A Novel Starch Digesting Halo-Acid-Alkali-Tolerant and Surfactant Stable Amylase from Newly Isolated Halophilic Bacterium Bacillus Siamensis Sp. F2: Application in Waste Valorization to Ethanol Fermentation

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Abstract

The extracellular amylase production level by the moderate halophile *Bacillus siamensis* sp. F2 was optimized, and the enzyme was biochemically characterized. The culture parameters for NaCl, carbon, nitrogen, pH, and temperature were optimized for high titers of amylase production. Growing *Bacillus siamensis* sp. F2 cultures in Great Salt Lake-2 medium with additions of (in g/l) NaCl (100), starch (30), yeast extract (2), KNO_3 (2), and $MgSO_4$ (1) at pH 8, 30°C resulted in the maximum amylase production (4.2 U/ml). The amylase was active across a wide range of salinities (0 to 30% NaCl), pH (5.0–10.0), and temperatures (20–70^oC), and showed good stability with surfactants (SDS and Triton X-100); hence identified as halo-acid-alkali-tolerant and surfactant stable. Temperature, pH, and salinity were optimal for amylase activity at 50°C, pH 7, and 5% NaCl, respectively. It also generates amylase utilizing agricultural wastes like sugarcane bagasse, wheat bran, and rice husk. Based on the performance of *Bacillus siamensis* sp. F2 using sugarcane bagasse and synthesizing amylase, the current study attempted to produce bioethanol by co-culturing with baker's yeast, which yielded 157 g/L of bioethanol. This novel amylase may have many potential applications in industrial waste-valorization and biorefinery sectors.

Statement Of Novelty

Amylase is a critical enzyme explicitly used in the bioconversion of starch-based waste in the food, textile, and pharmaceutical industries. Our research group has isolated and characterized a rare moderate halophile *Bacillus siamensis* sp. F2, which produces high titers of halo-acid-alkali-tolerant and surfactant stable amylase (4.2 U/mL) by utilizing starch. It also utilizes agricultural waste such as sugarcane bagasse and produces amylase. Based on the success of *Bacillus siamensis* sp. F2 using sugarcane bagasse and producing amylase, the current study aimed to produce bioethanol by co-culturing with baker's yeast, which yielded 157 g/L of bioethanol. This is the first report from the halophilic *Bacillus siamensis* sp. F2 shows high yields of amylase titers and valorization of starchy waste to ethanol.

Introduction

In recent years, it has been consistently noticed that more than 1.3 billion tonnes of starch-based waste are wasted by hotels, kitchens, eateries, bistros, and farmer communities worldwide. Squander is transported in large quantities, and it has traditionally been not easy to dispose of it [1]. Rapid technological development resulted from industrialization, offering new research areas. One of the most searched industrial goods is microbial enzymes, with amylases being one of the most well-known [2]. α-amylase (EC 3.2.1.1, 1, 4-α-D-glucan glucanohydrolase) catalyzes the hydrolysis of α-1, 4- glycosidic linkages in starch and related polysaccharides. Endocytic amylases produce compounds with variable glucose lengths and α-anomeric structures.

Amylase-producing halophilic bacteria can be found abundantly in the environment. These amylolytic bacteria may be easily identified and analyzed for amylase production in a laboratory setting. Certainly, only a few α-amylases from halophilic origins have been investigated, viz., *Natronococcus amylolyticus*

(0.12 U/ml) [3], *Haloferax mediterranei* (0.5 U/ml) [4], *Halorubrum xinjiangense* (0.7 U/ml) [5], *Micrococcus* sp. 4 (1.2 U/ml) [6], *Halobacterium holobium* (1.8 U/ml) [7], and *Halobacillus* sp. strain MA-2 (3.2 U/ml) [8]. The capability to create large quantities of amylase and the simplicity with which microbes may be regulated to produce an enzyme with extremophilic characteristics are two critical advantages of employing microorganisms for amylase production [9]. The *Bacillus* genus is well-known for producing amylase, and very few halophilic *Bacillus* strains, such as *Bacillus dipsosauri* (2.5-3 U/ml) [10] and *Bacillus sp.* strain TSCVKK (0.6 U/ml) [11], have been isolated and screened for producing amylase in past studies. *Bacillus* sp. is a potential candidate for commercial production of microbial enzymes due to its quick fermentation cycles, low-risk handling, the convenience of manipulation, homogeneity, effective enzyme activity under stressful circumstances, and environmentally favourable features [12, 13].

Amylase enzyme is used in food, fermentation, textile, paper, brewing, sugar, baking, soap, distillation, and pharmaceutical sectors [14]. Amylase is also essential in waste management; they are capable of degrading agricultural wastes such as wheat straw, corn starch, oat hulls, and sugarcane bagasse; residues from logging and wood milling; defective products and food processing wastes; and urban solid trash such as paper, cardboard, kitchen, and garden refuse. The use of α-amylase in various industrial sectors is predicted to drive up demand in the upcoming years. Starch saccharification to produce maltooligosaccharides, detergent formulations, starch liquefaction, and bioremediation of wastes from extreme saline environments containing starch and organic solvents are among the most common uses for halophilic amylases [15]. Recently, co-culturing of enzyme-producing and fermentative organisms were employed using a consolidated bioprocessing fermentation process to yield more ethanol from agricultural waste. Multiple researchers conducted one-pot cultivation tests with two different microorganisms, *Pachysolen tannophilis* and *Saccharomyces cerevisiae* [16], *Escherichia coli* and *S. cerevisiae* [17], *Zymomonas mobilis* and *Pichia stipitis* [18], *Acremonium cellulolyticus* and *S. cerevisiae* [19] and *Z. mobilis* and *P. tannophilus* [20], showing ethanol production and demonstrating that bioconversion is viable and potentially efficient.

The current study produced high amounts of unusual halo-acid-alkali-tolerant and surfactant stable amylase by a moderately halophilic bacterium, *Bacillus siamensis* sp. F2, obtained from the soil sample taken from a fish processing tank in the Vishakhapatnam district of Andhra Pradesh, India. We also showed efficient agricultural waste (sugarcane bagasse) conversion to ethanol by co-culturing with baker's yeast. This is the first report from the halophilic *Bacillus siamensis* sp. F2 shows high yields of amylase titers and valorization of starchy waste to ethanol.

Materials And Methods

Isolation and culture conditions of bacterial strain:

Six samples were collected from different habitats in the Vishakhapatnam area of Andhra Pradesh, India, including beach soil, lake soil, lake water, sewage water, and water from the fish processing facility. The samples were preserved in sterile zip-lock bags, labelled with collecting information, transferred to the lab

for analysis, and stored in the refrigerator at 4°C until further use. 10 g of each soil sample collected from the various habitats indicated above was suspended in 90 mL of sterile distilled water, vortexed vigorously, and serially diluted in sterile distilled water for amylase-producing bacteria isolation. After that, dilutions ranging from 10^{-6} to 10^{-8} were plated on GSL-2 medium [21] with the following composition (g/l): starch, 10; citric acid, 0.5; tryptone, 2; yeast extract, 2; $MgSO_4.7H_2O$, 10; NaCl, 50; KCl, 5; NH_4Cl, 2; $NaHCO_3$,1; KH_2PO_4, 0.5; agar, 20. Before autoclaving, the pH of the medium was adjusted to 7 with 1% NaOH. On the plates, well-formed colonies were developed. Colonies with notable morphological changes were chosen and re-streaked on nutrient agar plates containing 5% NaCl to obtain pure cultures. The microbes were cultured in an incubator shaker at 30°C for 72 hours at 150 rpm in a 250 ml Erlenmeyer flask containing 50 ml of medium, and the purified colonies were stored at 4°C on slants of the same media.

Screening of amylolytic bacteria based on starch hydrolysis test

The cultures were screened by starch hydrolysis on GSL-2 plates [13]. Pure soil cultures were subcultured on starch GSL-2 agar plates and incubated for 72 h at 37°C before being flooded with Lugol's iodine solution. When iodine reacts with starch in the medium, it turns blue. The clear zone next to growth was observed as starch was hydrolyzed. A visible halo zone around the colony was detected by removing Lugol's iodine solution from the plates. Amylase production is indicated by a distinct halo zone around the colony (Fig. 1).

Biochemical and morphological characterization of the bacterial strain

Bergey's Manual of Systemic Bacteriology [2, 22] was used to conduct morphological and biochemical characterization tests. Gram staining, catalase test, indole test, gelatin hydrolysis test, oxidase test, urea hydrolysis test, acid-fast staining, Methyl Red and Voges-Proskauer test (MRVP), H_2S production test, and spore staining were all performed on the bacterial strain.

