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Research Article

Potential antimicrobial and fruit juice clarification activity of amylase enzyme from Bacillus strains

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ABSTRACT

The hydrolytic enzyme, amylase possesses wide industrial applications and its production from bacterial sources by submerged fermentation is much simplified and economical. The research aimed to characterize amylase-producing bacteria and evaluate their potential for amylase activity regarding antimicrobial and fruit juice clarification. In current study, *Bacillus licheniformis, Bacillus amyloliquifaciens, Bacillus cereus, Bacillus subtilis* and *Bacillus paramycoides* was identified by 16S rRNA sequencing. After submerged fermentation, amylase activity of bacteria was measured by 3, 5-dinitro salicylic acid (DNS) assay. A substantial amount of amylase (423.47 mg/ml) in crude extract was measured by Bradford protein assay. Later, ammonium sulfate (80 %) precipitated partially purified amylase showed 1.6 times enhanced amylase activity (1484.94 U/ml) compared to crude amylase (973.23 U/ml). For highest amylase production, 72 h of optimum fermentation period was recorded at pH 7 with 2 % starch as substrate. Potent thermophilic amylase activity was observed at 65 °C. In apple juice clarification activity of amylase, turbidity of juice was reduced to 54.18 %. Potential antimicrobial property of amylase was detected with largest zone of inhibition against *Escherichia coli* ATCC 25922 (22.36 mm) and *Mucor* sp. ATCC 48559 (22.45 mm). Considering promising amylase properties, amylase-producing Bacillus strains from rice mill soil can be fermented for large scale amylase production providing application for industrial purposes including fruit juice clarification and antimicrobial activities. It will also overthrow the requirement of employing expensive and harmful chemicals in fruit juice clarification and combating pathogens.

1. Introduction

Though amylase can come from diverse sources like animals, plants, and microorganisms; from microbial sources, the bacterial amylases dominate commercial amylase production attributable to their capability to uphold pressure-induced shape, chemical stability, wide availability, environmental security, cost-effectiveness, and easy production on large scale [1]. The manufacture of microbial amylases from bacterial sources relies on the type of microbial species, composition of the medium, incubation period, pH, temperature, method of cultivation, cell growth, and requirement for the nutrients [2]. The enzyme α-amylase utilized in the industry comes chiefly from bacterial sources (83 % of total). Among bacterial strains, *Bacillus* species such as *B. subtilis, B. licheniformis, B. amyloliquefaciens, B. methylotrophicus, B. stearothermophilus, B. megaterium* and *B. circulans* are the most regularly utilized bacterial workhorses for commercial amylase production [3,4]. The genus *Bacillus* solely manufactures 60 % of industrial enzymes because of its rapid growth rate which requires short fermentation phases, high ability for protein production into the extracellular medium, and biosafety [5,6].

At present, α-amylase is extensively comprised of 25–30 % of the global enzyme market being the second largest category of enzymes utilized worldwide [7]. The production of amylase has attained 65 % of the world enzyme market and the usage is constantly rising [8]. It was estimated to USD 6.30 billion and is expected to grow at a CAGR of 6.8 % from 2018 to 2023 [9]. Among other enzymes, amylase possesses the

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highest prospective for utilization in diverse industrial and medicinal purposes. Amylases could be potentially efficient in a range of industries like detergent industries, textiles, food industries, paper industries, fine-chemical industries, and also, in the pharmaceutical management of digestive disorders [10].

To prevent unwanted turbidity, haziness, and cloudiness together with enriching juice quantity, and duration for storage, the application of enzymes has been spread considerably in different fruit juice industries [11,12]. Processed or properly clarified apple juice is the preferred fruit juice for most people in the world. The haziness or cloudiness of apple juice is mainly because of the presence of starch and pectin. Apple juice may have contained a substantial quantity of starch, for instance, unripe apples contain about 15 % starch. Amylase reduces the amount of starch accumulating with pectin and therefore, removes haziness. Thus, a huge amount of amylase is required in fruit juice industry during the clarification process daily [13,14].

Enzymes must be stable and endure rough conditions of reaction like extreme pH, elevated temperature, strong alkaline, strong acid, high salinity, and bear high concentrations of substrate/product [15]. To beat such conditions, several approaches have been accomplished like screening enzymes from natural sources, immobilization, and random mutations [16]. The process for identifying extremely stable types of enzymes is to extract microbial organisms from natural environments. Soil receiving the rice mill wastes could be one of the affluent sources of amylase producing microorganisms as it encompasses predominantly starchy substrate which constructs a favorable environment to grow them in a large population. By screening microbes that make the desired enzyme and optimizing the required growth conditions, industrial quantities can be achieved. This method renowned for over 3000 years, is named fermentation including submerged fermentation (SmF) method which has been conventionally utilized for the fabrication of industrial enzymes. Due to the ease of control of various parameters like pH, temperature, oxygen transfer, aeration, and moisture content, SmF is the most favorable and convenient to manage for bacterial growth [17].

The price of amylase generally exhibits 24 % of the entire production cost, causing increased interest in the decrease of amylase production costs by enriching enzyme yield rate and/or activity [18]. Amylase production from microbes can decrease the production cost by large-scale production through fermentation processes [19]. This research evaluated rice mill soil for the abundance of native amylase-producing bacteria by analyzing their morphological, biochemical, and molecular properties. Subsequently, the potential for the production of amylase enzyme through optimizing fermentation parameters in the submerged fermentation process, partial purification, along with antimicrobial and fruit juice clarification activity of amylase were tested.

