Isolation, characterization, partial purification of α-amylase from *Bacillus species* M7 and its applications.

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INTRODUCTION

Enzymes can be used as biocatalysts in a wide range of chemical processes. One of the most significant enzymes in a variety of sectors is α -amylase. It catalyzes the breakdown of substrate starch to monosaccharides, disaccharides, dextrin and simple sugars. α - amylase is made by a wide range of living creatures, including bacteria, plants, and humans. All variety of living organisms like plants, animals, humans, fungi and bacteria, are produced amylase enzyme. In industrial level, commercially amylase enzyme is mostly produced from bacteria and fungi. Bacteria and fungi produce α-amylase as extracellular enzyme. These α-amylase producing microbes are generally present in soil to degrade carbohydrates present in the waste or soil to simple sugars to be utilized by others. They convert soluble end products from insoluble starch. α-amylase act on α-1,4 glycosidic bonds of starch to form maltose, maltotrioses, maltotetroses, glucose and a mixture of malto-oligosaccharide (Anupama and Jayaraman, 2011). Amylase enzymes are divided into three types: α-amylase, β- amylase, and γ-amylase. Amylase production now accounts for up to 30% of the global chemical market and is growing at a rapid pace. Because of their quick growth rates that Result in short development cycles, simplicity, ability to transfer proteins into extracellular medium, greatest yield, and overall management success, amylase extracted from microbes has varied applications in biotechnology (Yaseen et al., 2021).

The production level of amylase by microorganisms is varies, even they have same genus, species, and strain. Also, the production level of amylase differs dependent on the microorganism's origin, i.e. amylose rich environments or starch strains obviously have higher amounts. Production of amylase also depends on various factors like, temperature, carbon, pH and nitrogen sources, particularly in fermentation processes. Microorganisms can also be altered to produce efficient amylases that are thermostable and stable at strict conditions. This

also reduces the background protein contamination and also reduces the time of reaction, and this leads to less energy spending in the amylase reaction (Sundarram et al., 2014).

Current molecular techniques such as genetic engineering and recombinant DNA technology are used to stimulate efficient enzyme production (Cai et al., 2011). By using these technology, higher yields of amylase are possible due to the high-copy numbers of the gene promoter (Son et al. 2016). Previous studies conducted by deleting amyR (encoding a transcription factor) from *A. niger* CICC2462, that leads to the production of enzyme/protein specifically with lower background protein secretion.(Zhang et al., 2016) and also they have generated a new strategy to express the α-amylase from *Pyrococcus furiosus* in B. *amyloliquefaciens*.(Wang et al., 2016) By optimization studies, we can enhance the amylase production and this can be achieved either experimentally or by using design of experiments (DOE). (Garc´ıa et al., 2002 and Chang et al., 2002).

Production of amylase from microbes is economical since it is easy to cultivate and produce enzymes and proteins extracellularly, under appropriate cultural conditions. In industry, the significant amylase producing organisms include *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus cereus* and *Bacillus megaterium* and parasites like *Penicillium*, *Aspergillus niger*, *Cephalosporium, Neurospora* and Rhizopus. Other organisms like actinomycetes, yeast and fungi can be used for production of amylase (Kizhakedathil et al., 2021).

Applicability of the amylase:

Among the all enzymes, amylase marks up nearly 25% of the world enzyme market (Beg et al., 2001). It is primarily utilized in the manufacturing of materials, paper, paint, food, and food preparation. Amylase appears to be a promising invention in bioethanol production and an enzymatic enzyme that allows for extremely precise maturation and substrate hydrolysis cycles, resulting in higher returns (Bhatt et al., 2020). In food industry its applications involve the production of syrups (maltose, corn, glucose), juices, alcohol fermentation and baking (Beg

et al., 2001) and also used for making detergents. In wine industry used in beer and liquor brewing from sugars (based on starch).

Now a day, α-amylase enzyme is important for many industries, its demand increases day by day due to industrial development and increase in population. Many researchers are trying to isolate new microbial strain and new processes to increase the production of enzyme. So, in this work, we made an attempt to isolate high amount of α-amylase enzyme producing bacteria from soil and analyzed the production of enzyme in large quantity and find out the optimum condition for high enzymatic activity and to determine its wash performance (Basma et al., 2015).

MATERIALS AND METHODS

Sample collection

Soil samples were collected from Musuti land farm, beside Dharwad- Uppinbetageri, District-Dharwad of Karnataka state. Soil samples were collected from the depth of 4-5 cm using a sterilized spatula and kept in sterilized polythene bags and brought to laboratory. In the laboratory, all samples were kept refrigerated until isolation (Rakaz et al., 2021).