Identification of isolated bacteria (F2) by 16S rRNA gene sequencing

Universal primers (5' AGAGTTTGATCCTGGCTCAG 3' and 5' GGTTACCTTGTTACGACTT 3') were used to polymerase chain reaction (PCR) amplify the 16S rRNA gene. The sequence of the amplified gene was aligned and compared to sequences in GenBank. MEGA, version 5.10, was used to create the phylogenetic tree, which used a neighbour-joining strategy [23].

Amylase assay and biomass detection

The 3,5-dinitrosalicylic acid (DNS) method was used to calculate amylase activity with minor modifications [24, 25]. One mL of assay reaction mixture comprising 1% soluble starch and 0.1 mL of

cell-free supernatant in potassium phosphate buffer (pH 7; 100 mM) containing 5% NaCl incubated at 50°C for 15 min. The amount of reducing sugar released was quantified using DNS with glucose as standard at 540 nm using a Shimadzu UV-1800 spectrophotometer (Nakagyo-Ku, Kyoto, Japan). One unit of amylase activity (U) is the quantity of enzyme that releases one µmole of reducing sugar as glucose equivalents in 1 minute under specified assay conditions. Optical density at 600 nm was used to estimate cell growth. Using lowry's technique and bovine serum albumin (BSA) as a reference, an increase in extracellular protein was evaluated every 24 h [24, 26]. Estimation of total carbohydrate was done using the modified anthrone method [27] to assess substrate utilization by *Bacillus siamensis* sp. F2 for amylase production.

Effect of culture conditions on Bacillus siamensis **sp. F2 for growth and amylase production**

Effect of NaCl, pH, and temperature

Bacillus siamensis sp. F2 was cultivated at various salt concentrations (0 to 30%) with pH 8 at 30°C; different pH (4–10) with 10% NaCl at 30°C; different temperatures (20–50°C) with pH 8 containing 10% NaCl; and different inoculum levels (2, 4, 6, 8, and 10%) with pH 8 containing 10% NaCl at 30°C to see how different NaCl concentrations, pH, temperatures, and inoculum levels impacted amylase production. Each medium is inoculated with 2% *Bacillus siamensis* sp. F2 inoculum and incubated for 48 h [24]. The enzyme activities were determined as previously stated.

Effect of carbon, nitrogen source, salts, and metal ions

Starch in the GSL-2 medium was replaced with 1% (w/v) disaccharide and polysaccharide (sucrose, maltose, lactose, maltodextrin, and pectin) to investigate carbon source's impact on amylase production. The GSL-2 medium was substituted with 0.2% (w/v) casein enzyme hydrolysate, yeast extract, urea, peptone, and potassium nitrate (KNO_3) instead of tryptone and ammonium chloride. Each medium is inoculated with 2% *Bacillus siamensis* sp. F2 inoculum and then incubated for 48 h at 37°C. The enzyme activities were calculated as described earlier.

The salts' effect was tested by substituting NaCl with other salts (10%) viz., KCl, Na_2SO_4, $NaNO_3$, sodium acetate ($C_2H_3NaO_2$,) and trisodium citrate ($Na_3C_6H_5O_7$). Metal ions like $FeSO_4$, $MgSO_4$, $HgCl_3$, $CuSO_4$, $BaCl_2$, and $CaCl_2$ with 10% NaCl were investigated at 0.1% (w/v).

Effect of chelating agent (EDTA) and surfactants (SDS, Triton X-100)

To investigate the effects of chelating agents such as ethylenediaminetetraacetic acid (EDTA) and surfactants such as sodium dodecyl sulfate (SDS) and Triton X-100 on growth and amylase production, 1 and 5% of EDTA and surfactants (SDS and Triton X-100) were added to the medium separately.

Effect of different concentrations of starch (carbon source) and yeast extract, KNO_3, and combination of yeast extract and KNO_3 (nitrogen source)

The effect of different concentrations of a carbon source such as starch (0.5, 1, 2, 3, 4, and 5%) and different concentrations of nitrogen source such as yeast extract (0.2, 0.4, 0.6, 0.8, and 1%), KNO_3 (0.2, 0.4, 0.6, 0.8, and 1%) and a combination of yeast extract and KNO_3 with GSL-2 medium containing 10% NaCl, pH 8, at 30^{o}C were tested for optimum growth and amylase production.

Final time course with the optimized conditions

The final time course was performed with the parameters that yielded high titers of amylase production for every 24 h, GSL-2 medium with additions of (in g/l) starch (30), yeast extract (2), KNO_3 (2), NaCl (100), $MgSO_4$ (1) at pH 8, 30°C.

Effect of NaCl, pH, and temperature on amylase assay

The maximal activity for crude amylase was tested for various temperatures, pH, and NaCl concentrations. The crude amylase was assessed at distinct temperatures (10, 20, 30, 40, 50, 60, 70, and 80°C) in 100 mM phosphate buffer, pH 7 containing starch (1%) and 5% NaCl; different pH with various buffers (100 mM; 5% NaCl) of the following: citrate buffer, pH 5; phosphate buffer, pH 6 and 7; Tris buffer, pH 8; and different NaCl concentrations (0, 5, 10, 15, 20, 25, and 30%) in 100 mM phosphate buffer, pH 7 containing starch (1%) at 50°C.

Partial purification of amylase by acetone precipitation

The supernatant was partially purified by precipitating 50 mL of each culture-free supernatant collected at 48 h of culture with two volumes of cold acetone. After the precipitation stage, the precipitate was centrifuged at 10,000 rpm for 20 min at 4°C. The resulting pellet was solubilized in 10 mL of 100 mM phosphate buffer, pH 7, containing 5% NaCl, and amylase activity was determined.

Native-PAGE and zymogram activity of amylase enzyme

Native-PAGE (Biobase, Shandong, China) of 10% and zymogram activity for amylase were performed accordingly with slight modifications [24, 25].

Effect of NaCl, pH, and temperature on the stability of partially purified amylase

The acetone precipitated enzyme was used to evaluate the enzyme's stability at different NaCl concentrations, pH, and temperatures. The assay was performed at different NaCl concentrations (0, 5, 10, 15, 20, 25, and 30%) in 100 mM phosphate buffer, pH 7 containing 1% starch at 50°C; different pH with various buffers (pH 4–10) containing 5% NaCl; and different temperatures (50, 60, and 70°C) in phosphate buffer (100 mM), pH 7 containing 5% NaCl [24].

Effect of metal ions on amylase

The impact of several metal ions ($CuSO_4$, $FeSO_4$, $MgSO_4$, $ZnSO_4$, $HgCl_2$, $CaCl_2$, $BaCl_2$, and $FeCl_3$) on the partially purified acetone precipitated amylase was investigated by adding metal ions concentrations (2,

5, or 10 mM) to the enzyme assay mixture. Following that, the enzyme assay was performed as previously described.

Effect of EDTA and surfactants

EDTA and surfactants (SDS, Triton X-100) on the partially purified amylase were studied by adding 10 mM EDTA and 0.1% surfactants to the enzyme assay mixture, and the assay was done as described earlier.

Utilization of agricultural waste as substrate for amylase production by Bacillus siamensis **sp. F2**

The utilization of agricultural waste (rice husk, rice straw, sugarcane bagasse, wheat bran, and sweet potato peel) as a substrate for amylase production of *Bacillus siamensis* sp. F2 was examined, replacing starch in the GSL-2 medium with the optimized conditions (10% NaCl, pH 8, at 30^{o}C).

Bioethanol production through consolidated bioprocessing of sugarcane bagasse using Bacillus siamensis **sp. F2 and baker's yeast**

Consolidated bioprocessing was carried out on sugarcane bagasse by co-culturing *Bacillus siamensis* sp. F2 and baker's yeast to produce bioethanol. The optimized medium containing 5% w/v of sugarcane bagasse was used for the fermentation. The medium was autoclaved, and after cooling to room temperature, 4% v/v of the inoculum from a 24 h broth culture of *Bacillus siamensis* sp. F2 and 6% v/v of baker's yeast were added to the biomass suspension and incubated at 37°C. The fermentation broth was performed for twenty-one days, and the ethanol estimation was done by potassium dichromate, followed by the redox titration method [28]. Further, the distillate of the fermented sample was sent for ^{1}H (proton) Nuclear Magnetic Resonance (NMR) analysis for ethanol detection [29].

Results And Discussion

Identification and characterization of amylase producing bacteria

From soil samples, bacterial strains were isolated and evaluated for amylase production in starch agar plates. Halo zones around the colonies were observed for the samples collected from the various habitats. The culture isolated from the fish processing plant displayed a better halo zone; based on this, we have taken this culture and proceeded further to optimize culture and assay conditions for amylase production (Fig. 1). The bacterial strain was identified as amylase-producing bacteria and was further characterized morphologically and biochemically. In biochemical and morphological characterization, the isolate was positive for gram staining, catalase, oxidase, indole, and gelatin hydrolysis test; and negative for acid-fast staining, urea hydrolysis, MRVP, H_2S production, and spore staining test; suggesting the isolated strain belonged to the genus *Bacillus*. Many starch hydrolyzing bacteria belonging to the genus *Bacillus* were isolated in the past [11, 30–32].