2. Materials and methods

2.1. Microorganisms and growth condition

Bacteria were isolated on starch agar plate from three local rice mill soil of Khulna District, Bangladesh. Starch agar media were prepared containing soluble starch 0.1 %, yeast extract 0.2 %, peptone 0.5 %, NaCl 0.1 %, $MgSO_4$ 0.1 %, $CaCl_2$ 0.02 %, and agar 2 %. The pH of the media was adjusted to 7 and incubated for 24 h at 37 °C.

2.2. Starch degrading index determination

In qualitative test of starch iodine test, dark blue color with a clear zone surrounding the colonies was observed for the positive test of starch degradation activity [3,20]. The proportion of the total clear zone produced around the bacterial colonies and diameter of colony of bacteria; starch degrading index (SDI) was calculated in quantitative test.

2.3. Biochemical characterization

Bacterial colonies were characterized biochemically by oxidase test, catalase test, indole test, urease test, methyl red test, Vogues Proskauer's test, triple sugar iron test, citrate utilization test and sugar fermentation test (starch, sucrose, lactose, maltose, glucose and glycerol). Parameters reading tolerance to different harsh conditions; NaCl concentrations (2 %, 4 %, 6 %, 8 %, 10 % and 11 %), pH (pH 2, 4.0, 6.0, 7.0, 8.0, 9.0 and 10.0), temperatures (10 °C, 20 °C, 30 °C, 37 °C, 45 °C, 50 °C, 60 °C and 70 °C) and incubation periods (24 h, 36 h, 48 h, 72 h, and 84 h) were analyzed.

2.4. Molecular identification

Genomic DNA was isolated using Maxwell Blood DNA Kit and bacterial 16 s rRNA gene was amplified using 10 μM of each 27F (AGAGTTTGATCMTGGCTCAG) and 1492 R (CGGTTACCTTGTTACGACTT) primers using Hot Start Green Master Mix. PCR products were visualized on a 1 % agarose gel using Wizard SV Gel. 16S rRNA sequencing was performed by Sanger sequencing and gene sequences were used as input for the Basic Local Alignment Search Tool (BLAST). Sequences were then submitted to National Center for Biotechnology Information (NCBI) GenBank to get accession numbers and phylogenetic tree was constructed using UPGMA in MEGA X 7.2 software [10].

2.5. Fermentation medium and crude enzyme preparation

For amylase production, 3.0 % (v/v) of bacterial seed culture was inoculated into 1 L of the liquid medium [21]. Incubation was done in a rotary shaker at 150 rpm for 3 days at 37 °C. Bacterial cells were separated by centrifugation at 10,000 rpm for 15 min to get crude enzyme as cell-free supernatant [5].

2.6. Enzyme assay

Enzyme activity was measured by the DNS method following the modifications of Bernfeld protocol using starch as the substrate [19,22]. The reaction included 1 ml of amylase and 1 ml of 1 % soluble starch dissolved in 1 ml of sodium phosphate buffer (pH 7) for 10 min at 37 °C. The reaction was stopped by supplying 1 ml of DNSA reagent (3, 5-dinitro salicylic acid). Absorbance was read at 540 nm where pure α-amylase (500 mg/ml, 250 mg/ml, and 125 mg/ml) was used as standard. One unit of amylase activity was defined as the enzyme amount that releases 1 μmol of maltose per minute under the specific assay conditions and enzyme activity was expressed as U/ml.

2.7. Protein concentration determination

Concentration of protein in crude extract was determined by Bradford method where bovine serum albumin (BSA) was considered as standard protein [23]. Optical density was read at 600 nm in spectrophotometer.

2.8. Optimization of fermentation parameters

Effect of fermentation temperature (37 °C, 45 °C, 50 °C, 55 °C, 60 °C, and 65 °C) and incubation time (0, 8, 16, 24, 48, 72, 88 and 96 h) on bacterial enzyme production was analyzed.

2.9. Partial purification of enzyme

Ammonium sulfate precipitation was used for partial purification of crude enzyme [7]. In brief, crude enzyme was purified in an ice bath by ammonium sulfate fractionation to 80 % saturation. Centrifugation was done at 10,000 rpm for 10 min at 4 °C and precipitated protein was then dissolved in 0.1 M phosphate buffer (pH 7).

2.10. Effects of enzyme

2.10.1. Apple juice clarification test

1 ml of amylase enzyme was mixed with 6 ml of freshly prepared apple juice while 1 ml pure commercial α- amylase was used as control. Incubation was done for 150 min in water bath at 50 °C and the clarity of the juice was measured in a spectrophotometer (600 nm) every 30 min. Unclarified raw apple juice was considered as 'blank' and its turbidity was considered 100 % [6,12].

2.10.2. Antibacterial and antifungal activity test

Agar well diffusion method [24] was utilized to detect antimicrobial activities of amylase against *Salmonella typhi* ATCC 1408, *Staphylococcus aureus* ATCC 49,775, *Escherichia coli* ATCC 25922, *Vibrio cholera* ATCC 51394, *Shigella* sp. ATCC 12022, *Aspergillus niger* ATCC 16404, and *Mucor* sp. ATCC 48559. Pathogenic strains (10^7 CFU/ml) were grown on the surface of nutrient agar plate and 100 µL of amylase enzyme was placed in each plate. Incubation was done overnight at 37 °C and zones of inhibition were measured. Ciprofloxacin and Nystatin were used as a positive control for bacterial and fungal antagonistic activity, respectively.

