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Isolation of a Novel Thermophilic *Anoxybacillus flavithermus* SO-13, Production, Characterization and Industrial Applications of its Thermostable α-Amylase

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Abstract

In this research paper, a novel thermophilic bacteria named strain SO-13 was isolated from hot spring mud sample in Afyonkarahisar (Omer). Based on morphological and biochemical tests, 16S rRNA gene sequence analysis, G-C content and DNA-DNA hybridization analysis, the thermophilic isolate belonged to *Anoxybacillus* genus and it was closely related to *A. flavithermus*. The effects of different solid state fermentation conditions such as different substrate, incubation time, temperature, pH and surfactants on α-amylase production were experimented. In addition to these, different parameters such as temperature and temperature stability, pH and pH stability, detergents and surfactants, different starches and metal ions on effect of partially purified enzyme characterization were examined. The optimum temperature and pH of thermostable α-amylase was 80°C and 7.0, respectively. Thermostable α-amylase hydrolyzed the 76% and 87% of soluble starch content in red and green apple juice at 80°C in 30 min.

Keywords: α-amylase; *Anoxybacillus flavithermus*; Apple juice industry; Detergents; Solid state fermentation; Thermostable

Introduction

Due to the need for the effective, economic, and biotechnological techniques, enzyme technology has progressed a lot. Thanks to biotechnology, it is possible to produce bulk, amount of new enzymes in an economical way. The usage of an enzyme in any industrial area depends heavily on costs of the enzyme that is supposed to be low, being able to use in various areas and the most important one is not having a toxic and allergenic effect [1]. The enzymes which are used almost in every field of industry are derived from microorganisms. The reason for this, compared to enzymes originating from plant and zoological sources, is that the enzymes derived from microorganisms do not create waste, have high catalytic activities and can be attained in excess quantities. Today, enzymes that are used in industry are mostly coming from microbial resources as usage of microorganisms has increased in the industry [2].

The world-wide usage of industrial enzymes was 1 billion dollars in 1995, but in 2000 this number increased to 1.5 billion dollars [3]. 29% of the world enzyme industry comes from food segment, 15% is provender and 56% of it from general technical areas [4].

Biotechnological usage of thermophilic bacteria has come into prominence in recent years; these bacteria are refractory and generate from hot spring water. These microorganisms can produce unique biocatalyst under the extreme circumstances. The enzymes that are produced by these microorganisms have been used in the field of textile, food, detergent, cosmetic and molecular biology [5]. Thermostable enzymes which are procured by being insulated from thermophilic microorganisms have lots of commercial application areas [6]. Enzymes which are produced by these microorganisms have been arousing so much interest because of not being denatured [7].

World-wide usage of microbial enzymes consists of 25% of alkaline proteases, 25% of other protease, 10% of rennin, 3% of lipase, 3% of other carbohydrases, 10% of analytic and pharmaceutics enzymes, and 18% of amylases [8]. Among these enzymes Amylases have an industrial importance as they break up starch to be able to produce dextrin, oligosaccharide, glucose molecules [9].

From food to fermentation, textile to paper industry, amylases have a broad practice area in today's technology. Even though these enzymes come from plant, zoological or microbial sources, microbial enzymes are preferred in the industrial usages. Nowadays a lot of microbial enzymes are available and they have taken their place in starch industry instead of chemical hydrolysis [10]. α-Amylases, which constitute a class of industrial enzymes, keep 25% of world enzyme marketing [11]. α-Amylases are part of endoamylases which randomly hydrolyzes glycosidic bond of starch. α-Amylase ((1,4), glucan, glucanhydroxylase, E.C 3.2.1.1) has a key role in conventional technology by making starch being useful for other amylases [11]. Besides starches hydrolyze, microbial α-amylases are used in chemistry, textile, pharmacy, food, and detergent industry [9].

SSF (Solid-State Fermentation) is described as growth of microorganisms in wet and solid substrate which has no water flow. The presence of humidity is needed in SSF and complex liquid is present that are absorbed by solid particles [12]. Since evaporation and metabolic activity change during fermentation process, the level of humidity of substrate is important [13]. All the biological activities stop at 12% humidity. 12% humidity is recognized as the lowest level of humidity for the SSF [12]. By means of SSF method, the amount of humidity causes the change of structure and stickiness of substrate [12,14].

It can be said that the most favorable microorganisms are yeast and fungi since there is no free water flow at SSF. Because of high need for the water of bacteria, it has been thought that they are not appropriate for SSF. On the other hand, based on the researches there have been some prosperous results of SSF techniques that used bacteria cultures [13,15,16]. It has been proved that especially *Bacillus* genus is appropriate for SSF culture. Because *Bacillus* genus forms a fibrous structure, they benefit from the water in the substrate particules and might reproduce in SSF methods [12].

In SSF technology, the natural structure of substrates is extremely important. Substrates are usually water, insoluble cellulosic polymers and starchy substances. Even though they are kind of substances that come into existence naturally, due to not being refined, these substances' heterogenic nature may affect kinetic reactions of an organism in a negative way in SSF methods. In SSF method, substrate not only provides an organism to have enough nutrients, but it also procures cells to hold on to each other [13,15,16].

Because SSF comprises more production, simple technique, low capital investment, low contamination risk, low energy need, more concentrated production and lower waste water, it has more advantage than SmF (Submerged Fermentation) method [14,15]. Moreover, SSF is important for recycling agricultural wastes [16,17].

Bacterial characterization and determination are based on 16S rRNA gene sequencing. That it has presence in all bacterial species, evolutionary unchanged region and enough length makes the 16S rRNA gene the best classification tool [18]. Additionally, DNA-DNA hybridization was used in bacterial characterization to define a new species and for exact assignment of a strain with changeable characteristic to right taxonomic unit [19].

In this research paper, isolation and identification of thermophilic bacteria was experimented. In addition to these, optimization of α-amylase production and characterization of partially purified thermostable α-amylase were tested. The availability of thermostable α-amylases in the detergent industry, starch hydrolysis and apple juice clarification was experimented.