Identification of amylase-producing bacterial isolate (F2) using the 16S rRNA gene sequencing analysis

The DNA of the amylase-producing isolate F2 was extracted, and the 16S rRNA gene was amplified using PCR. The sequencing and amplification of the 16S rRNA gene demonstrated 97% similarity with *Bacillus siamensis* strain APPMB25; the isolate F2 will be referred to as *Bacillus siamensis* sp. F2. The 16S rRNA gene sequence was submitted to GenBank with the accession number ON383536. A phylogenetic analysis of the isolate *Bacillus siamensis* sp. F2's relationship to other species was constructed using MEGA X version 5.1, and molecular techniques for the 16S rRNA gene were used to perform contemporary taxonomy for distinguishing between seldom observed bacterial strains (Fig. 2).

Effect of culture conditions on Bacillus siamensis **sp. F2 for growth and amylase production**

Effect of NaCl, pH, temperature, and inoculum levels

The effect of various NaCl concentrations on amylase production was investigated. The *Bacillus siamensis* sp. F2 grew under a diverse range of salinity conditions, from 0–30%. The amylase activity was drastically reduced in the absence of salt, showing that the isolate was a stringent halophile. However, at a concentration of 10% NaCl, the higher cell growth and amylase activity of 1.7 U/ml were attained, and at 30% NaCl, almost 20% amylase activity was retained, suggesting extreme halo-tolerance of the organism (Fig. 3a). The finding of current studies agrees with the other halophilic *Bacillus sp*. strain TSCVKK [11] and showed superiority to amylase-producing halophile, *Halomonas meridiana* [33], which reported the maximum growth at 5% NaCl concentrations. Similar to the current finding, previous research also suggests that amylase activity decreases as NaCl concentrations in the medium increase by more than the optimum of 10% NaCl [34–36].

The effect of pH was investigated under different pH conditions. At pH levels ranging from 4 to 10, the *Bacillus siamensis* sp. F2 strain was capable of producing amylase. However, maximum amylase activity was observed at pH 8 (1.75 U/ml). At pH 4, almost 45% of the activity was displaying, and at pH 9, greater than 85% of amylase activity was retained (Fig. 3b). It can be declared acid-alkali-tolerant based on the enzyme tolerance levels in extreme conditions (acid and alkaline environments). Several researchers looked at the impact of pH on halophilic organism's amylase production. Other halophilic organisms such as *Halobacillus sp* strain MA-2 (pH 7.8) [8] and *Bacillus* strain TSCVKK (pH 8) [11] demonstrated similar results as the current study, and *Bacillus siamensis* sp. F2 was superior to halophilic *Marinobacter sp*. EMB8 (pH 7) [37] indicates the amylase produced from the *Bacillus siamensis* sp. F2 has the peculiar characteristic of showing activity under extreme conditions (acid and alkali).

Under various temperature circumstances, the impact of temperature on amylase production was examined. *Bacillus siamensis* sp. F2 produced amylase within the temperature range from 20–50°C. However, at 30°C, the highest amylase activity was observed, viz., 1.75 U/ml, and at 50°C, almost 18% of amylase activity was retained (Fig. 3c). This observation agrees with halophilic organisms such as *Halobacillus sp* strain MA-2 [8] and *Bacillus* strain TSCVKK with an optimum to be 30^{o}C, and a decline in

production was noticed with the increase in temperature [11]. Few halophilic organisms, such as *Chromohalobacter sp*. TVSP101 [38] and *Marinobacter sp*. EMB8 [37] reported the highest amylase production at 37 and 35°C, respectively.

The effect of various inoculum levels of *Bacillus siamensis* sp. F2 (2, 4, 6, 8, and 10%) in the GSL-2 medium containing 10% NaCl was investigated for amylase activity. The optimum amylase activity was observed at 2% inoculum viz is 1.73 U/ml (Fig. 3d). Few authors reported similar findings for the current study for amylase-producing halophiles such as *Bacillus cereus* [39] and *Bacillus vallismorti* [40].

Effect of carbon, nitrogen, salts, metal ions, chelating agent, and surfactants

The impact of different carbon sources on optimum growth and amylase production was studied using various carbon sources (sucrose, maltose, lactose, starch, maltodextrin, and pectin) in the GSL-2 medium containing 10% NaCl. The observation revealed starch as the best source out of all the carbon sources with the highest amylase production (1.7 U/ml) (Fig. 4a). Similarly, several researchers have identified starch as the optimum carbon source for halophilic organisms such as *Halobacterium salinarum* [41], *Halomonas Meridiana* [33], and *Marinobacter sp*. EMB8 [37].

Using several nitrogen sources (yeast extract, casein enzyme hydrolysate, urea, peptone, and KNO_3) in the GSL-2 medium containing 10% NaCl, the effect of nitrogen source on amylase production was examined. In the current study, the best nitrogen source was yeast extract, followed by KNO_3 with the maximum amylase production viz., 1.68 and 1.35 U/ml, respectively. (Fig. 4b). Similar to current findings, several researchers reported yeast extract as the best nitrogen source for amylase production of halophilic organisms such as *Rhodothermus marinus* ITI 990 [42] and *Bacillus* strain TSCVKK [11].

The impact of salts and metal ions on *Bacillus siamensis* sp. F2 amylase production and growth were investigated. Maximal amylase production was recorded in the presence of 10% NaCl (control) followed by KCl viz., 1.7, and 1.6 U/ml, respectively (Table 1). Other salts, such as Na_2SO_4 and $NaNO_3$, did not influence the growth of the bacteria. However, $C_2H_3NaO_2$ and $Na_3C_6H_5O_7$ hindered the growth of *Bacillus siamensis* sp. F2. Similar results were reported for the halophiles. *Halomonas meridiana* [33] and *Bacillus sp. strain* TSCVKK [11] reported the optimum amylase production at 5 and 10% of NaCl salt, respectively, and *Chromohalobacter sp.*TVSP101 [38] recorded optimal amylase production at 15% KCl salt. $MgSO_4$ ions displayed the maximum amylase activity (1.7 U/ml) (Table 1). Other metal ions like $CaCl_2$ and $BaCl_2$ did not influence growth and amylase production; other halophiles like *Chromohalobacter* sp. TVSP101 reported similar results [38]. However, $HgCl_3$, $CuSO_4$, and $FeSO_4$ inhibited the amylase production, agreeing with the halophile *Halobacillus sp*. strain MA-2 [8].

Table 1
Effect of salts, metal ions, chelating agent, and surfactants on amylase production by *Bacillus siamensis* sp. F2

Additions	Optical density at 600 nm	Amylase activity (U/ml)
Salts (10%)		
KCl	0.66	1.6
Na_2SO_4	0.58	1.4
$NaNO_3$	0.5	1.2
$C_2H_3NaO_2$	0.08	0.2
$Na_3C_6H_5O_7$	0	0
Metal ions (0.1%)		
$MgSO_4$	0.7	1.7
$BaCl_2$	0.58	1.4
$CaCl_2$	0.58	1.4
$FeSO_4$	0.2	0.5
$CuSO_4$	0.2	0.5
$HgCl_3$	0.2	0.5
Chelating agent		
EDTA (1%)	0.15	0.24
EDTA (5%)	0.1	0.12
Surfactants		
SDS (1%)	0.19	0.37
SDS (5%)	0.19	0.37
Triton X-100 (1%)	0.22	0.49
Triton X-100 (5%)	0.15	0.24
Control	0.71	1.7

For investigating the effect of salts, the cells were grown in the GSL-2 medium containing 1% starch, pH 8, at 30°C, and for metal ions, chelating agent, and surfactants, the cells were grown in the GSL-2 medium containing 10% NaCl, 1% starch, pH 8 at 30°C. In control, the cells were grown in the GSL-2 medium containing 10% NaCl, 1% starch, pH 8 at 30°C. Two batches of growth were examined in duplicates. The standard deviation was less than 0.05%.

The effect of chelating agents and surfactants was tested by adding 1 and 5% of EDTA, SDS, and Triton X-100 in the medium. This study reveals the growth of *Bacillus siamensis* sp. F2 was heavily affected by EDTA, SDS, and Triton X-100 and showed limited growth and amylase (Table 1). The observation of the current study is in close agreement with other halophiles such as archaeon *Halococcus* strain GUVSC8 [35], and *Haloferax mediterranei* [4], the presence of EDTA was found to cause a significant reduction of amylase activity.