Isolation of organism from soil sample

Serial dilution method was used for isolation. Briefly, 0.25g of soil was dissolved in 25mL of sterilized distilled water in conical flask. Six test tubes containing 5 ml of sterilized distilled water were used for serial dilution (10-1-10-6). A known volume of sample from 10-5and 10-6 test tube was spread on Nutrient agar plate and incubated at 37˚Cfor 1-2 days. The colonies having distinct differences were picked and purified. The purified isolates were sub-cultured and preserved in refrigerator for further analysis and studies (Yaseen et al., 2021).

Screening and selecting amylase-producing bacterial isolates

Starch hydrolysis (1%) method was used for screening of isolates. A loopful of isolates were inoculated on starch agar plates and incubated at 37˚C for 24 hours. Later, it was flooded 1%

iodine solution. Plates were undisturbed for few minutes and the solution was discarded. The plates were observed for the formation of clear zone around the colonies and the diameter of clearance was measured. The isolates exhibiting largest clear zones around them were selected for further studies (Yaseen et al., 2021)

Identification of amylase-producing bacterial isolates

Colony Characterization.

Different colony characteristics like the colour, shape, size, elevation, pigmentations were observed. (Rakaz et al., 2021).

Microscopic Observation.

Gram staining, Endospore staining, Capsule staining and motility of the cells were performed to observe the general microscopic features (Rakaz et al., 2021).

Amylase production:

Fermentation media.

Fermentation media containing starch, peptone, $MgSO_4$, NaH_2PO_4, $(NH_4)_2SO_4$, and KCl was used. 1ml of Freshly prepared inocula was inoculated in media and kept in rotary shaker incubator with the speed of 7000rpm for 8 days. 1ml of the inoculum was withdrawn each day which served as enzyme source and enzyme assay was carried out (Rakaz et al., 2021).

Enzyme assay.

Alpha amylase activity was determined using dinitrosalicylic acid (DNS) method. Briefly, to the tube labelled blank: 100µl of enzyme and 900µl buffer solution, 1ml of starch was added and then denatured by boiling it in hot water bath. To the test sample, 1ml substrate, 500µl of enzyme and 500µl of buffer was added and incubated at 25˚C for 15minutes and 1ml of DNS and kept in boiling water bath for 5 minutes. 7 ml of water was added and absorbance was recorded at 540nm. Amount of maltose produced was calculated using the standard graph and the activity of enzyme was calculated. Maltose was used for determination of activity (Desai

et al., 2008). Enzyme assay is defined as the amount of enzyme required to produce one micromole of product in one minute.

Estimation of Soluble Protein.

Soluble protein concentrations were determined Lowry's method using Bovine Serum Albumin(BSA) as standard. Briefly, two test tubes were taken and labelled as blank and test. To the test, known volume of enzyme and distilled water was added. 1ml of distilled water was added. To both tubes, 5ml of Alkaline Copper Sulphate (ACS) was added and incubated for 10 min. Then, 0.5ml of Folin-Ciocalteau reagent (FCR) added and incubated for 30 min in dark condition. After incubation the volume was made up to 10ml by additing distilled water. The absorbance was recorded at 660nm. The protein concentration was determined by using standard graph of BSA (Desai et al., 2008).

Partial purification of α-amylase.

The partial purification of amylase enzyme was carried out using ammonium sulphate precipitation and dialysis. All the steps were carried out at 4˚C. Different saturation (0-60%, 60-70%, and 70-80%) of ammonium sulphate was used. The crude enzyme was treated with ammonium sulphate and kept for overnight in a magnetic stirrer. The precipitate obtained were collected by centrifugation (10,000g for 15min).It was dissolved in 0.1M phosphate buffer, pH 7.0.

Dialysis for the salt precipitated enzyme was carried out using the same (Kikani and Singh, 2011) for 12h with several changes of buffer to remove the salt. Enzyme assay was carried out by the method described earlier (Singh et al., 2014, David et al., 2017).

Characterization of α-amylase.

Enzyme was characterized for various parameters. Optimum temperature of enzyme was determined using different temperatures like 7˚C, 17˚C,27˚C, 37˚C, and 47˚C (4). The optimum pH of α-amylase determines using buffers of different pH ranges. acetate buffer (pH3.5-5.5),

phosphate buffer (pH 6.0-7.5), and Tris-HCl (pH 8.0 to 9.0) (6). To determine kinetic parameters, the substrate starch was taken in the range of 0.1 to 1.0 ml and same procedure was followed. Kinetic parameters K_m and V_{max} were calculated by drawing Michelis-Menton and Lineweaver-Burk plots (Xian et al., 2015). The catalyst activity of enzyme was analyzed using different chemicals like NaCl, Kcl, $CaCl_2$, and EDTA**.** To evaluate the effect of these activators or inhibitors, 5mM of these solutions was incubatedwith enzyme at 37˚C and then assayed for enzyme activity in the presence of metal ions (Tabassum et al., 2014).

Amylase applicability.

The partially purified enzyme was used to study its applicability by using it in the Laundry detergent formulation. White cotton cloth pieces (7 × 7 cm) were were soiled with spinach gravy and chocolate. The stained cloth pieces were kept undisturbed for 12 hrs and then used for experimentation. Different expermiments were carried out as follows.