3. Results

3.1. Qualitative and quantitative starch degrading assay

A total of twenty-two (22) bacterial isolates were harvested by colonic observation during periodic subculture on starch agar plate. Based on qualitative and quantitative starch degrading properties, fifteen (15) isolates were primarily screened as amylase-producing bacteria which produced SDI > 4 in starch iodine test (**Figure S1**).

3.2. Morphological and biochemical characterization

Fifteen (15) bacterial isolates (r1, r3, r4, r6, r7, d2, d3, d5, d6, d7, f1, f2, f4, f5, and f7) were observed as Gram-positive, rod-shaped with chain forming appearance. During biochemical characterization, all bacteria fifteen (15) showed positive reaction in oxidase, catalase, citrate, and Voges-Proskauer test while negative reaction in indole, methyl red, and urease test. Bacterial isolates (except r4, r6, d2, f2, and f5) were able to ferment glucose, lactose, sucrose, starch, maltose and glycerol in sugar fermentation test (Table 1). Optimum pH, time and temperature for all bacterial (except r4, r6, d2, f2, and f5) growth were observed at pH 7, 72 h of incubation, and 37 °C temperature, respectively (Fig. 1**A-C**). Five bacterial isolates (r4, r6, d2, f2, and f5) showed poor growth at optimum conditions and couldn't tolerate high temperatures. Bacterial isolates (except r4, r6, d2, f2, and f5) were able to withstand up to 10 % NaCl concentration in NaCl tolerance test (Fig. 1**D**). Due to poor growth pattern in different biochemical conditions, five bacterial isolates (r4, r6, d2, f2, and f5) were not considered for further analysis.

Table 1
Biochemical features of starch hydrolyzing bacteria isolated from different rice mill soil.

Biochemical tests	Observations
Oxidase test	+
Catalase test	+
Indole test	–
Citrate utilization test	+
Methyl red test	–
Voges–Proskauer test	+
Urease test	–
H_2S production	–
Carbohydrate fermentation test: Glucose, lactose, sucrose, starch, maltose and glycerol	+
Optimum pH	7
Tolerance to NaCl	10 %
Tolerance to temperature	60 °C
Growth rate	72 h

Legends: (+) means positive reaction; (-) means negative reaction.

3.3. Molecular identification and phylogenetic analysis

According to NCBI BLAST result, bacterial isolates showed close homology with *Bacillus licheniformis, Bacillus cereus, Bacillus amyloliquifaciens, Bacillus subtilis*, and *Bacillus paramycoides* (Table 2). Accession numbers of corresponding bacteria were acquired from NCBI GenBank. For the construction of the phylogenetic tree, sequences were aligned by employing the Clustal W method in MEGA X software (version 10.1.8). The relationship among the aligned sequences was determined using UPGMA (Fig. 2). The phylogenetic tree was constructed using the Bootstrap method with 1000 replications. The pair-wise genetic distances were measured using the maximum composite likelihood method.

3.4. Fermentation parameters optimization

3.4.1. Effect of protein concentration on enzyme activity

In DNS assay, maximum enzyme production was recorded when crude enzyme without any dilution was employed for the assay. *Bacillus subtilis* (f1) expressed the highest enzyme activity, 810.88 U/ml among other enzyme producing strains (Fig. 3**A**). A substantial amount of enzyme was present in crude extract (for *Bacillus subtilis* f1) measured by Bradford assay (Fig. 3**B**).

3.5. Effect of fermentation time on enzyme activity

Crude enzyme extract obtained from the bacterial strains was subjected to fermentation for different incubation time intervals. Enzyme activities showed by bacterial strains were enhanced with increasing time of fermentation. Enzyme activity was also compared with growth pattern of each strain for specific incubation period. The highest growth rate and enzyme activity was observed after 72 h of incubation. The maximum growth rate and enzyme activity (860.69 U/ml) was recorded for *Bacillus subtilis* (f1) (Fig. 4**A–D**).

3.6. Effect of fermentation temperature on enzyme activity

Maximum enzyme activity was recorded for at a range of temperature 55 °C to 65 °C (Fig. 5). The enzyme activity was increased with increasing temperature and *Bacillus cereus* (r3) showed highest enzyme activity (806.89 U/ml) at temperature 65 °C. For both *Bacillus licheniformis* (r1) *and Bacillus amyloliquifaciens* (d5) the optimum fermentation temperature observed at 60 °C.

3.7. Amylase activity of partial purified enzyme

After 80 % ammonium sulphate precipitation, partially purified amylase showed 1.6 times enhanced amylase activity compared to crude amylase at optimized fermentation condition. Partially purified amylase from *Bacillus licheniformis* (r1) showed highest amylase activity 1484.94 U/ml while it was reduced to 973.23 U/ml for crude enzyme (Fig. 6).

3.8. Effects of amylase enzyme

3.8.1. Apple juice clarification

Clarification activity of amylase enzyme in apple juice was determined by measuring optical density (OD) in spectrophotometer at 600 nm. The turbidity of juice was gradually decreased with time after being treated with partially purified amylase. Most turbidity reduction performance (54.18 %) was observed for by *Bacillus cereus* (r3) (Fig. 7).

