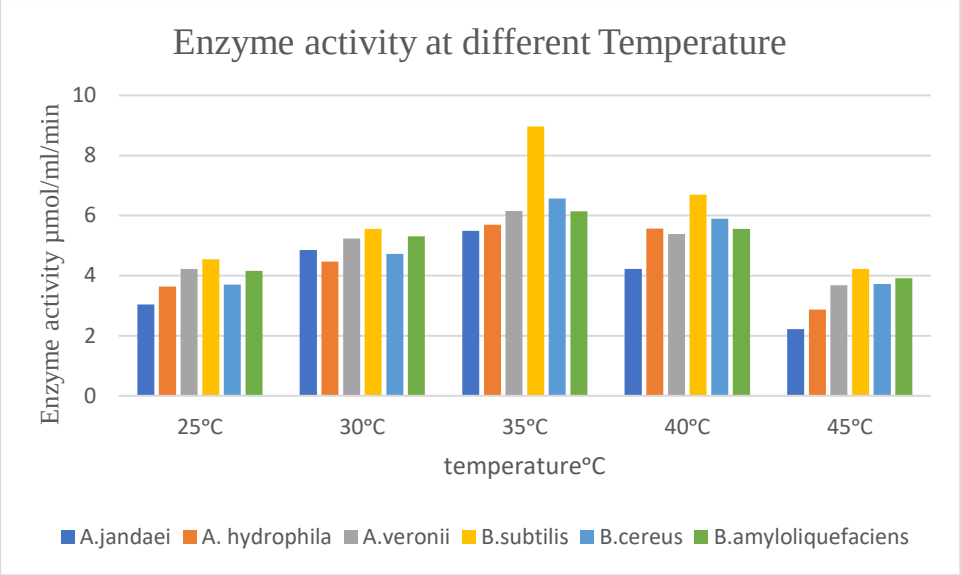
Graph 3: Enzyme activity of all isolated strains at different incubation time

3.6.3 Enzyme activity at different temperature

All the strains were incubated at different temperature and after thar enzyme activity was calculated and mentioned in table number 7.

Table 7: Enzyme activity of all isolated strains at different temperature

Temp.	*A.jandaei*	*A. hydrophila*	*A.veronii*	*B.subtilis*	*B.cereus*	*B.amyloliquefaciens*
25°C	3.04	3.64	4.22	4.54	3.7	4.16
30°C	4.85	4.469	5.24	5.55	4.72	5.31
35°C	5.49	5.69	6.15	8.96	6.57	6.14
40°C	4.22	5.57	5.38	6.7	5.9	5.55
45°C	2.22	2.87	3.68	4.22	3.72	3.91



Graph 4: Enzyme activity of all isolated strains at different temperature

. Conclusion

From this study we observed that among both the genus bacillus species showed slightly higher enzyme activity than Aeromonas species. Bacillus subtilis which exhibit the maximum enzyme activity was isolated from the rice field and rice is major food grain in Jharkhand. So there is a potential of research work in this area. There is extensive work done on the amylase worldwide but no paper has been published from Ranchi till date. The findings from this research underscored the amylolytic potential of all the isolates, all of which were isolated from dairy, fruit and vegetable and rice field waste dump sites. These bacteria have potential to help in bioremediation. With optimization processes, the production of α-amylase by these isolates can be further enhanced, making them promising candidates for industrial amylase production in Jharkhand.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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