Material and Methods

Morphological and biochemical tests

In this study, a thermophilic bacterium was isolated from Omer hot-spring mud samples, Afyonkarahisar in Turkey. Mud suspension in sterilized NaCl solution (0.9%) was diluted, poured and spread onto nutrient agar plates, which were incubated at 50°C for 24 h. The different colonies that were found on the plates were transferred one by one onto another nutrient agar consisting of 1% soluble starch. These plates were also incubated at 50°C for 24 h. Several bacterial colonies that yielded a high level of α-amylase were selected after flooding the plates with iodine solution. The morphological and biochemical identification of the thermophilic bacteria were experimented. To determine the characteristics of the bacterium Gram, spore-staining methods and motility tests were tested. The hydrolysis of starch, gelatin and casein were examined. In addition to these, the catalase and lipase activities were assayed for determination of some characteristics of the bacteria.

DNA isolation and Polymerase Chain Reaction (PCR) and phylogenetic analysis of 16S rRNA

To isolate genomic DNA, bacterial sample was incubated at 60°C in rotary shaker at 120 rpm for 16 h in 50 mL liquid broth agar medium that was prepared with tap water. Total genomic DNA isolation was then performed by using Fast DNA Spin Kit for Soil (MP Bio, USA) following instruction's protocol. After that, DNA was measured using Qubit fluorometer (Invitrogen, USA). Lastly, total DNA was run in 1% (0.5 g agar/50 mL 1X TAE buffer) agarose gel at 110 Volt and visualized under Gel Doc (BIORAD, USA) imaging system.

For amplification 16S rRNA gene, PA/PH primer sets (PA: 5'- AGAGTTTGATCCTGGCTCAG-3', PH: 5'-AGGGAGGTGATCCAGCCGCA-3') were used in PCR [20]. PCR was constructed with 1U i-StarTaq™ DNA polymerase in the buffer provided by the manufacturer (INTRON Biotechnology, Inc, USA), 1 μM of each primer, 0.2 mM dNTP and 10 ng of DNA as template in a 40 μL PCR volume totally. PCR reaction was performed with A C1000™ Thermal Cycler (BIORAD, USA) and PCR conditions were settled as 5 min at 95°C, of 30 s at 95°C, 35 cycles 30 s at 55°C, 45 s at 72°C, and extension for 10 min at 72°C. After the polymerization reaction, PCR product was purified by QIAquick PCR Purification Kit (Qiagen, Germany) following manufacturer's protocol and measured using Qubit fluorometer (Invitrogen, USA). Then, purified DNA was analyzed with 1% (0.5 g agar/50 mL 1X TAE buffer) agarose gel at 110 volt and visualized under Gel Doc (BIORAD, USA) imaging system. Finally, sequencing of 16S rRNA gene was performed with PA primer with an AB1373 Automated Sequencer (Invitrogen, Carlsbad, USA) at Iontek Company (Istanbul, Turkey).

Phylogenetic analysis

16S rRNA gene sequence of the new isolate was submitted to Gen Bank database and deposited under the accession number is KJ095000.1. Homology search was performed with Basic Local Alignment Search Tool (BLAST) server of the National Centre for Biotechnology Information by using the BLAST algorithm. Nucleotide sequence was queried against sequence database (blast) and most closely related 16S rRNA genes of species were determined.

The evolutionary history was inferred using the neighbor-joining method [21]. The percentage of replicate trees in which the associated taxa are clustered together in the bootstrap test (500 replicates) is shown next to the branches [22]. The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [23] and are in the units of the number of base differences per site. The analysis involved 5 nucleotide sequences (sequenced nucleotid (sample 13, Accession no. KJ095000.1), the closest nucleotide (Accession no. KF952570.1) and tree close species nucleotide sequences which were taken from NCBI). All positions containing gaps and missing data were eliminated. There were a total of 982 positions in the final data set. Evolutionary analyses were conducted in MEGA6 [24].

Determination of GC contents of DNA by HPLC and DNA-DNA hybridization

GC contents of newly isolated thermophilic bacteria and DNA-DNA hybridization were tested in DSMZ, Germany. For this purpose, cells were cultivated and then disrupted by using a Constant Systems TS 0.75 KW instrument (IUL Instruments, Germany). The DNA was purified on hydroxyapatite according to the procedure of Cashion et al. [25]. The DNA was hydrolyzed with P nuclease and the nucleotides dephosphorylated with bovine alkaline phosphates. The resulting deoxyribonucleosides were analyzed by HPLC. GC was calculated from the ratio of deoxyguanosine and thymidine according to the method

of Mesbah et al. [26]. *Bacillus subtilis* DSM 402 (43.518 mol% G+C), *Xanthomonas campestris* pv. *campestris* DSM 3586[T] (65.069 mol% G+C), *Streptomyces violaceruber* DSM 40783 (72.119 mol% G+C) and non-methylated Lambda-DNA (49.858 mol% G+C; Sigma) were used as references DNA.

HPLC apparatus: The HPLC system (Shimadzu Corp., Japan) consisted of the following modules: LC-20AD solvent delivery module, DGU-20A3 online degasser, CTO-10AC column oven, SIL-20 automatic sample injector, and SPD-20A UV spectrophotometric detector. Chromatograms were analyzed by using the CLARITY (Version 2.4.1.93) software package (DTA Apex Ltd., Czech Republic). The analytical column was a VYDC 201SP54, C_{18}, 5 µm (250 × 4.6 mm) equipped with a C_{18} guard column.

Chromatography conditions: Temperature 45°C, 10 µL sample, solvent 0.3M (NH_4)H_2PO_4/acetonitrile, 40:1(v/v), pH 4.4, 1.3 mL/min [27].

DNA-DNA hybridization: Cells were disrupted by using a Constant Systems TS 0.75 KW (IUL Instruments, Germany) and the DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion [25]. DNA-DNA hybridization was experimented as described by De Ley [28] under consideration of the modifications described by Huss et al. [29] using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian).