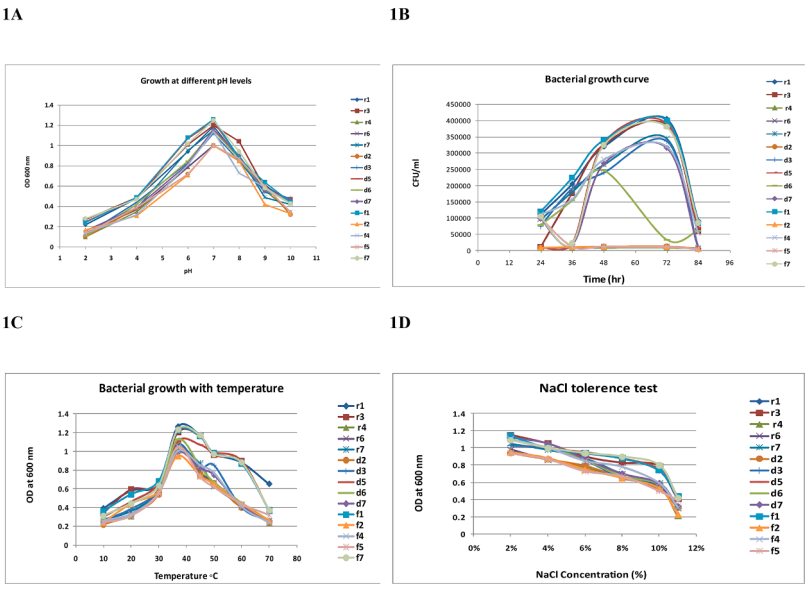


Fig. 1. A-D Growth pattern of starch degrading bacteria in presence of different ranges of pH, incubation time, temperature, and NaCl.

Table 2
Molecular identification profile of amylase producing bacteria using BLAST results and NCBI GenBank accession numbers.

Internal code	Microorganisms	GenBank accession no.	Gene Bank reference bacteria	Reference accession no.	%Homology
r1	Bacillus licheniformis	MZ675464.1	*Bacillus licheniformis* strain ATCC 14580	NR 074923.1	97.46 %
r3	*Bacillus cereus*	MZ675460.1	*Bacillus cereus* strain CCM 2010	NR 115714.1	97.5 %
r7	*Bacillus paramycoides*	MZ676026.1	*Bacillus paramycoides* strain MCCC 1A04098	NR 157734.1	97.36 %
d3	*Bacillus paramycoides*	MZ676059	*Bacillus paramycoides* strain MCCC 1A04098	NR 157734.1	97.58 %
d5	*Bacillus amyloliquifaciens*	MZ675463.1	*Bacillus amyloliquefaciens* strain BCRC 11601	NR 116022.1	98.34 %
d6	*Bacillus paramycoides*	MZ831321.1	*Bacillus paramycoides* strain MCCC 1A04098	NR 157734.1	96.02 %
d7	*Bacillus paramycoides*	MZ675584.1	*Bacillus paramycoides* strain MCCC 1A04098	NR 157734.1	97.99 %
f1	*Bacillus subtilis*	MZ675461.1	*Bacillus subtilis* strain DSM 10	NR 027552.1	97.40 %
f4	*Bacillus paramycoides*	MZ831320.1	*Bacillus paramycoides* strain MCCC 1A04098	NR 157734.1	95.71 %
f7	*Bacillus paramycoides*	MZ676060.1	*Bacillus paramycoides* strain MCCC 1A04098	NR 157734.1	98.83 %

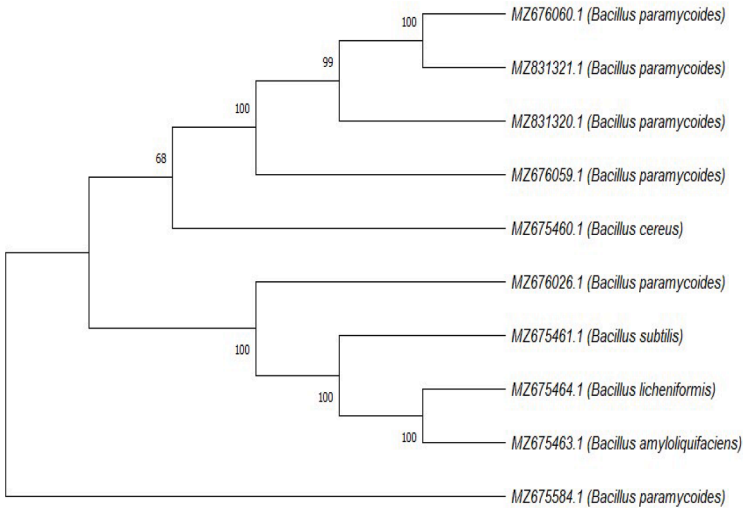
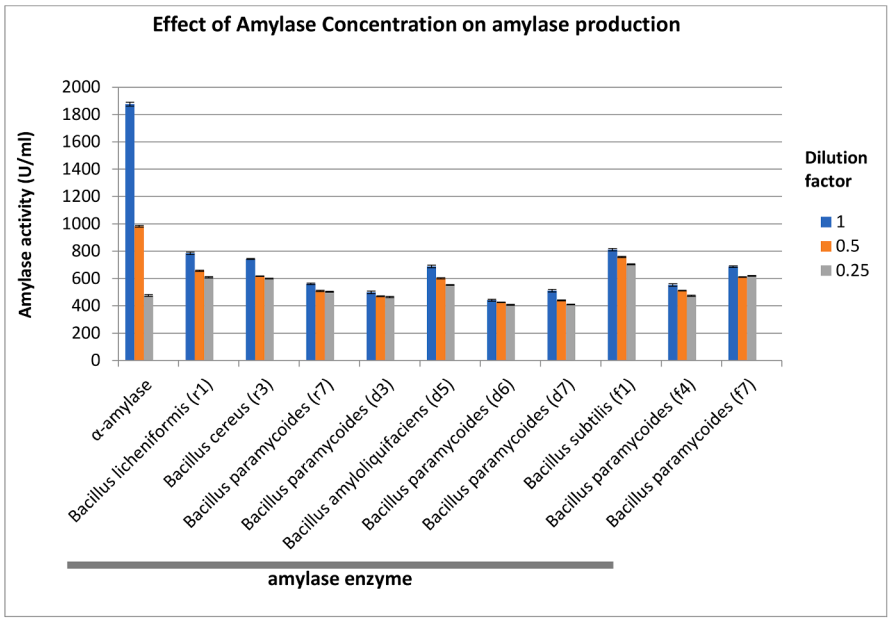