Effect of different concentration levels of carbon (starch) and nitrogen (yeast extract and KNO_3)

The impact of different concentration levels of starch solutions (0.5, 1, 2, 3, 4, and 5%) on amylase production was investigated. As shown in Table 2, optimum amylase production was observed at 3% starch substrate concentration, 2.75 ± 0.08 U/ml. However, an increase in amylase production was observed with 0.5–3% starch concentration, and a decline in production was observed with an increase in a concentration above 3% substrate. Perhaps this is due to the culture's ability to metabolize starch in such a short amount of time when the starch content was increased [43]. The current study's findings are superior to other halophilic organisms such as *Bacillus cereus* MS6 (2% starch) [44] and *Haloferax* sp. HA10 (1% starch) [45].

Table 2
Impact of different concentrations of carbon (starch) and nitrogen sources (yeast extract, YE and potassium nitrate, KNO_3) on amylase production by *Bacillus siamensis* sp. F2

Additions	Optical density at 600 nm	Amylase activity (U/ml)
Carbon		
Starch (%)		
0.5	0.32	0.79
1	0.71	1.7
2	1.12	2.7
3	1.14	2.75
4	0.31	0.75
5	0.29	0.7
Nitrogen		
YE (%)		
0.2	0.7	1.7
0.4	0.61	1.5
0.6	0.49	1.2
0.8	0.32	0.8
1.0	0.28	0.7
KNO_3 (%)		
0.2	0.54	1.3
0.4	0.48	1.15
0.6	0.45	1.1
0.8	0.29	0.7
1.0	0.25	0.6
YE (%) + KNO_3 (%)		
YE (0.2) + KNO_3 (0.2)	1.04	2.5

For carbon source, the cells were grown in the GSL-2 medium containing 10% NaCl, pH 8, at 30°C, and for nitrogen source, the cells were grown in the GSL-2 medium containing 10% NaCl, 1% starch, pH 8 at 30°C. In control, the cells were grown in the GSL-2 medium containing 10% NaCl, 1% starch, pH 8 at 30°C. Two batches of growth were examined in duplicates. The standard deviation was less than 0.05%.

Additions	Optical density at 600 nm	Amylase activity (U/ml)
YE (0.4) + KNO_3 (0.4)	0.8	2
YE (0.6) + KNO_3 (0.6)	0.54	1.3
YE (0.8) + KNO_3 (0.8)	0.49	1.2
YE (1.0) + KNO_3 (1.0)	0.41	1.0
Control	0.7	1.7
For carbon source, the cells were grown in the GSL-2 medium containing 10% NaCl, pH 8, at 30°C, and for nitrogen source, the cells were grown in the GSL-2 medium containing 10% NaCl, 1% starch, pH 8 at 30°C. In control, the cells were grown in the GSL-2 medium containing 10% NaCl, 1% starch, pH 8 at 30°C. Two batches of growth were examined in duplicates. The standard deviation was less than 0.05%.		

Further, different concentrations of yeast extract, KNO_3, and a combination of both were tested for optimum growth and amylase production. The maximum amylase production was recorded at 0.2% KNO_3, 0.2% yeast extract, and with 0.2% yeast extract and 0.2% KNO_3 viz., is 1.3, 1.7, and 2.5 U/ml, respectively (Table 2). Similar to current findings, a few researchers observed an increase in enzyme activity with the combination of different nitrogen sources [24, 46].

Final time course with the optimized conditions

The final time course of growth of *Bacillus siamensis* sp. F2 with the optimized parameters (that yielded high titers of amylase) in GSL-2 medium with additions of (in g/l) starch (30), yeast extract (2), KNO_3 (2), NaCl (100), $MgSO_4$ (1) at pH 8, 30°C was attempted. A three-fold increase in amylase activity was observed throughout the time course, and the optimum amylase activity was recorded at 48 h with 4.2 U/ml (Fig. 5).

Amylase assay

The amylase secreted by *Bacillus siamensis* sp. F2 showed activity from 0 to 30% NaCl. However, the maximal amylase activity was observed at a 5% NaCl concentration, and at NaCl of 30% concentration, almost 30% of amylase activity prevailed. At 0% NaCl, 60% activity was retained, indicating the broad halo-tolerance capability of the amylase (Fig. 6a). Similar findings were shown in the literature about halophilic amylases in which they reported that amylases produced were active within 1–2 M NaCl (5.8–11.6% NaCl) [47], suggesting amylase secreted by *Bacillus siamensis* sp. F2 is moderately halotolerant.

The amylase produced by *Bacillus siamensis* sp. F2 was active across a broad range of pH (5–10) and displayed maximal activity at pH 7. It showed pH resistance throughout a wide range, with a retainment of 60% activity at pH 5 and pH 10 (Fig. 6b). The current study's findings agree with other amylases

secreted from halophiles such as *Halomonas meridiana* [33] and *Bacillus* strain TSCVKK [11], indicating amylase enzyme produced by *Bacillus siamensis* sp. F2 is acid-alkali tolerant.

The amylase assay was tested for the enzyme activity at various temperatures (10–80°C). The amylase secreted by *Bacillus siamensis* sp. F2 showed good activity from 20 to 70^{o}C. However, it exhibited maximum activity at 50°C and at 70°C, almost 50% amylase activity was retained **(**Fig. 6c**)**. The observation is in agreement with the findings of some researchers who demonstrated the activity of amylase enzyme obtained from halophilic organisms viz., *Halobacillus sp*. strain MA-2 [8] and *Bacillus* strain TSCVKK [11] and showed superiority to the findings of an amylase-producing organism obtained from solid waste, viz., 40°C [48].

Native PAGE and zymogram activity of amylase enzyme

The partially purified enzyme was subjected to native-PAGE (10%) to determine the molecular weight of amylase produced by *Bacillus siamensis* sp. F2. The molecular weight of partially purified amylase appears to be around 40 kDa **(**Fig. 7a, **Lane F2)**. In previous research, α-amylases with a molecular mass range of 12.5–70 kDa have been reported for the pH ranges of 6.5–8, while amylases are acid-stable (pH 3.0–6.0) and have a more extensive molecular weight range, ranging from 41 to 160 kDa [25]. Figure 7b illustrates the zymogram displaying the clearance zone of amylase activity in the native-PAGE gel, represented by the protein band at 40 kDa.

Effect of pH, NaCl, and temperature on the stability of amylase assay

Bacillus siamensis sp. F2 amylase was 100% stable across a wide pH range of 4–8 and slightly decreased at high pH for 48 h **(**Fig. 8a**).** The current study's findings agree with other halophilic organisms such as *Micrococcus halobius* [49] and *Natronococcus sp.* strain Ah-36 [50] amylases.

Even after 48 h of incubation, the acetone precipitated amylase demonstrated good halo-stability, demonstrating 100% activity at 0 to 15% NaCl **(**Fig. 8b**)**. This observation is similar to halophilic amylase from *Haloferax mediterranei* [4]. The halo-stability of *Bacillus siamensis* sp. F2 amylase is superior to other halophilic amylases such as *Natronococcus sp.* strain Ah-36 [50]. However, a slight decrease in residual activity was observed above 15% NaCl concentration **(**Fig. 8b**).**

The *Bacillus siamensis* sp. F2 amylase showed good thermostability, exhibiting 100% activity at 50°C for 45 min, 85% at 60°C, and 80% at 70°C for 5 min **(**Fig. 8c**).** The *Bacillus siamensis* sp. F2 amylase showed better thermostability than other halophiles like *Micrococcus halobius* and *Natronococcus sp.* strain Ah-36 [49, 50].

Effect of metal ions, EDTA, and surfactants on acetone precipitated amylase

The effect of several metal ions on acetone precipitated amylase ($CuSO_4$, $FeSO_4$, $MgSO_4$, $ZnSO_4$, $HgCl_2$, $CaCl_2$, $BaCl_2$, and $FeCl_3$) was investigated. The amylase activity was elevated up to 25% when $CaCl_2$ (5 mM) was added and inhibited by almost 40% in the presence of $CuSO_4$. The other metal ions, such as $FeSO_4$, $MgSO_4$, $ZnSO_4$, $HgCl_2$, $BaCl_2$, and $FeCl_3$, had no discernible impact on amylase activity **(**Table 3**).** The observation of the current study agrees with other halophile-producing amylases, viz., *Amphibacillus sp*. NM-Ra2 [51], *Marinobacter sp.* EMB8 [37] *Nesterenkonia sp.* strain F [52], and *Chromohalobacter sp.* TVSP 101 [38].