Solid-State Fermentation (SSF): Rice husk (RH), banana husk (BH), melon husk (MH), water melon husk (WMH), lentil bran (LB), wheat bran (WB), pistachio husk (PH) and maize oil cake (MOC) were obtained from a traditional markets in Siirt, Turkey. These agro-wastes were utilized as solid-state fermentation substrates and their influences on the production of α-amylase were determined. The best agro-wastes for α-amylase production was selected and used in following experiments.

Three grams of dry agro-wastes, which passed through sieve of 1.500 µm, were put into 100 mL Erlenmeyer flasks. To adjust moisture levels (% by mass per volume), 0.1M Tris HCl (pH 7.0) was added. After autoclaving at 121°C for 15 min, and cooling to room temperature, the flasks were inoculated with 3 mL spore suspension (3 × 10^8 CFU/mL) and incubated at 55°C at 120 rpm.

Optimization of process parameters

The effect of different physico-chemical parameters for the optimization of production of α-amylase by *Anoxybacillus flavithermus* sp. nov. was experimented in SSF. The strategy was to optimize each parameter independently and we tested optimum conditions subsequently in all experiments. Incubation time (24-144 h), temperature (25-80°C), pH (5.0-11.0), and surfactants (0.025-0.1% of SDS and Tween 40) were optimized.

α-Amylase enzyme activity assay

Enzyme activity is determined based on Bernfeld [30] method. According to this method, 100 µL enzyme solutions and 200 µl 0.5% starch solution was incubated in 50°C for 30 min. At the end of 30 min, to stop the reaction by adding 400 µL 3,5-dinitrosalicylic acid, it was kept waiting in hot water bath for 5 min. On one hand, 3,5-dinitrosalicylic acid goes into reaction with the reducing end of sugar and stops the reaction, on the other hand it procures color formation. After that, enzyme activity was determined by spectrophotometric measurement at 489 nm. One unit of enzyme activity was defined as the amount of enzyme that produced 1 mmol of reducing sugar as maltose per minute under the assay conditions.

Partially purified of enzyme

Partially purified of enzyme has been done by Ozdemir et al. [31] based on 2011.

Effect of temperature on α-amylase activity and stability

The effect of temperature on enzyme activity was experimented by using partially purified enzyme. With this purpose, based on Bernfeld method, α-amylase activity was tested via temperature between 30 to 90°C with 10°C increasing intervals. The thermal stability of enzyme was tested by using partially purified enzyme at different degree of temperature. For that, at 50, 60 and 70°C α-amylase activity was experimented for 0, 30, 60, 90, 120, 720 min [32].

Effect of pH on α-amylase activity and stability

The effect of pH on enzyme activity was examined by using partially purified enzyme. The starch which was used as substrate was prepared in order of citrate acid (0.1M pH 4.0, 5.0 and 6.0), Tri-HCl (0.1M pH 7.0, 8.0 and 9.0) and carbonate/bicarbonate (0.1M pH 10.0 and 11.0) buffer. Later, optimum pH was estimated by measuring α- amylase activity. pH stability of enzyme was studied at different pH ranges by using partially purified enzyme. For that, the enzyme activity was examined between pH 5.0 to 9.0, for 0, 30, 60, 90, 120, 240, 360 min pre-incubated.

Effect of detergents and surfactants on α-amylase activity

A 0.5% of detergent solutions were prepared. 200 µL detergent solution, 200 µL 0.5% of starch and 100 µL enzyme solution were added to each tube. Later, enzyme activity was done as mentioned above.

Effect of some metal ions on α-amylase activity

To determine the influence of some metals, which were $MnCl_2$, $ZnCl_2$, $MgCl_2$, $CoCl_2$, $CuCl_2$, $CaCl_2$, $FeCl_2$, $CdCl_2$ and $HgCl_2$ on α-amylase activity, was studied. The stock metal solutions were prepared as concentration of 50 Mm. The metal solution was added to make sure that the concentration of final concentration became 1.5 mM. The group that was tested and did not include metal solution was assumed as control group and enzyme activity was tested.

Effect of different starches on α-amylase activity

For the purpose of testing the effect of α-amylase activity on wheat, potato, corn, and rice starches, 100 µL partially purified enzyme and 200 µL 0.5% of starch solution were incubated under the optimum conditions for 30 min. To stop the reaction, 400 µL 3,5-dinitrosalicylic acid was added and waited in boiling water for 5 min. Later, dilution was done via 3 mL distilled water and spectrofotometric measurement was done at 489 nm.

Starch content in unripe red and green apples and testing of utilization of partially purified α-amylase within clarification industry of apple juice

The determination of the amount of starch in unripe apple juice was tested according to Carrín et al. [33], and the usage of partially purified α-amylase in the industry of clarification of apple juice was assayed.

Results and Discussion

Morphological and biochemical tests, 16S rRNA gene sequence analysis, GC content and DNA-DNA hybridization

According to the morphological analysis, the isolate was Gram (+), motile, terminal spore-forming, rod-shaped and forming a pale yellow

colony. The isolated strain was catalase and lipase positive. The starch was hydrolyzed. Thermophilic isolate could grow at temperatures and pHs from 25 to 85°C and 5.0 to 10.0, respectively.

The DNA was extracted from the bacterial sample numbered as 13. The amount of DNA was quantified as 51 µg/mL and the extracted DNA was visualized by GelDoc™ (Biorad, USA) (Figure 1a). Furthermore, the result of PCR which amplifies 16S rRNA gene region by PA/PH primer set was visualized by agarose gel (Figure 1b). After PCR purification, the amount of DNA was quantified as 24.7 µg/mL.

The sequence was compared to related bacteria sequences by using BLAST. The results demonstrated that the most related species to our new isolated bacteria (Accession no: KJ095000.1) based on 16S rRNA gene region sequence is *Anoxybacillus flavithermus* species (Accession no: KF952570.1) which has 99% similarity to the bacterium DNA and also has 99% similarity with *Anoxybacillus kestanbolensis* (Accession no. KC310452.1), 94% similarity with *Geobacillus stearothermophilus* (Accession no. AY608948.1) and 94% similarity with *Bacillus vireti* (Accessing no. EU221371.1). Figure 2 shows the phylogenetic tree analysis of 6 nucleotide sequences which include our bacterial sample nucleotide sequence (Accession no. KJ095001), the closest bacteria nucleotide sequences and four related bacterial sequences.