Fig. 2. The phylogenetic tree of bacterial isolates based on 16S rRNA gene sequences. The Sequences were aligned by ClustalW method and evolutionary relationship was inferred employing UPGMA using MEGA X v 10.1.8 (bootstrap value = 1000).

3.8.2. Antagonistic activity of amylase enzyme

In case of antibacterial activity, amylase enzyme showed varying zones of inhibition depending upon the particular pathogen. Amylase from *Bacillus cereus* (r3) showed most inhibitory activity with largest zone of inhibition 22.36 mm against *E. coli* ATCC25922 (Fig. 8**A**). In case of antifungal activity, amylase enzyme produced by *Bacillus licheniformis* (r1) showed most inhibitory activity with largest zone of inhibition 22.45 mm against *Mucor* sp. ATCC 48559 (Fig. 8**B**).

3A



3B

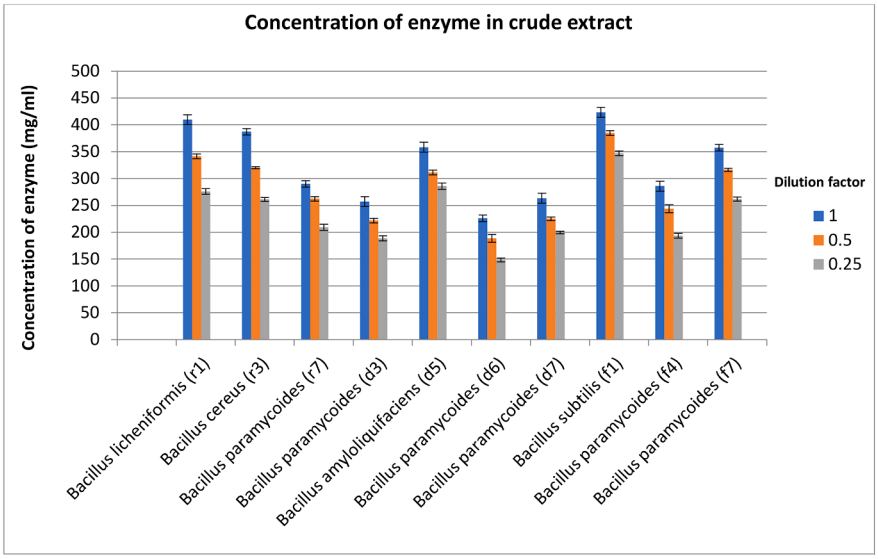


Fig. 3. A. Enzyme activity of crude amylase produced by amylase producing bacteria. In the assay, pure α amylase was used as positive control. **B.** Concentration of amylase present in crude extract measured by Bradford protein assay. Results are expressed as the mean ± SEM of the triplicate experiments.

4. Discussion

Isolation, characterization and molecular identification of soil microorganisms that are able to express good hydrolytic activity may contribute to potential amylase discovery for utilization in different suitable industrial and biotechnological sectors [25]. Soil receiving the rice mill wastes could be one of the affluent resources of starch degrading microorganisms as it encompasses predominantly starchy substrate which constructs a favorable environment to grow them in a large population. In the present study, 22 bacterial isolates were harvested on starch agar plate from different Rice Mills of Khulna District, Bangladesh. Among them, fifteen isolates were further screened based on starch degradation index (SDI) and characterized as gram-positive rod-shaped bacteria. In line with our study, 6 isolates among 30 different bacterial strains were reported to hydrolyze starch on the starch agar isolated from rice mill soil [26]. Amylase producing bacterial strains were also isolated from different sources like brick kiln soil [27], mud from thermal spring [28], soil of fruit stalls [29], and cassava peel dump-site [30].

Amylase enzymes with advantageous industrial attributes including enhanced activity, specificity to the substrate, a wide range of pH tolerance profile, thermal stability, and appropriate resistance against denaturing materials and heavy metals, high salinity, and high concentrations of substrate/product are considered for utilizing in different industrial sectors [31]. There has been ongoing interest in isolation, identification and optimization of new *Bacillus* strains with potential amylase-producing activity that are also suitable for several industrial purposes. The bacilli isolated from soil sources are considered as the best source for bulk extracellular amylase production, in fact, they produce around 60 % of commercial enzymes [32,33]. In current study, *Bacillus licheniformis, Bacillus cereus, Bacillus subtilis, Bacillus amyloliquifaciens* and 6 strains of *Bacillus paramycoides* were identified by 16S rRNA