Flask Number	Distilled water(mL)	Detergent(1%)	Enzyme solution
1	100	--	--
2	98	2	--
3	96	2	2

Experiments carried out using amylase enzyme to study wash performance

The above flasks with stained clothes kept in separate flasks were incubated at 28˚C for 30 min in a rotary shaker, later they were rinsed with cold tap water and dried. Untreated cloth pieces stained with gravy and chocolate were taken as control (Tallapragada et al., 2017).

Statistical analysis.

One-way analysis of variance (ANOVA) and regression analysis were carried out. Data will be expressed as means ±standard deviation (SD). The differences among mean will be assessed via least significance differences (LSD) tests. The significance level will be set $p<0.05$ (Simair et al., 2017).

Experimental design.

All the experiments will be carried out in duplicate. For optimization, OFAT (one factor at a time) will be followed.

RESULTS AND DISCUSSION

Isolation and Screening of Amylase-Producing Bacteria from Soil.

In this study, soil for isolation of microorganisms were gathered from hot temperature region Musuti land ranch, next to Dharwad-uppinbetageri, District-Dharwad of Karnataka state. A total of seven distinct colony were observed which were labelled as M1 to M7 (Fig 1). These isolated colonies were purified and preserved. These seven colonies were screened for extracellular amylase activity using iodine solution and checked for zone of clearance.

The size of the clear zones generated varied greatly among the isolates which ranged from 1.9 mm to 4.2 mm. M7 colony showing greater zone of clearance (4. 2mm) indicated higher enzymatic activity (Elmansy et al., 2018) and hence selected for further studies (Fig:2). M 7 isolate on starch agar plates revealed hydrolysis zones.

Identification and Characterization of the Selected Bacterial Isolates.

Themorphological characterization of the bacterial isolates are summarized as follows (Table 1). The findings of Gram's staining of seven isolates are also shown in Figure 3. The color indicated that the isolate is a Gram-positive, cells organized in chains, they were spore-forming bacterial species, indicating that they belonged to genus "*Bacillus.*" (Yassin et al 2021). M7 isolate (Fig 4) is gram positive, rod-shaped and belongs to *Bacillus species.*

Production of α-amylase.

Optimization of the incubation period was found to be very critical for maximum production of amylase. In this study, for Bacillus sp M7, maximum activity was found to be 0.73 U/ml, when incubated for 6 days in the production media (Table 2, Fig 4). The activity of α-amylase declined by further increasing the incubation period. After 8 days of incubation, the activity

was extremely reduced. Similar activity was obtained for production of amylase for B. licheniformis was 0.136 U/mL/min and for B. cereus 0.122 U/mL/min at 24h after inoculation and decreased rapidly thereafter. The standard graph of maltose was used for calculation.

The enzyme activity from Bacillus.sp M7 produced was detected after 24hours after inoculation, but it was low. This could be because the bacterium entered the stationary phase. As the fermentation progresses, the activity increased. In the most studies, amylase activity increased after 72 hours after incubation. Similar observation was obtained by Rakaz et al. (Rakaz et al 2021).

Partial Purification of α-amylase Enzyme.

Bacillus species M7 inoculated in broth, kept for 6 days was centrifuged to obtain homogenate. This was used as enzyme source for salt precipitation. Various percentage of ammonium salt was used and maximum activity of amylase was obtained at 80% saturation (0.48U/ml). Table 3 summarizes the several stages ofammonium sulphate precipitation for partial purification of α-amylase from Bacillusspecies. In the present study, the ammonium sulfate precipitation (60-80% saturation) of crude amylase yielded 64% of the yield with 2.4 - fold purification. A 1.3-fold purification with 55% yield of α-amylase was obtained after ammonium sulphate precipitation using Thermobifida fusca NTU22 (27). Whereas 1.3-fold-purifiaction of amylase was obtained from Geobacillus LH8 strain producing 4.29% yield using ammonium sulphate fractionations. (Singh et al., 2014, Mollania et al., 2010). The enzyme was purified four times, and 31.35 % of its original amylase activity was recovered (Bhatt et al., 2020).

Enzyme Characterization.

Effect of pH.

The effect of pH was investigated at pH levels ranging from 5.0 to

9.0. pH 7.0 (0.85U/ml) was obtained to be the optimal pH with maximum activity 0.85U/mL(Fig 7)l. At either sides, the activity decreased indicating that at a particular pH,

enzymes are at optimum ionic state to yield the maximum acitivty. This result matched the characteristics of the most bacterialand contagious alpha-amylases (Simair ey al., 2017)

Effect of Temperature.