The isolated bacteria strain of 13 was found to be 39% G-C content. These isolates were found to show 61.9% similarity with DSM 2641T *Anoxybacillus flavithermus* ssp. *flavithermus* after the DNA-DNA hybridization results. The hybridization result is lower than the recommended threshold value of 70% DNA-DNA similarity for the definition of bacterial species by the *ad hoc* committee [34] are considered. According to the low similarity of DNA-DNA hybridization between strain 13 and the *Anoxybacillus flavithermus* ssp *flavithermus* DSM 2641^T (ID 12-1132), phylogenetic analysis, and biochemical differences, we recommend that strain 13 should be placed in the genus *Anoxybacillus* as the type strain for the novel species *Anoxybacillus flavithermus* sp.

Production of α-amylase by using different substrates

In SSF studies, different industrial and agricultural wastes such as rice, wheat, millet, corn and soybeans are usually used as the main substrate. Works of producing enzyme in an environment of SSF by using thermophilic microorganisms have been increasing in recent years and have started to become an alternative technique to SmF. Increase in worldwide reuse of agricultural wastes is very important ecologically and economically. It is known that agricultural waste can be used for areas such as animal feed, plant food, etc. However, by using SSF technique, it is possible to have more benefits from these wastes. In our study, the banana husk (BH), water melon husk (WMH), lentil bran (LB), wheat bran (WB), melon husk (MH) and maize oil cake (MOC), rice husk (RH), pistachio husk (PH) were used as SSF medium. The highest enzyme production (2967.8 U/mg) was obtained with SSF medium containing the rice husk at 24 h by *A. flavithermus* sp. nov. SO-13 (Figure 3). It was noticed that RH was found as the best substrate for α-amylase production by thermophilic isolate [35]. As the maximum α-amylase production activity was obtained in rice husk, in our further studies the rice husk was used as SSF of substrate. For the same incubation time the low production (129.5 U/mg) was obtained in the pistachio husk. Because rich content of vitamins and minerals are present in rice husk and wheat bran, growth of microorganisms might be facilitated and increase their capacity to produce the enzyme. With this study, rice husk can be used as the solid substrate for the production of industrial enzymes by thermophilic *A. flavithermus* sp. nov. SO-13 and also leads to environmental problems, both

economically and safety related.

Determination of incubation time for production of α-amylase

This study was experimented to determine the effect of incubation time on enzyme production. From 24 h till 120 h, enzyme activity was assayed for every 24 h. The enzyme production at incubation time of 144, 120, 96 and 72 h were found as 2516.3, 3211.7, 3421.8 and 3714.6 (U/mg), respectively. For α-amylase production the most appropriate incubation time was determined as 48 h (3871.2 U/mg) with rice husk. After this time, a decline was observed in enzyme production (Figure 4). As there is food consumption and production of the secondary metabolites in the fermentation medium at the microbial growth stationary phase, the enzyme synthesis and activity decrease [36]. The synthesis and activity of the enzyme also reduce due to the interaction with other compounds in the medium occurring because of enzyme denaturation [37]. These results are in line with previous studies. In addition to these, decreasing of enzyme production after 48 h is thought to be due to the presence of the protease enzyme.

Effect of incubation temperature on α-amylase production

To determine the effects of the incubation temperature on α-amylase production, the SSF medium which contained rice husk was incubated at 25, 30, 40, 50, 60, 70 and 80°C. The results were represented in Figure 5. When the fermentation temperature increased from 25°C to 40°C, enzyme production rose from 101.4 U/ mg to 3506.9 U/ mg. The optimal production temperature for the α-amylase production was determined as 50°C (4012.7 U/ mg). As the temperature increased, a decline in the production of enzymes occurred. The influence of temperature on α-amylase production is related to the growth of the organism. The decreasing of production of the enzyme below and above 50°C is thought to be low bacterial growth.
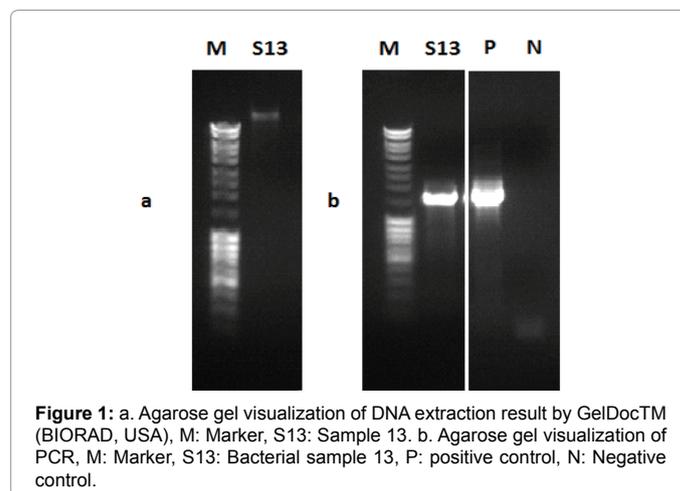