Table 3
Effect of metal ions on amylase activity

Metal ion	Relative activity (%) ± standard error		
	2 mM	5 mM	10 mM
None	100 ± 0.1	100 ± 0.07	100 ± 0.14
$CuSO_4$	60 ± 0.18	65 ± 0.09	60 ± 0.19
$FeSO_4$	95 ± 0.19	92 ± 0.08	85 ± 0.11
$MgSO_4$	92 ± 0.21	85 ± 0.08	98 ± 0.15
$ZnSO_4$	68 ± 0.17	93 ± 0.11	75 ± 0.21
$HgCl_2$	85 ± 0.16	93 ± 0.07	80 ± 0.11
$CaCl_2$	99 ± 0.15	125 ± 0.14	110 ± 0.13
$BaCl_2$	80 ± 0.17	99 ± 0.1	80 ± 0.21
$Fecl_3$	80 ± 0.21	98 ± 0.12	70 ± 0.16

On acetone precipitated amylase, the effects of EDTA and surfactants such as Triton X-100 and SDS were investigated. After the addition of EDTA, Triton X-100, and SDS, the relative activity was 50, 92.5, and 92.5%, respectively. The observation shows that the addition of EDTA inhibited the amylase activity. This observation agrees with other halophiles such as *Haloferax mediterranei* [4]. Surfactants such as Triton X-100 and SDS had almost no effect on activity, suggesting that the amylase secreted from *Bacillus siamensis* F2 is surfactant-stable.

Utilization of agricultural waste as substrate for amylase production by Bacillus siamensis **sp. F2**

The utilization of agricultural waste (rice husk, rice straw, sugarcane bagasse, wheat bran, and sweet potato peel) as a substrate for amylase production of *Bacillus siamensis* sp. F2 was investigated. In the current investigation, *Bacillus siamensis* sp. F2 utilized various agricultural waste for its amylase production, viz., sugarcane bagasse (2.1 U/ml), wheat bran (1.9 U/ml), rice husk (1.6 U/ml), rice bran (1.5

U/ml), and sweet potato peel (1.2 U/ml). Similar to the current study, the halophilic *Bacillus cereus* [53] isolated from salt pan soil collected from Tuticorin Coast, Tamil Nadu, India, reported the maximum amylase production using sugarcane bagasse substrate, suggesting sugarcane bagasse as a cheap and readily available source for amylase production.

Bioethanol production through consolidated bioprocessing of sugarcane bagasse using Bacillus siamensis **sp. F2 and baker's yeast**

Bioethanol production through consolidated bioprocessing was carried out on sugarcane bagasse by co-culturing *Bacillus siamensis* sp. F2 and baker's yeast [54]. An increase in ethanol concentration and ethanol yield was observed in the fermentation medium cocultured with *Bacillus siamensis* sp. F2 and baker's yeast (157g/L) compared to the fermentation medium fermented by only baker's yeast (71 g/L) **(**Fig. 9a**)**. Similar to current study, an increase in ethanol concentration were observed in one-pot cultivation tests conducted by multiple researchers with two different microorganisms, *Pachysolen tannophilis* and *Saccharomyces cerevisiae* [16], *Escherichia coli* and *S. cerevisiae* [17], *Zymomonas mobilis* and *Pichia stipitis* [18], *Acremonium cellulolyticus* and *S. cerevisiae* [19] and *Z. mobilis* and *P. tannophilus* [20].

The broth samples were fermented by *Bacillus siamensis* sp. F2 and baker's yeast were examined for the presence of ethanol by NMR spectroscopy. The ^{1}H NMR spectral analysis representing 4, 3, 2, and 1 proton at chemical shifts 4.7, 3.7, 2.2, and 1.2 ppm, respectively, exhibited the characteristic signals for two different groups, namely methylene (-CH_2) and methyl (-CH_3) groups. Figure 9b shows the ^{1}H NMR spectrum of a sample co-cultured with *Bacillus siamensis* sp. F2 and baker's yeast, with the signal of ethanol, were indicated. Few researchers obtained similar spectra for ethanol, which gave triplet signals at 1.2 ppm and multiplet signals at 3.7 ppm produced by the protons of methylene groups, confirming the synthesis of ethanol by one-pot cultivation (coculturing of *Bacillus siamensis* sp. F2 and baker's yeast) displaying the waste (sugarcane) valorization [29].

Conclusion

The current research focuses on developing and optimizing amylase secreted by *Bacillus siamensis* sp. F2 is a moderate halophile and acid-alkali-tolerant bacterium. The highest amylase production, viz., 4.2 U/ml (3-fold increase), was obtained by growing *Bacillus siamensis* sp. F2 cultures in GSL-2 medium with additions of (in g/l) starch (30), yeast extract (2), KNO_3 (2), NaCl (100), $MgSO_4$ (1) at pH 8, 30°C. The amylase was active in a diverse range of salinity (0–30%), pH (5–10), and temperature (20–70°C); and also showed good stability with surfactants (SDS and Triton X-100), hence identified as halo-acid-alkali-tolerant and surfactant stable. In the current study, *Bacillus siamensis* sp. F2 utilized agricultural wastes such as sugarcane bagasse, wheat bran, and rice husk to generate amylase. Further, based on the outcome of *Bacillus siamensis* sp. F2 using sugarcane bagasse and making amylase, the study aimed to produce bioethanol by co-culturing with baker's yeast, which yielded 157 g/L of bioethanol. Further

optimization of one-pot cultivation of *Bacillus siamensis* sp. F2 for obtaining better ethanol yields are in progress.

Declarations

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Author contributions

BGR: He carried out >80% experiments, analysis, and assisted in data curation. SP: She carried out >20% experiments. VGP: He designed the experiments, involved in data curation, analysis, overall supervision of the entire research, and prepared the manuscript.

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Data Availability

The data supporting this study's findings are available from the corresponding author upon reasonable request. However, data have been fully presented in the manuscript, no additional data to disclose.

Code Availability

Not applicable.

Conflict of interest

The authors declare that there is no conflict of interest.

Ethics Approval

Ethics approval was not required for this research.

Consent to Participate

Not applicable.

Consent for Publication

Not applicable

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Figures

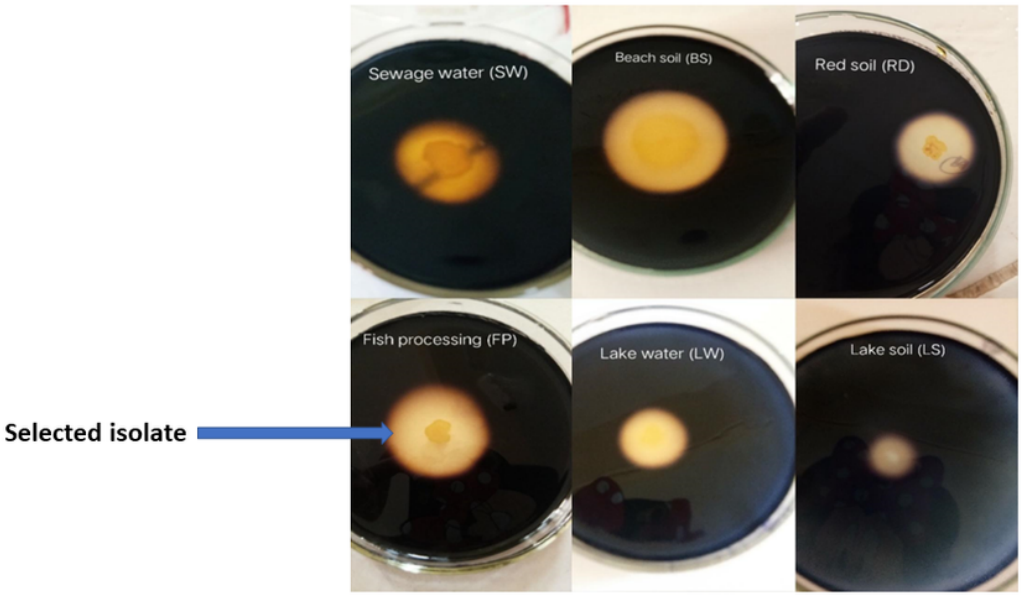

Figure 1

Amylase activity on a starch agar plate with a clear halo zone in the center surrounding a single bacterial colony isolated from several habitats, beach soil, red soil, lake soil, lake water, sewage water, and water from the fish processing facility near Vishakhapatnam area of Andhra Pradesh, India. Blue arrow indicates selected isolate F2 isolated from the fish processing facility.