The effect of temperature on the amylase enzyme from M7 was investigated. The temperatures ranged from 7 to 47°C (Fig 8). The maximum activity (0.34U/ml) was obtained at 27°C, and the activity was significantly reduced at temperatures below or above 27°C. Denaturation of proteins occurat temperatures over 27°C, which may have slowed the activity. The temperature-dependent features of this enzyme are comparable to those of *Bacillus sp* α-amylase generated by recombinant E. coli, which was active site temperatures ranging from7-47˚C and had optimal activity at 27˚C (Tabassum et al., 2014). The best temperature for retaining α-amylase activity (100% relative activity) from *Nocardiosis sp* was 70˚C, and the enzyme retained 50% of its initial activity at 90˚C. Amylase has a specific activity of 13.14μmol/mg/min at 40˚C (Raul et al., 2014). α-amylase has been observed to have greatest activity at temperatures ranging from 30˚C and 60˚C (Stamford et al., 2001). Enzyme activity was measured periodically 20 min time interval (Al-Agamy et al., 2021). At its ideal growth temperature of 70˚C, *Bacillus thermooleovorans* NP54 generated amylase at the highest rate (Malhotra et al., 2000).

Effect of Enzyme Concentration.

The determination of effect of enzyme concentration on activity was analyzed (0.1 to 1.25μM/ml) on cell growth and extracellular amylase secretion (Fig 9). Highest amylase activity was achieved at 1.0 m^3. As enzyme concentration increases, the activity increases. After a particular concentration, the activity remains same and then declines. Our results are in line with literature for enzyme activity from various strains in terms of enzyme production (Elmansy et al., 2018).

Effect of Substrate Concentration.

Amylase enzyme activity increased when the starch concentration increased from 0.1 to 1.0%, then decreased (Fig 10,11).The amylase activity was highest at 0.6% starch. From LB plot he kinetic parameters of enzyme like K_m and V_{max} were found to be 0.31% and 0.68 µM/ml/min, respectively.

When the starch concentration increased beyond 0.7%, the enzyme activity decreased. This could

be owing to the isolate's metabolic capacity during the brief period when the starch content was elevated(Yassin et al., 2021). In addition, excessive starch concentrations made the broth culture thick, obstructing oxygen transfer and decreasing the amount of dissolved oxygen accessible for microbial growth. Similarly, researchers showed that when the starch concentration was increased, in a *Bacillus* strain isolated from kitchen wastes,there was improvement both in terms of cell growth and α-amylase production, with the highest at a starch concentration of 0.6%, the enzyme yield occurs (Elmansy et al., 2018, Demirkan et al., 2005, Matsuzaki et al., 1974).

Effect of Inhibitors/ activators.

In comparison to control, greatest activation was seen with $CaCl_2$ and NaCl (0.98 and 0.97 µM/ml/min) and least activation was seen with KCl (0.92 µM/ml/min) (Fig.12). In general, high concentrations of both monovalent and divalent salts activated the enzyme. The changes depend on the nature of the salt, either due to anionic strength effect or specific cation effect, depending on the type of the salt (Tabassum et al., 2014). Addition of chelating agents to the reaction mixture interferes with the active site of the enzyme causing inhibition or activation of enzyme activity.

Chelating substances in the reaction mixture cause enzyme activity to be decreased by interfering with the active site of the enzyme. Enzymatic activity is often reduced or lost entirely

when metal ions are removed. Inorganic modifiers like metal ion has the ability to both speed up and slow down enzyme-catalyzed processes (Tallapragada et al., 2017).

Application of α-amylase.

Wash performance analysis of amylase from *Bacillus* Sp.M7 was carried out with two different stains on white cotton cloth. Spinach curry was the first used stain and Chocolate was the second stain. The α-amylase from *Bacillus sp* exhibited high efficiency for the removal of Spinach curry and Chocolate stains in combination with commercial detergent (Surf excel) at 20˚C (Fig 6). In our study, washing efficiency was significantly improved, when crude enzyme plus detergent was used. In tap water, the washing efficiency was poor, but it improved with the addition of detergent and enzyme alone with detergent. Initially, these pieces are removed from the cloth surface using either a detergent matrix component or just water. The enzymatic detergent helps the fabric feel softer to the touch than other cotton cloths cleaned with detergent and starch stains. The efficiency was calculated using washout and may be represented as the higher the starch concentration, the higher the efficiency.

When detergent and crude enzyme was employed in this investigation, washing efficiency increased considerably. Water, detergents, enzyme and detergent plus enzyme were used to wash the starch damaged cotton fabric pieces (Simair et al., 2017). Because of its capacity to break down residues from starchy meals like gravies, chocolate, and other foods, the amylase enzyme is used in a variety of detergents (Tallapragada et al., 2017).

CONCLUSION

This present study shows that studies on *Bacillus* Sp.M7 isolate, production of α-amylase from the isolate, characterization, and partial purification of enzyme. Furthermore, morphological and microscopic analysis of the organism revealed that they belonged to the *Bacillus species*. It was designated as *Bacillus* Sp.M7. it was Gram-positive, spore-forming.