Figure 1: a. Agarose gel visualization of DNA extraction result by GelDocTM (BIORAD, USA), M: Marker, S13: Sample 13. b. Agarose gel visualization of PCR, M: Marker, S13: Bacterial sample 13, P: positive control, N: Negative control.
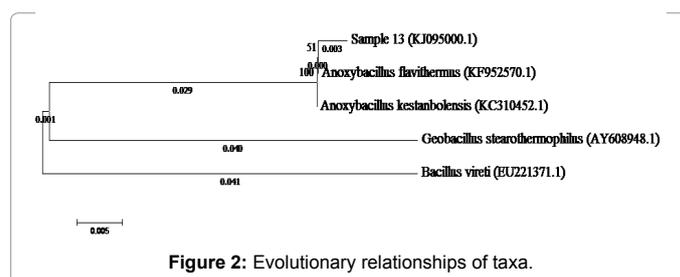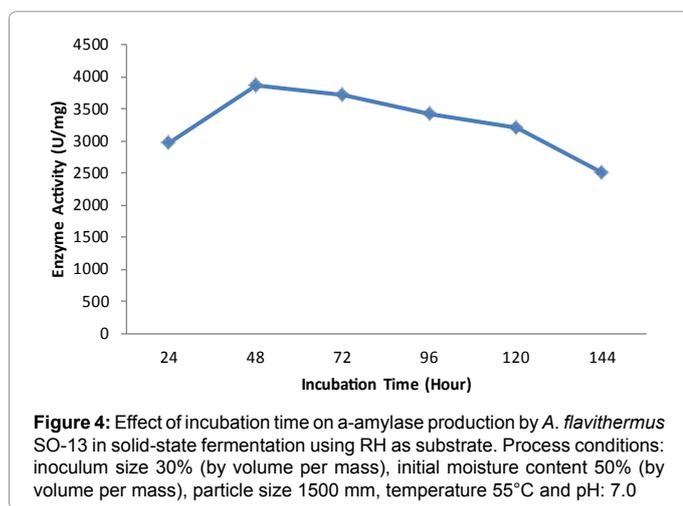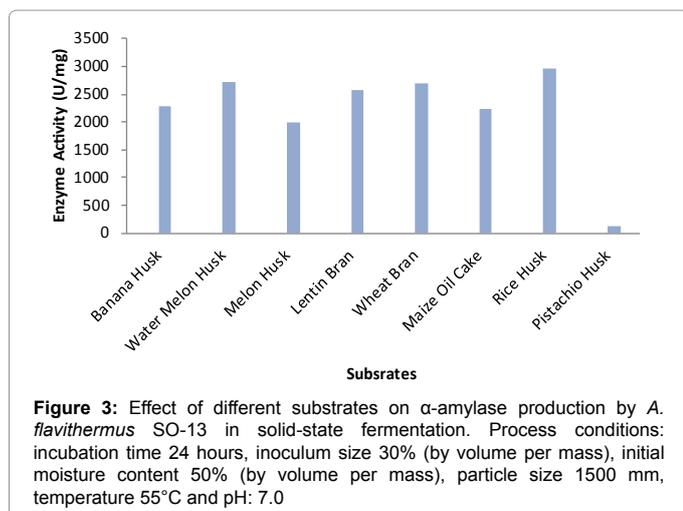

Figure 2: Evolutionary relationships of taxa.

Figure 3: Effect of different substrates on α-amylase production by *A. flavithermus* SO-13 in solid-state fermentation. Process conditions: incubation time 24 hours, inoculum size 30% (by volume per mass), initial moisture content 50% (by volume per mass), particle size 1500 mm, temperature 55°C and pH: 7.0

Figure 4: Effect of incubation time on a-amylase production by *A. flavithermus* SO-13 in solid-state fermentation using RH as substrate. Process conditions: inoculum size 30% (by volume per mass), initial moisture content 50% (by volume per mass), particle size 1500 mm, temperature 55°C and pH: 7.0