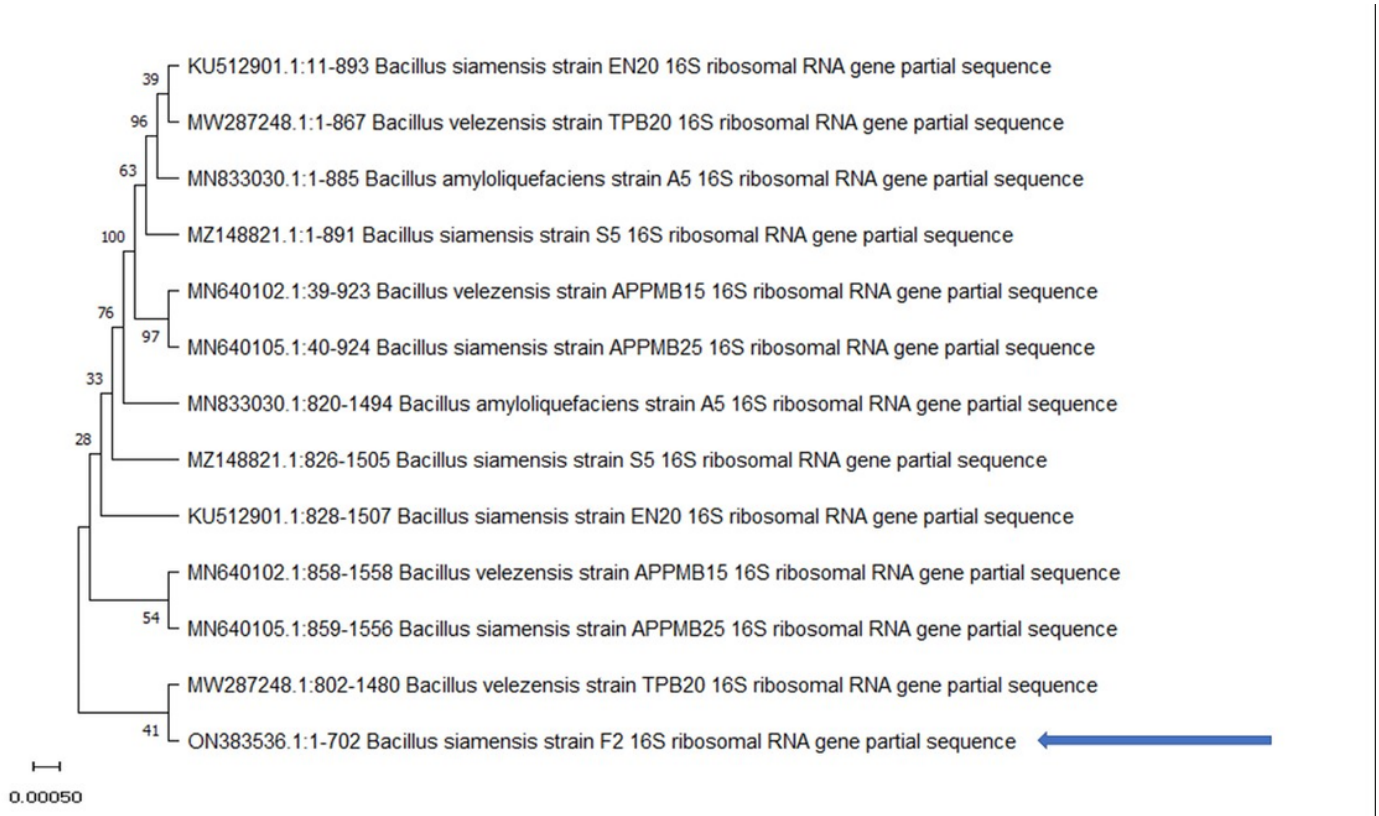

Figure 2

Phylogenetic study of amylase-producing bacteria (*Bacillus siamensis* sp. F2) using MEGA X software and the 16S rRNA sequence generated using the neighbor-joining method. A 0.05 nucleotide mutation per sequencing location corresponds to the scale bar. At the nodes, the boot strap values (%). Blue arrow indicates *Bacillus siamensis* sp. F2 in this study

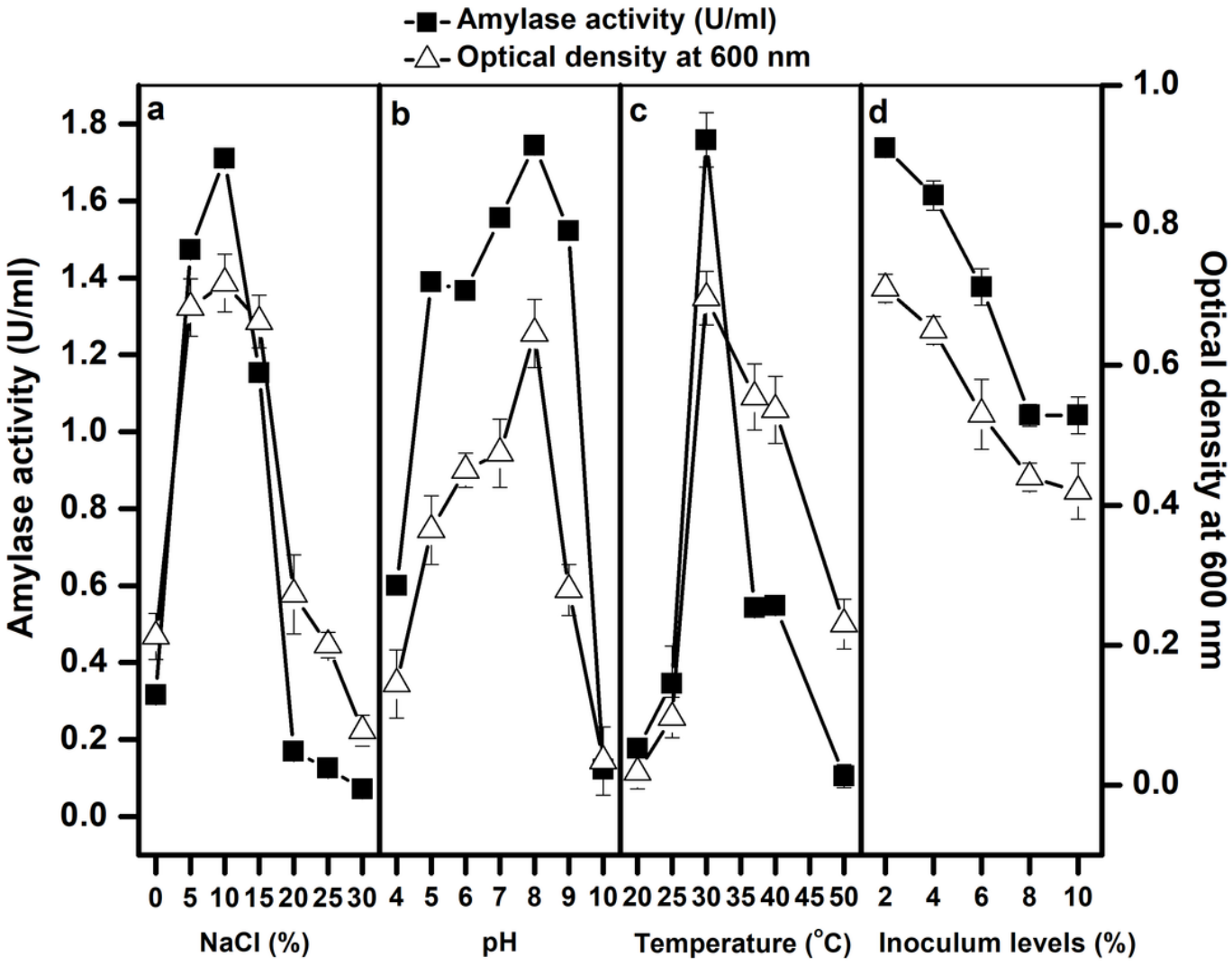

Figure 3

Effect of culture conditions on *Bacillus siamensis* sp. F2 for growth and amylase production; **a**. NaCl, **b.** pH, **c.** temperature, and **d.** inoculum levels. Two batches of growth were examined in duplicates. The standard deviation was less than 0.05%.