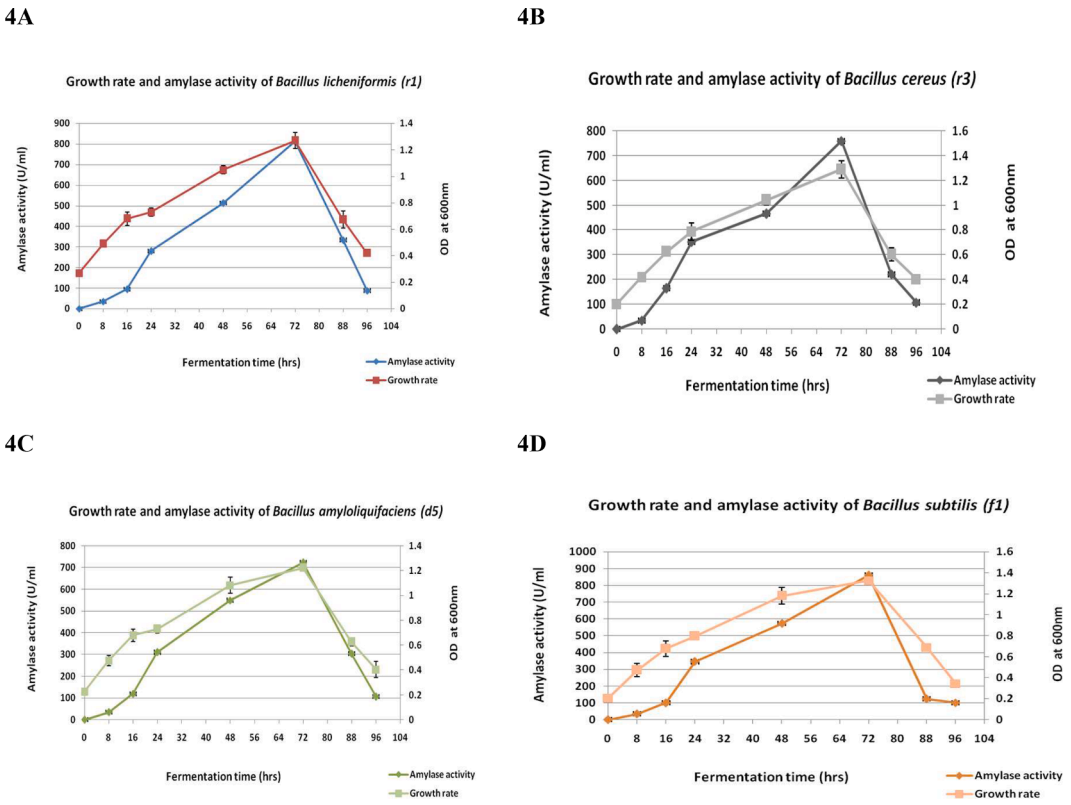


Fig. 4. A-D Growth rate and amylase activity of amylase producing bacteria over different fermentation periods. Results are expressed in mean ± SEM of the triplicate experiments.

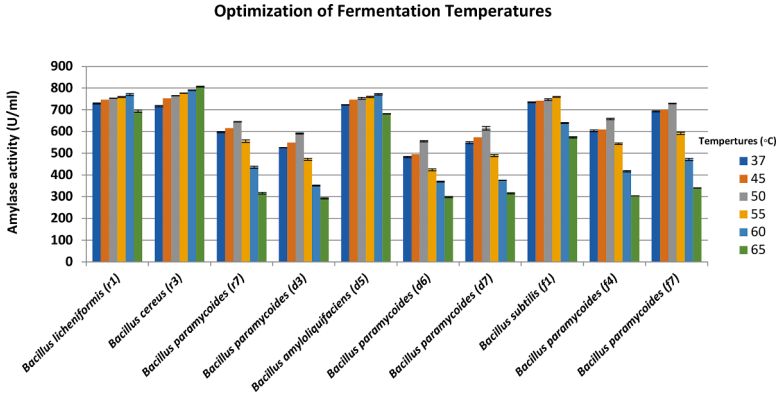


Fig. 5. Effect of fermentation temperature on amylase activity of bacterial strains. Results are expressed in mean ± SEM of the triplicate experiments.

sequencing. In consistent with present study, a number of studies have identified different amylase producing bacterial strains like *Bacillus cereus* and *Bacillus mycoides* [34,35].

Enhancing the rate of amylase yield and subsequent reduction of cost depends on the strains selection, optimization of the several factors that affect biosynthesis, kinetic studies, genetic improvement, and biochemical characterization of enzyme [35]. Their productivity can simply be enhanced by optimizing pH, temperature, incubation period and/or carbon sources, etc. The effects of temperature, pH, incubation time, inoculum amount, starch concentration, nitrogen sources, and small organic molecular inducers on amylase enzyme production by measuring the amylase activity of the fermentation supernatant [36,37]. In the present study, during fermentation period optimization, maximum amylase activity 860.69 U/ml was obtained after 72 h of submerged fermentation period from *Bacillus subtilis* (f1). In accordance with the results of present study, optimum fermentation time observed for *Bacillus licheniformis* was 72 h [38]. Other studies have reported an optimum 60 h of fermentation period for *Bacillus* sp. [4,25]. *Bacillus subtilis* [39] and *Bacillus cereus* [35] showed highest amylase activity at 24 h of incubation.