Effect of incubation pH on α-amylase production

To determine the influence of initial pH on α-amylase production was experimented. The fermentation medium pH was adjusted to 4.0 and pH 10.0 by using 0.1M HCl and 0.1M NaOH and 10 mL of prepared different pH of tap water was then added in SSF medium containing rice husk. After autoclaving the SSF medium, the bacteria cultivation was performed at 50°C. After 48 hours of incubation, the supernatant was taken and enzyme activity was estimated. The maximum enzyme activity was obtained as 4012.7 U/mg at pH 7.0 (Figure 6). Among the physical parameters, the pH of the fermentation medium has a significant role by growh of organism and in enzyme production. Most of the *Bacillus* strains used commercially for the production of bacterial α-amylases by SmF have an optimum pH between 6.0 to 7.0 for growth and enzyme production [10]. The α-amylase production decreased when the pH value was lower or higher than 7.0. This may be due to the low bacterial growth or denaturation of enzyme.
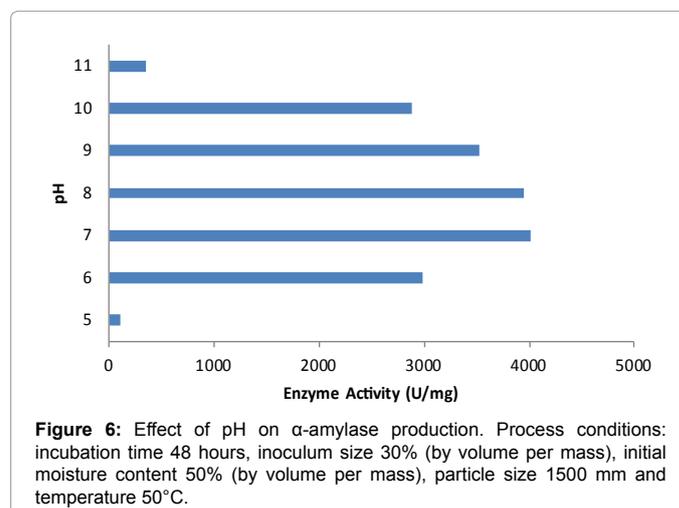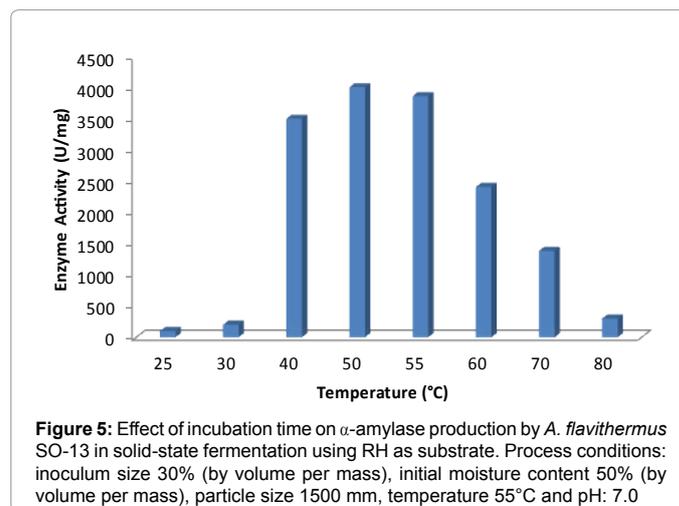
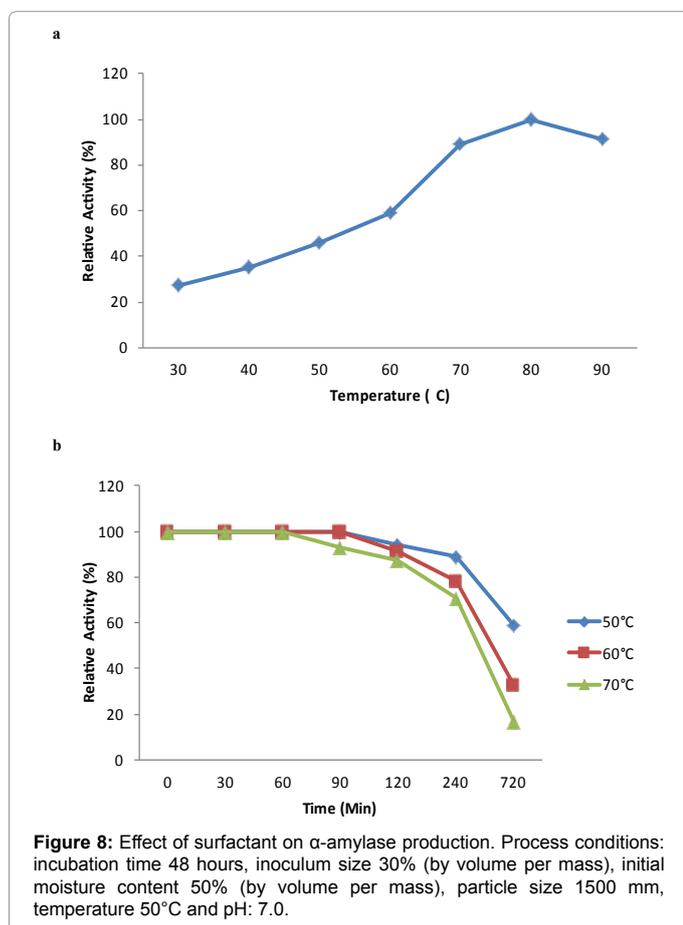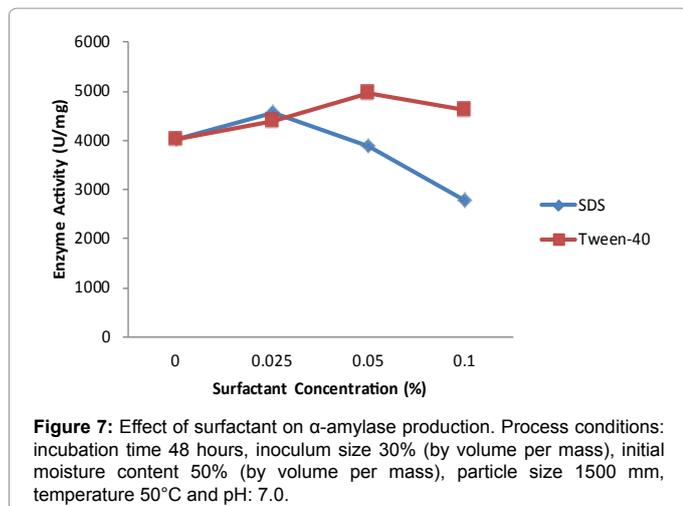## Effect of various surfactants on α-amylase production by *Anoxybacillus flavithermus* sp. nov SO-13

The effect of different concentrations of surfactants on production of α-amylase was shown in Figure 7. According to these results, it was observed that enzyme production was increased with a percentage of 9.6, 12.3 and 11.3% at the presence of 0.025, 0.05 and 0.1% Tween 40,

respectively. Goes and Sheppard [38], showed that Tween 80, Tween 20, and SDS significantly increased the production of α-amylase by *B. subtilis* ATCC 21556. The production of the enzyme showed an increase of 9.1% in the presence of 0.025% SDS. At the concentration of 0.05 and 0.1% of SDS enzyme production was found to be inhibited in the percentage of 3.2 and 11.4%, respectively. A decrease in enzyme production in high concentrations can be explained by the cytotoxic effect of surfactants (data not given). The increasing enzyme production in low concentrations can be clarified by expanding the pores on bacterial cell wall, so more enzymes secrets to the extracellular environment. These results are similar to earlier study [39].

Effect of temperature on α-amylase activity and stability

As depicted in Figure 8a, the enzyme activity showed an increase up to 80°C. The relative enzyme activities at 30, 40, 50, 60 and 70°C were obtained with the percentage of 27%, 35%, 46%, and 59%, respectively. The highest α-amylase activity was found to be 80°C. The similar results were reported by Burhan et al [39] and Mollania et al [40]. The enzyme activity decreased in the percentage of 9% at 90 °C. To determine the effect of different temperature values on α-amylase stability at 50, 60 and 70°C for 0, 30, 60, 90, 120, 240 and 720 min, α-amylase activity was performed. The relative enzyme activity at 50, 60 and 70°C for 720 min was obtained with 59%, 33% and 17%, respectively. As shown in Figure 8b, at all studied

Figure 5: Effect of incubation time on α-amylase production by *A. flavithermus* SO-13 in solid-state fermentation using RH as substrate. Process conditions: inoculum size 30% (by volume per mass), initial moisture content 50% (by volume per mass), particle size 1500 mm, temperature 55°C and pH: 7.0

Figure 6: Effect of pH on α-amylase production. Process conditions: incubation time 48 hours, inoculum size 30% (by volume per mass), initial moisture content 50% (by volume per mass), particle size 1500 mm and temperature 50°C.