To optimize the time of product recovery, it is necessary to determine the period of bacterial growth and amylase productivity. Under optimal conditions, the isolates produced a significant amount of amylase, according to the findings of the study. In this investigation, the optimal incubation time for α-amylase productivity was 6 days with productivity of 0.73 µM/ml/min. The enzyme activity of *Bacillus* Sp.M7 precipitated by 60% ammonium sulphate salt was found to be 51481 units. The partially purified enzyme was investigated for its properties. The maximum amylase activity was observed at pH 7 (0.85 µM/ml/min), temperature 27˚C (0.62 µM/ml/min) in the presence of Ca^{2+} ions, with enzyme concentrations of 1.25 µM/ml, substrate concentrations of 0.6% (0.70 µM/ml/min) and maximal activation in $CaCl_2$ (0.98 µM/ml/min). K_m and V_{max} of the enzyme was found to be 0.31% and 0.68 µM/ml/min. The use of *Bacillus* Sp.M7 amylase in the detergent industry was also examined, and it was discovered to be highly effective at eliminating stains from fabrics. The enzyme could be of potential use in the textile and food industries.

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CONFLICT OF INTERESTS

None

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the

authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects

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Table 1: Bacterial isolate's morphological characterization.

Bacterial isolates Morphology characteristics	M1	M2	M3	M4	M5	M6	M7
cell shape	Long, rod	Spherical	Long, rod	Spherical	Long, rod	Spherical	Long, rod
Cell arrangement	chain	chain	chain	chain	chain	chain	chain
Colonial pigmentation	Creamy	Creamy	Creamy	Creamy	white	white	white
Gram staining	+	+	-	+	+	+	+

Table 2: Production profile of α-amylase from Bacillus Sp.M7

	Day-1	Day-2	Day-3	Day-4	Day-5	Day-6	Day-7	Day-8
Test-1	0.06	0.09	0.16	0.25	0.28	0.40	0.28	0.23
Test-2	0.01	0.0	0.03	0.06	0.13	0.13	0.07	0.05
Activity	0.12	0.16	0.29	0.46	0.53	0.73	0.53	0.42

Table 3 : Purification of α-amylase produced by Bacillus Sp.M7 using different concentrations of ammonium sulphate salt.

	Volume (ml)	Activity (units)	Total protein (mg)	Total Activity (units∗ml)	Yield	Purification fold
Homogenate	12.50	42845	25.36	535563	81	1.4
60%	8.25	51481	18.25	424718	64	2.4
70%	4.30	48239	3.75	207428	32	11.0
80%	3.50	46578	1.28	163023	25	31.1

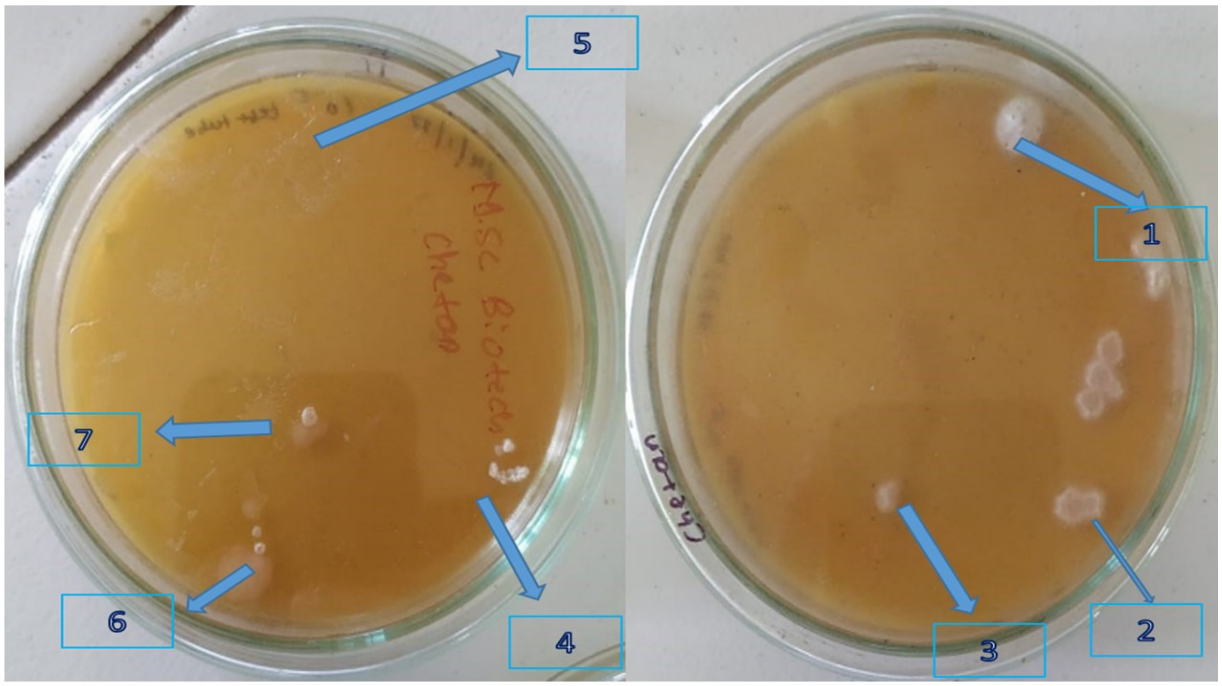

Fig 1: Seven distinct colony were observed from soil samples.