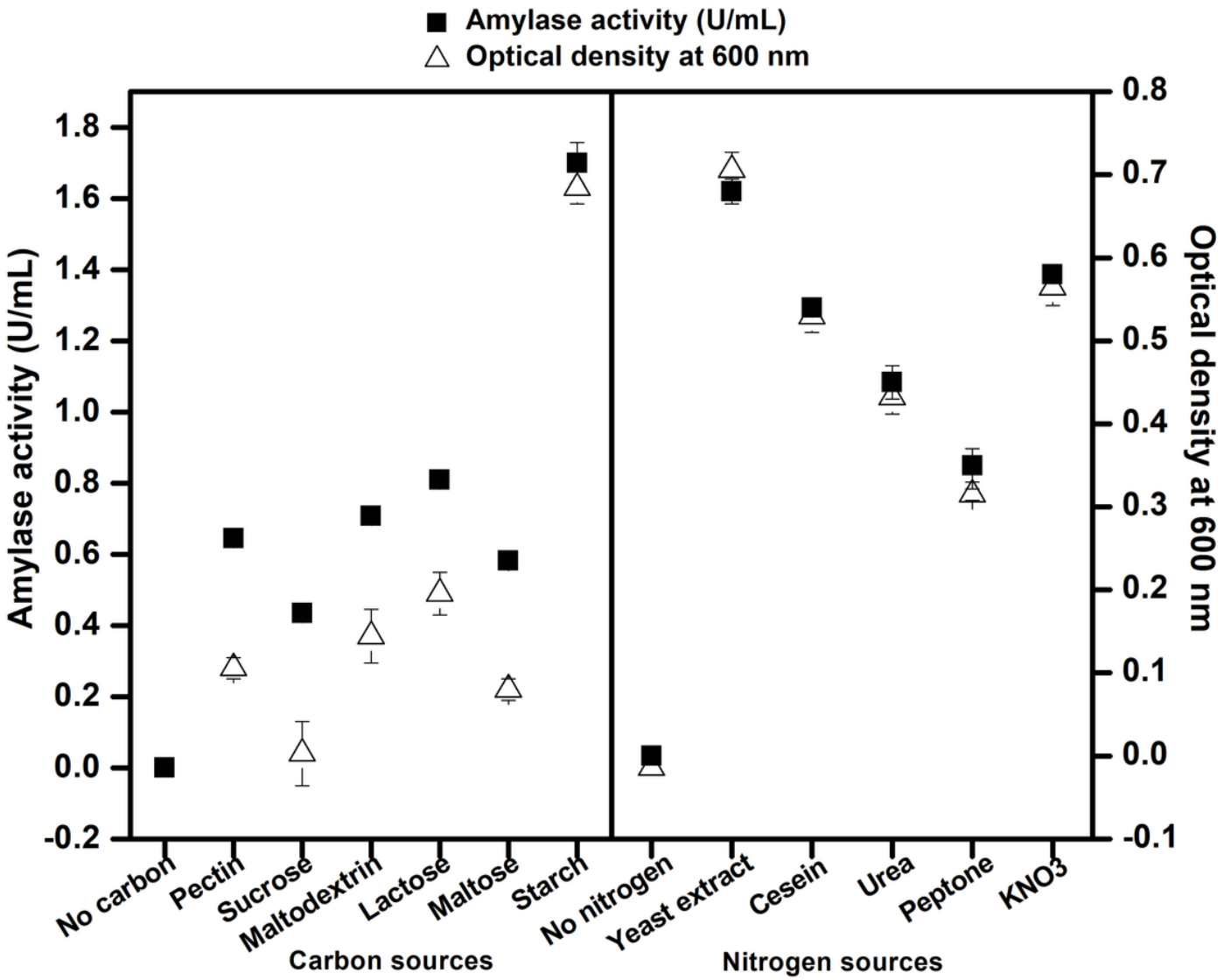

Figure 4

Effect of culture conditions on *Bacillus siamensis* sp. F2 for growth and amylase production; **a**. carbon sources, and **b.** Nitrogen sources. Two batches of growth were examined in duplicates. The standard deviation was less than 0.05%.

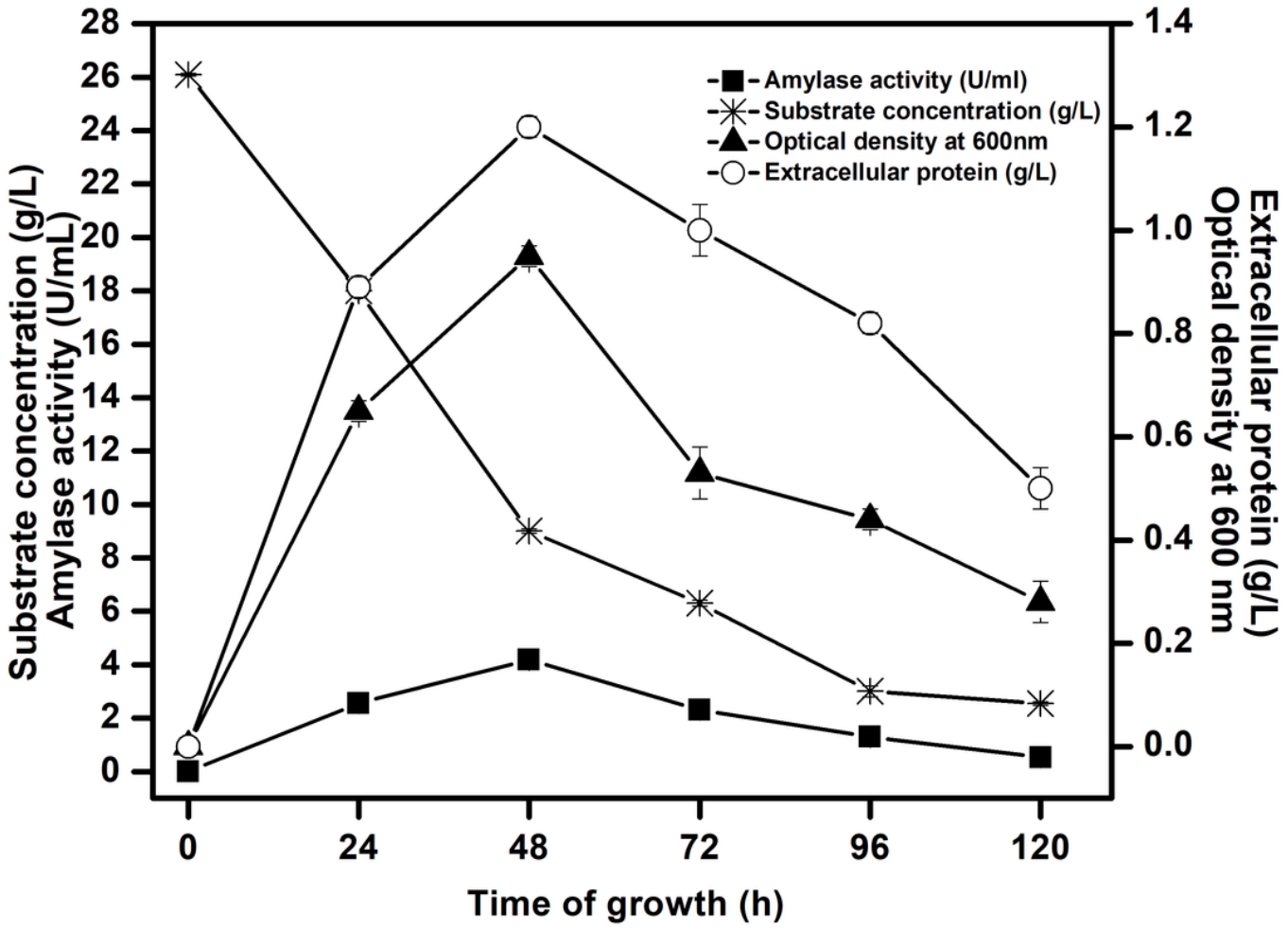

Figure 5

Final time course of growth of *Bacillus siamensis* sp. F2 on GSL-2 medium with additions of (in g/l) starch (30), yeast extract (2), KNO_3 (2), NaCl (100), $MgSO_4$ (1) at pH 8, 30°C. Two batches of growth were examined in duplicates. The standard deviation was less than 0.05%.

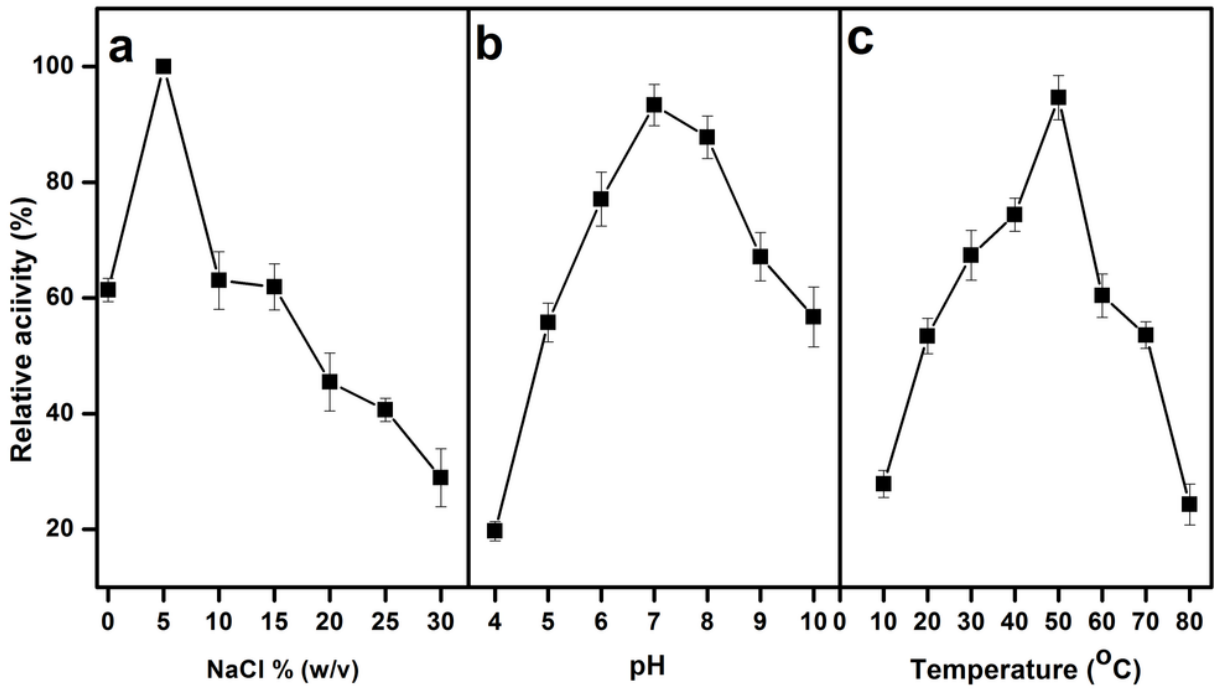

Figure 6

Effect of NaCl, pH, and temperature on amylase activity of *Bacillus siamensis* sp. F2.