Figure 7: Effect of surfactant on α-amylase production. Process conditions: incubation time 48 hours, inoculum size 30% (by volume per mass), initial moisture content 50% (by volume per mass), particle size 1500 mm, temperature 50°C and pH: 7.0.

Figure 8: Effect of surfactant on α-amylase production. Process conditions: incubation time 48 hours, inoculum size 30% (by volume per mass), initial moisture content 50% (by volume per mass), particle size 1500 mm, temperature 50°C and pH: 7.0.

temperatures showed no significant loss of activity up to 90 min. The half-live of our α-amylases was more than α-amylases from produced by *Geobacillus* sp. LH8 [40] and *B. licheniformis* NH1[41]. Our results showed that partially purified enzyme can be used in various biotechnological industries for its thermal stability character.

Effect of pH on α-amylase activity and determination of α-amylase stability at different pHs

To determine the effect of pH on α-amylase activity, starch (0.5%)

to be used as a substrate was prepared separately in the buffer of citric acid (0.1M pH 4.0, 5.0 and 6.0), Tris-HCl (0.1M pH 7.0, 8.0, and 9.0) and carbonate / bicarbonate (0.1M, pH 10.0, 11.0), respectively. Then α-amylase activity was experimented at the optimum temperature. The relative enzyme activity was 86%, 79%, 71%, 63% 59% and 49% at pH 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0, respectively. As shown in Figure 9a, the optimum pH was obtained as 5.0. Similar results were found by Liu et al [42] and Mollania et al [40]. To determine α-amylase stability at various pH values (5.0, 6.0, 7.0, 8.0 and 9.0), residual α-amylase activity was experimented at 0, 30, 60, 90, 120, 240 and 360 min. Up to 90 min., there was not a significant decrease in enzyme activity. As shown in Figure 9b, decreases in enzyme activity are observed at different pH values after 120 min. The high relative activity detected at 360 min as 83% as was obtained at pH 7.0. α-Amylases are generally stable over a wide range of pH from 4.0 to 11.0. The optimum pH and stability of α-amylase has been indicated to be the range of pH 6.0 to 7.0 [43,44]. Our results are consistent with these findings.

Effect of detergents (0.2 %) on α-amylase activity

The influence of detergents such as Omo, Ariel, Alo, SDS and Tween 40 on partial purified α-amylase activity in the time interval from 15 to 120 min is shown in Figure 10. According to these results, compared with the control, it was observed that all of the detergents inhibited enzyme activity during the 15 min. However, it was observed that the enzyme activity increased at the increasing period of time. As a result of the interaction of bacterial α-amylase with the detergent, it was achieved that it gained a significant stability together with increasing time. Our results are in accordance with the results of Saxena et al. [45], who reported α-amylase from *Bacillus* sp. PN5 showed more than 80% activity when incubated with sodium dodecyl sulphate. As
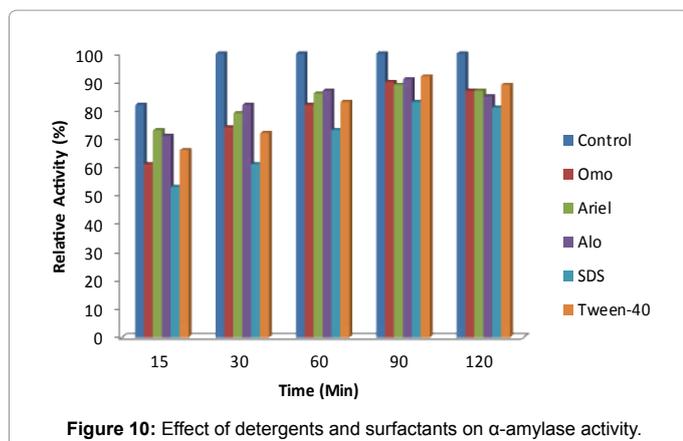

Figure 9: Effect of pH on α-amylase activity (a) and stability (b).
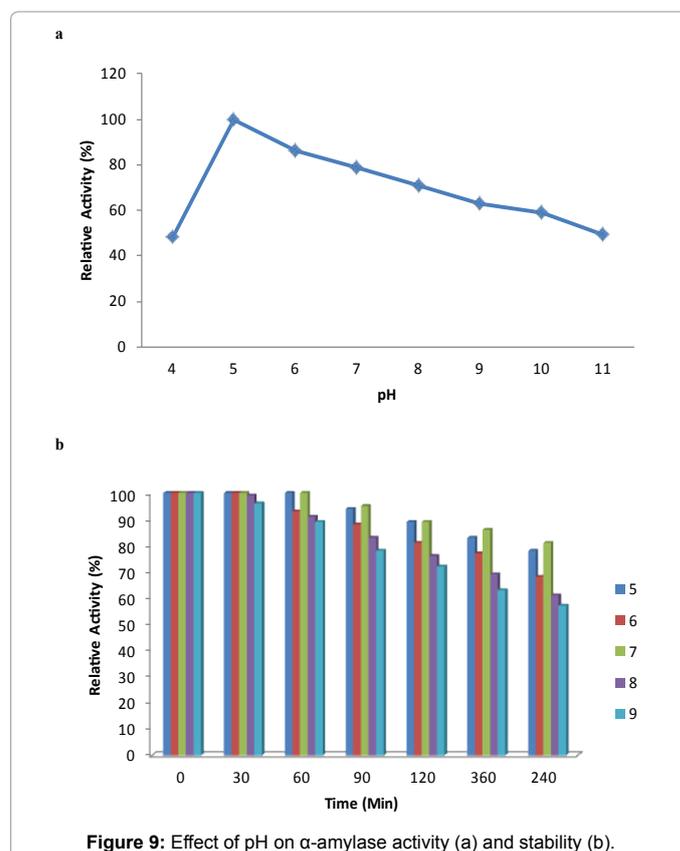
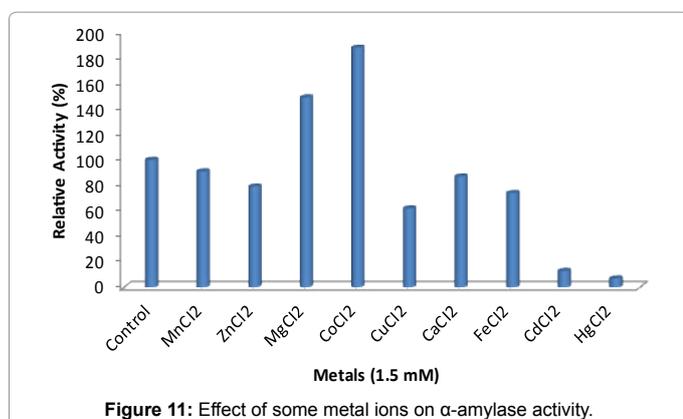**Figure 10:** Effect of detergents and surfactants on α-amylase activity.