In current study, *Bacillus licheniformis* (r1), *Bacillus amyloliquifaciens* (d5), *Bacillus cereus* (r3), and *Bacillus subtilis* (f1) was observed to withstand fermentation temperature 60 °C, 60 °C, 65 °C, and 55 °C, respectively. All six (6) *Bacillus paramycoides* strains expressed highest enzyme activity at fermentation temperature 55 °C which has correspondence with the findings of a study [27]. Several studies have identified different ranges of optimum fermentation temperatures (35 °C - 80 °C) regarding various strains of *Bacillus* sp. [30,35]. For instance, the maximum amylase production (538 U/ml) was observed from *Bacillus subtilis* at temperature around 32 °C [39]. Another researcher recognized optimum fermentation temperature for *Bacillus subtilis* at 80 °C [9]. For *Bacillus licheniformis*, it was found at 35 °C [28]. Optimum fermentation

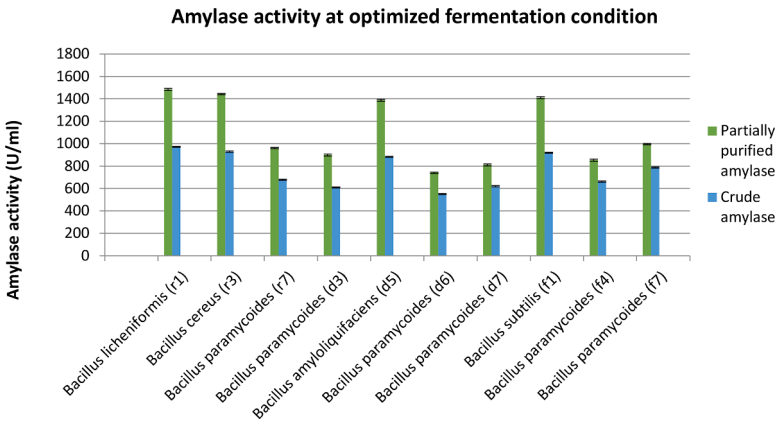


Fig. 6. Comparative amylase activity of partially purified and crude amylase produced by *Bacillus* strains in optimized fermentation condition for each strain. Results are expressed in mean ± SEM of the triplicate experiments.

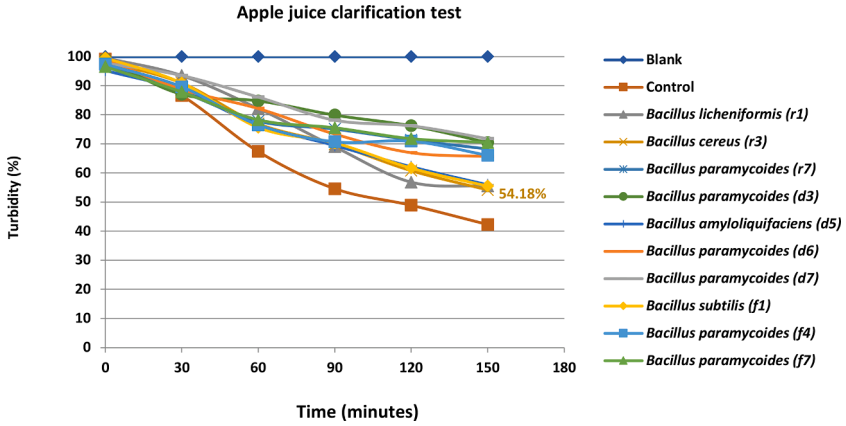


Fig. 7. Apple juice clarification activity test of amylase produced by *Bacillus* strains over time.

temperature for *Bacillus cereus* was found at 80 °C [35], and also at 50 °C [30]. Researchers have found optimum fermentation temperature for *Bacillus amyloliquifaciens* at 50 °C [40]. *Bacillus paramycoides* fermentation temperature was optimized at 50 °C [27].

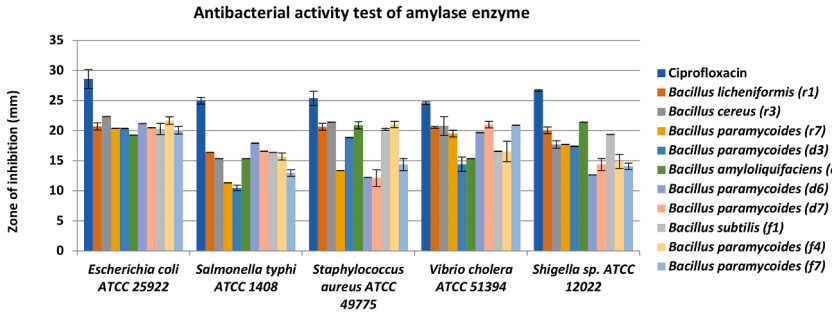
In the current study, amylase activity was enhanced for all *Bacillus* strains after maintaining optimized fermentation conditions for each strain. After ammonium sulphate precipitation, the amylase activity of partially purified amylase was considerably enhanced than crude amylase. Partially purified amylase produced by *Bacillus licheniformis* (r1) showed maximum amylase activity (1484.94 U/ml) which was 1.6 times more enzyme activity of crude amylase. In accordance with our study, amylase activity was higher in partially purified enzyme compared to crude enzyme [7,36].

The application of amylase enzyme in clarification of apple juice was evaluated in the present research. Since the clear appearance of juice is a determinant factor for particular consumers, to optimize this feature, the fruit juice industry is highly concerned and has been investing in methods that can optimize the feature [41]. Starch is a prospective contributor to the juice haziness along with filtration problems because of the gel formation by starch. Due to the presence of starch slow rate of filtration, formation of gel after concentration, membrane fouling, and post concentration haze formation can occur [42]. With the purpose of avoiding unwanted haziness, turbidity and cloudiness along with enhancing juice quality, yield, and storage durability and stability, utilization of enzymes could be a possible solution and recently it's been extendedly used in fruit juice processing industry [43]. Clarity is the lowest absorbance value recorded at highest enzyme concentration, and, here, lower absorbance points out a clearer juice was manufactured [44]. In the present study, amylase enzyme reduced turbidity of apple juice where most turbidity (OD) reduction performance (54.18 %) was observed for *Bacillus cereus* (r3). In accordance with our result, amylase enzyme was employed to clarify apple juice and it showed good clarification results [41].