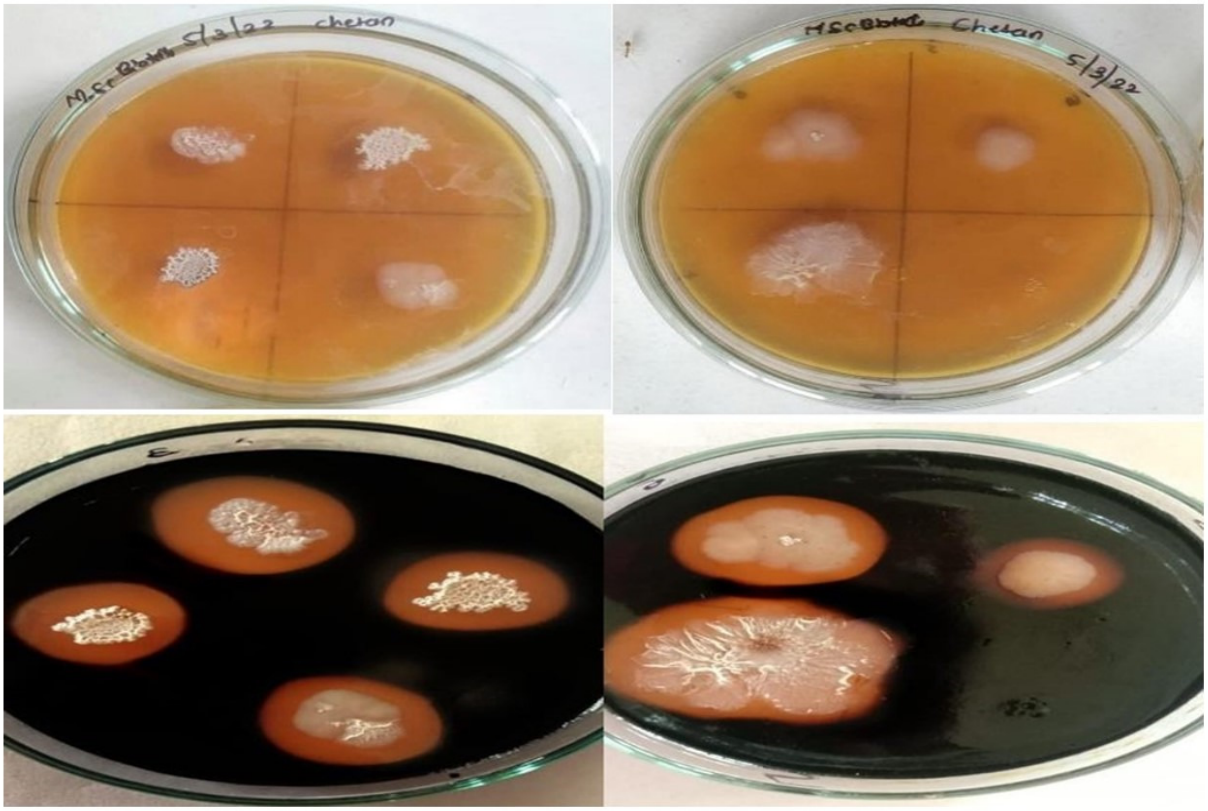

Fig 2: Zone of hydrolysis of starch by the selected isolates before (a) and after (b) addition of iodine solution.