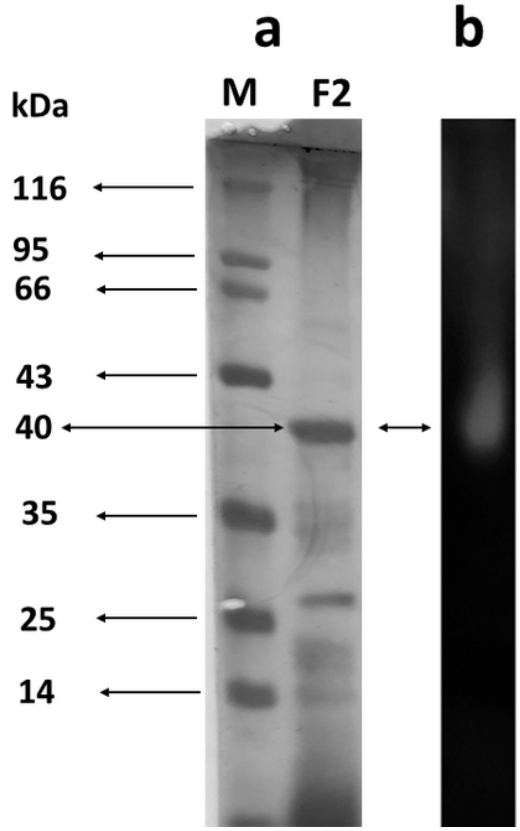

Figure 7

Native-PAGE and zymogram analysis of partially purified amylase **a.** the lanes displays the M, standard protein ladder samples, 14-116 kDa, and F2, acetone precipitated amylase obtained from *Bacillus siamensis* sp. F2, respectively. **b.** Zymogram activity staining of partially purified amylase obtained from *Bacillus siamensis* sp. F2 on Native-PAGE

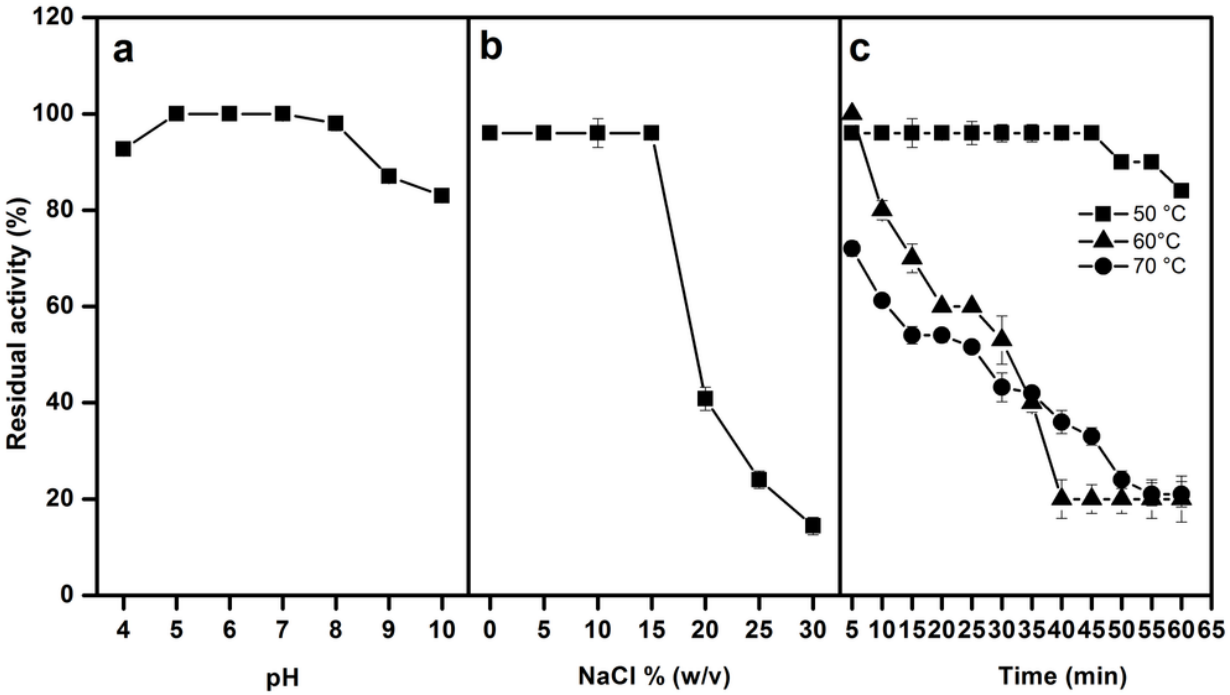

Figure 8

Effect on the stability of amylase assay **a.** NaCl, **b.** pH, and **c.** temperature.

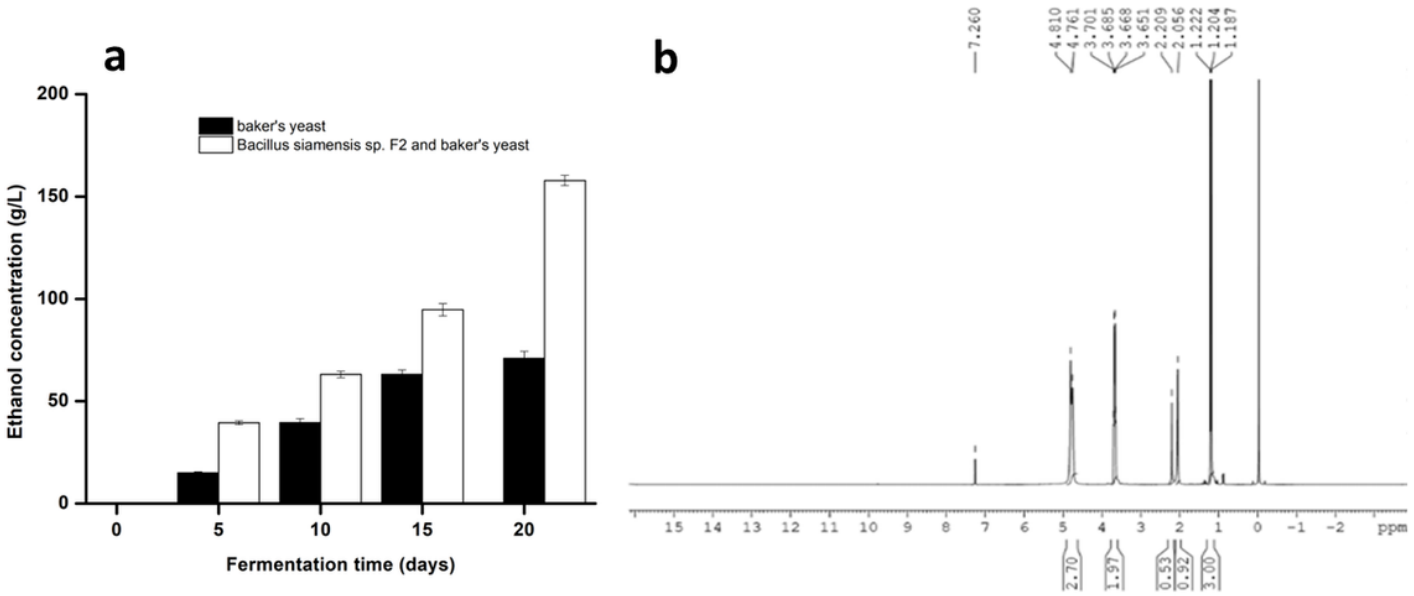

Figure 9

a. Ethanol concentration of fermented medium fermented by baker's yeast alone and *Bacillus siamensis* sp. F2 and baker's yeast combined. Two batches of growth were examined in duplicates, and the standard deviation was less than 0.05%, and **b.** Proton NMR spectra of the distillate fermented medium fermented by *Bacillus siamensis* sp. F2 and baker's yeast showing the presence of ethanol.