Antagonistic activity of amylase enzyme has expressed varying zones of inhibition ranged from 13.37–22.36 mm depending upon the particular pathogens studied in the current study. Most inhibitory activity of amylase enzyme with largest zone of inhibition was recorded against *E. coli* ATCC 25,922 and *Mucor* sp. ATCC 48,559. In line with the findings of current present study, antibacterial activity of amylase enzyme was measured against several bacterial (*B. subtilis, E. coli, S. aureus, Pseudomonas* sp., *Acinetobacter* sp., *Klebsiella* sp., *Shigella* sp., and *S. epidermiditis*) and fungal pathogens (*A. niger, A. flavus, Penicillium* sp., *A. tereus,* and *Mucor* sp.) [6,45,46]. Maximum inhibitory effect was recorded against *Klebsiella* sp. (33 mm) while minimum was against Methicillin-Resistant S. aureus (MRSA) (9 mm). Among all fungal strains, inhibitory effect against *Penicillium* sp. was reported maximum (40 mm) while minimum inhibitory activity was recorded against T. tonsurans (27 mm). [6]. Another study has found alpha-amylase enzyme as an excellent antibiofilm agent against biofilm forming clinical pathogens like *S. aureus, Vibrio cholera,* and *P. aeruginosa* [42]. Amylase enzyme also showed antibiofilm activity against bacterial pathogens [46]. Amylase extracted from *Bacillus tequilensis* has reported to degrade biofilm formed by pathogens like *Staphylococcus aureus* and *Pseudomonas aeruginosa* [47].

Optimization of other fermentation parameters and purification of produced amylase in future may drastically enhance amylase activity.

8A



8B

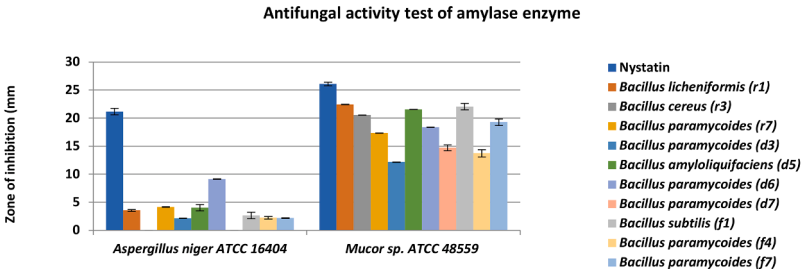


Fig. 8. A-B Antibacterial and antifungal activity of amylase produced by bacterial strains against common pathogens. Results are expressed in mean ± SEM of the triplicate experiments.

With increasing growth of enzyme market and amylase demand, the present study has been taken up with a view to explore the bacterial amylase activity from local rice mill soil. Due to the immense requirement of the amylase enzyme in several sectors of industries and medicinal purposes, production of this particular enzyme has enormous advantages. In aspect of the country like Bangladesh amylase production from bacteria could provide diverse sources of economic, social and environmental benefits. The promising results obtained in this study will help to initiate commercial industrial set up for amylase production from bacterial sources by fulfilling the demand of enzyme, reducing production cost, retaining foreign currency, creating job opportunities and protecting environment from harmful chemical substances used as a replacement of this enzyme.

5. Conclusions

With increasing growth of enzyme market and amylase demand, the present study explores bacterial amylase activity from local rice mill soil. Total ten amylase producing strains including *Bacillus licheniformis, Bacillus amyloliquifaciens, Bacillus cereus, Bacillus subtilis* and *Bacillus paramycoides* was identified from native rice mill soil. Crude extract produced by isolated strains contained a substantial amount of amylase enzyme (423.47 mg/ml for *Bacillus subtilis* f1*)*. >1.6 times higher amylase activity was recorded for partially purified amylase (1484.94 U/ml by *Bacillus licheniformis* r1) than crude amylase (973.23 U/ml by *Bacillus licheniformis* r1). All Bacterial strains required 72 h of fermentation period for highest amount of amylase production. *Bacillus licheniformis* (r1)*, Bacillus amyloliquifaciens* (d5) and *Bacillus cereus* (r3) produce amylase enzyme in hyperthermal fermentation condition like 60 °C - 65 °C temperatures. After treating with amylase from *Bacillus cereus* (r3), turbidity of apple juice was reduced to 54.18 %. Potent antagonistic activity of amylase against several common bacterial and fungal pathogens was also detected. Amylase activity demonstrated by specific *Bacillus* strains can be considered as a prospective candidate for future utilization in food industries as well as combating pathogens. It will also overthrow the requirement of employing expensive and harmful chemicals.

Author agreement statement

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CRediT authorship contribution statement

Khondoker Moazzem Hossain: Writing – review & editing, Supervision, Project administration. **Umama Khan:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **S.M. Mahbubur Rahman:** Writing – review & editing, Visualization, Resources, Project administration, Methodology, Conceptualization. **Md. Salauddin Khan:** Writing – review & editing, Validation, Software, Resources, Data curation.

Declaration of competing interest

There's no financial/personal interest or belief among authors.

Data availability

No data was used for the research described in the article.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2024.e00861.

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