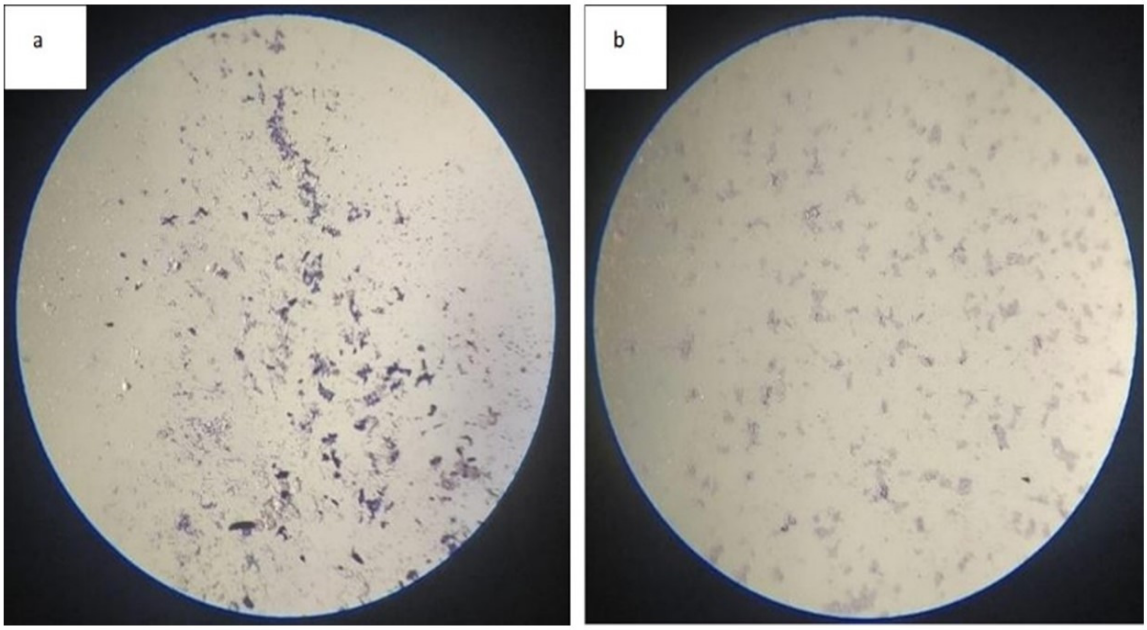

Fig 3: Gram's staining results for the isolates

Fig 4: M7 colony

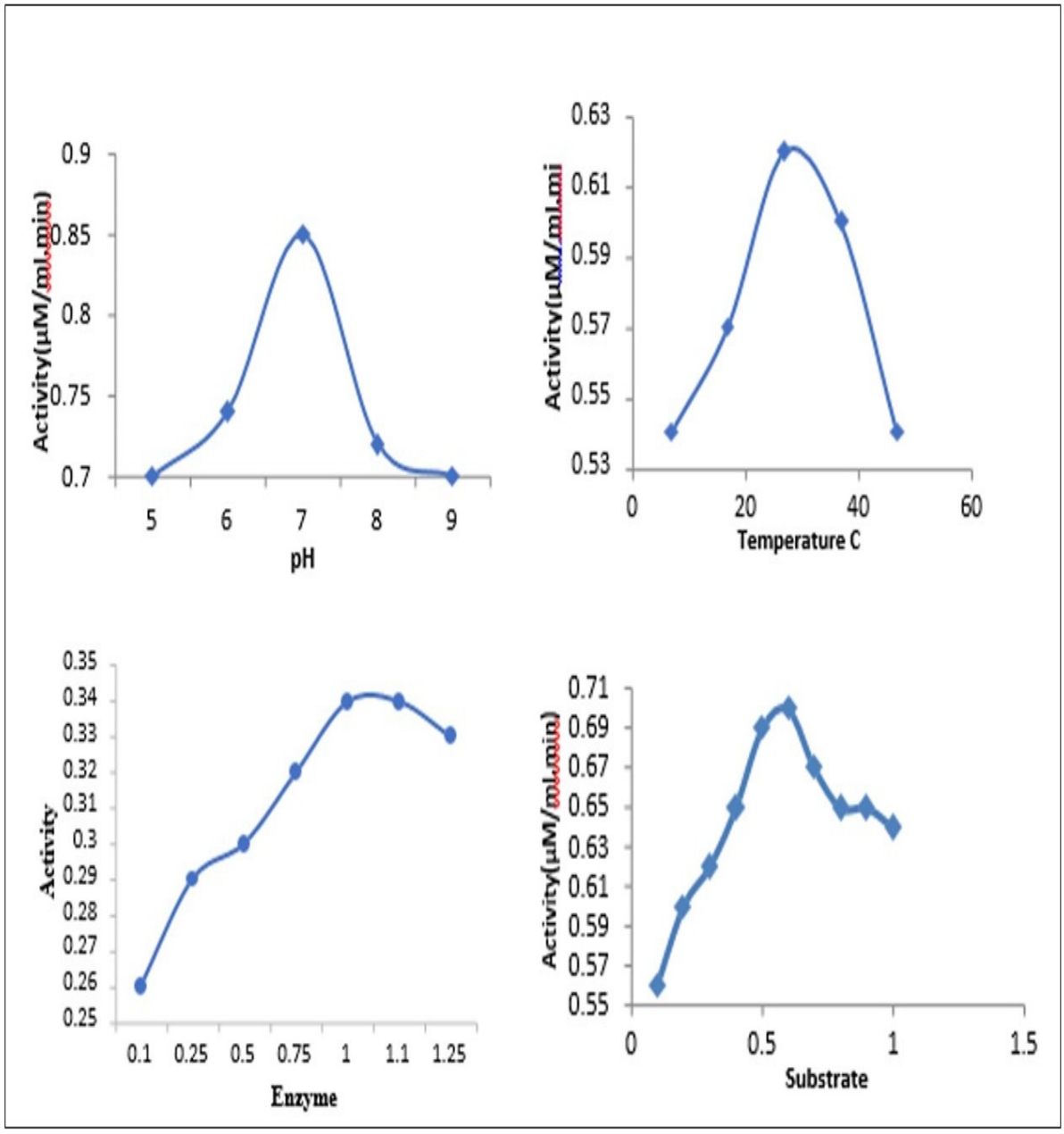

Fig 5: Effect of pH, temperature, enzyme and substrate on amylase enzyme activity.