Figure 11: Effect of some metal ions on α-amylase activity.
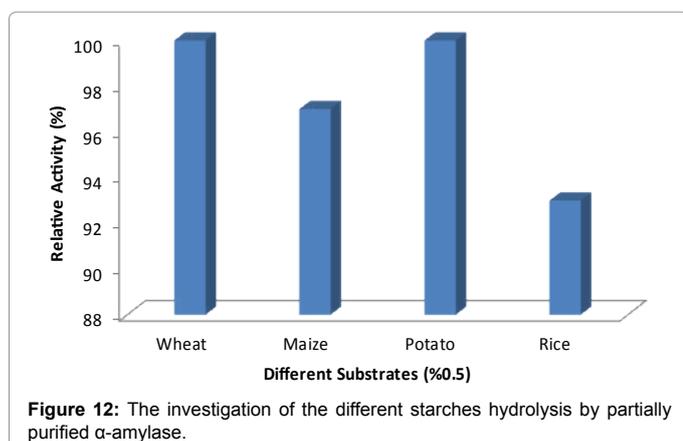

Figure 12: The investigation of the different starches hydrolysis by partially purified α-amylase.

a result of enzyme stability tests and tests conducted upon the effect of the detergents, the partially purified enzyme is commercially potential for use in the detergent industry.

Effect of some metals on α-amylase activity

The effect of some metals such as $MnCl_2$, $ZnCl_2$, $MgCl_2$, $CoCl_2$, $CuCl_2$, $CaCl_2$, $FeCl_2$, $HgCl_2$ and $CdCl_2$ on α-amylase activity is shown in Figure 11. As shown in Figure 11, the enzyme activities were found to be 188% and 149% in the presence of Co^{+2} and Mg^{+2}, respectively. However, these results did not accord with the results obtained by Asgher et al. [43]. When Cd^{+2}, Fe^{+2} and Cu^{+2} were incubated with partially purified enzyme, the relative enzyme activity decreased. It was

observed that the enzyme activity was almost completely lost in the presence of Hg^{+2}. It was similarly observed that activation of α-amylase with Co^{2+} was reported by Najafi et al. [46] and Saboury [47]. Other amylases that showed Ca^{2+} independent were also reported, viz., those of *B. thermooleovorans* NP54 [48] and *Bacillus* sp. KR-8104 [49]. Most of the results obtained in our study are in parallel with previous study [31].

Degradation of different starches by α-amylase

Degradation of 0.5% wheat, corn, potato and rice starches by partially purified α-amylase were tested (Figure 12). It was seen that α-amylase degraded these different starches higher than 90%. As a result, it is considered that the partially purified α-amylase may be used in starch industries to obtain glucose and maltose syrup.

Starch content in unripe red and green apples and testing of utilization of partially purified α-amylase within clarification industry of apple juice

Starch contents of unripe red and green apples were determined. According to these results, soluble and insoluble starch contents in the unripe red apples were found as 0.47 g/L and 6.48 g/L respectively. Soluble and insoluble starch contents in the unripe green apple were found as 0.38 g/L and 5.66 g/L respectively. Degradation of the soluble starch in unripe red apple by partially purified α-amylase was tested depending on time (0-30 min.). After 30 min of incubation, it was determined that partially purified α-amylase degraded 76% and 87% of soluble starch content in the unripe apple red and green respectively (Figure 13). Similar results had been seen in the studies done by Carrín et al. [33]. As a result, it can be said that this thermostable α-amylase can be used as an enzyme source in apple juice clarification industry.

Conclusion

In conclusion, a novel thermophilic *A. flavithermus* sp. nov. SO-13 was isolated and identified. SSF was used for thermostable α-amylase by using RH as substrate. The thermostable enzyme was characterized and some of its industrial applications were examined. Enzyme production was find maximum in presence of 0.025% SDS concentration, at 50°C and pH 7.0 for 48 hours. It was revealed that optimum temperature of activity was 80°C. The optimum pH activity was observed between 5.0. According to our results, α-amylase produced from *Anoxybacillus flavithermus* sp. nov. SO-13 can be used in different biotechnologic purposes such as detergents, apple juice clarification, etc.
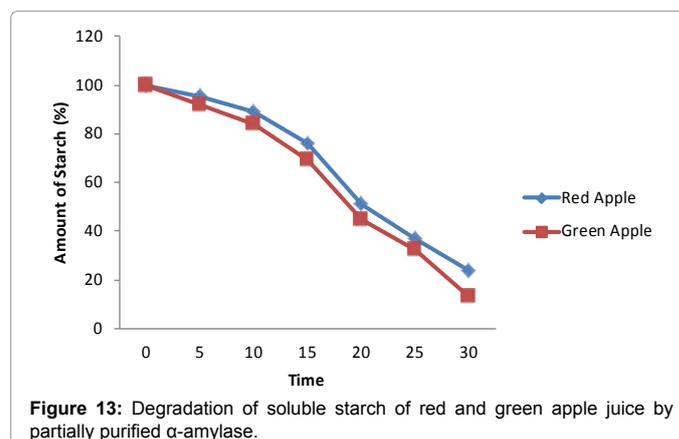
Acknowledgements

Figure 13: Degradation of soluble starch of red and green apple juice by partially purified α-amylase.

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