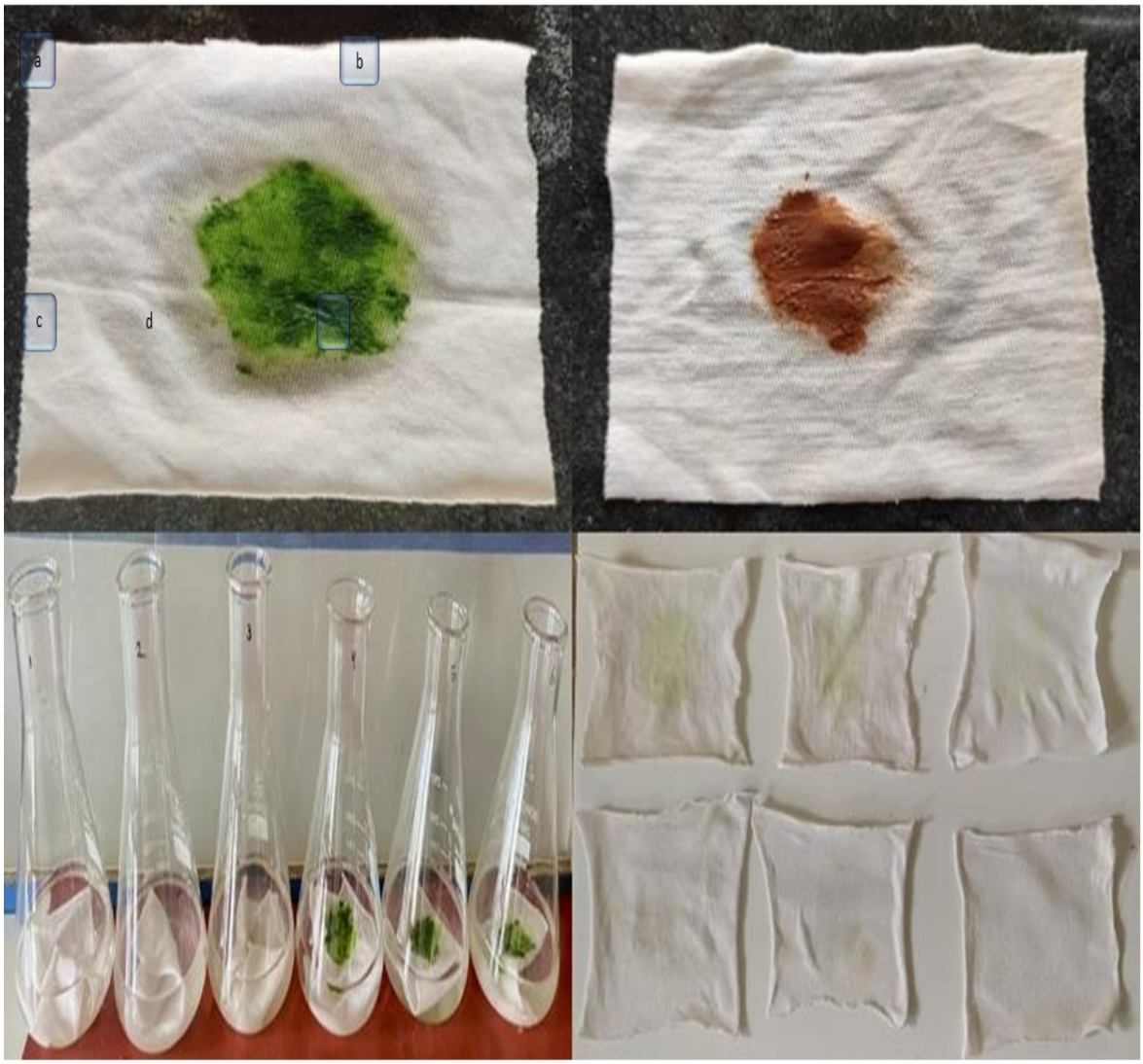

Fig 6: (a) A spinach curry-stained cloth. (b) A chocolate-stained cloth. (c) Flasks with (i) and (iv) controls, (ii) and (v) stained fabric with detergent, and (iii) and (vi) detergent and enzyme extract on a soiled fabric. (d) Monitor the discolored fabric's after-wash behavior. Control (ii) stained cloth with detergent, and (iii) detergent and enzyme extract on a soiled fabric.