Production of Thermostable α-Amylase Through Solid State Fermentation (SSF) by Using Thermophilic *Anoxybacillus* sp.

Termofilik *Anoxybacillus* sp. Kullanarak Katı Faz Fermantasyonu (SSF) ile Isılkararlı α-Amilaz Üretimi

Research Article

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ABSTRACT

The production of extracellular α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) by a newly isolated thermophilic bacterium *Anoxybacillus* sp. was studied in solid state fermentation (SSF). Bacterial strain was isolated from a thermal spring of Ömer, Afyonkarahisar in Turkey. Agricultural wastes such as banana husk, wheat bran, rice husk, apple bark, orange bark, maize oil cake, lentil bran and pistachio shell were used for α-amylase production as solid substrates. Growth on rice husk gave the highest α-amylase activity. The maximum enzyme activity obtained was 3.628 U/mg of under optimum conditions of an fermentation time of 48 h, an incubation temperature of 60°C, a pH of 6.0, a substrat particle size 1.500 μm, an initial moisture level of 60% and an inoculum level of 40% (v/w).

Key Words
Agricultural waste, α-amylase, solid-state fermentation (SSF), thermophilic bacterium.

ÖZ

Ekstrasellüler α-amilaz (1,4-α-D-glukan glukanohidrolaz, EC 3.2.1.1) üretimi yeni izole edilmiş bir termofilik bakteri olan *Anoxybacillus* sp. kullanılarak katı faz fermantasyon (SSF) yöntemiyle gerçekleştirilmiştir. Bakteri ırkı, Türkiye'de Afyonkarahisar, Ömer, termal kaplıcasından izole edilmiştir. α-Amilaz üretimi için, muz kabuğu, buğday kepeği, pirinç kabuğu, elma kabuğu, portakal kabuğu, mısır yağı pastası, mercimek kepeği ve antep fıstığı kabuğu gibi tarımsal atıklar katı substrat olarak kullanılmıştır. En yüksek α-amilaz aktivitesi, pirinç kabuğu üzerindeki mikroorganizma üremesinde elde edilmiştir. Maksimum enzim aktivitesi, 3.628 U/mg, 48 saatlik fermantasyon süresi, 60°C'lik bir inkübasyon sıcaklığı, pH 6.0, substrat parçacık boyutu 1.500 μm, başlangıç nem seviyesi %60 ve aşılama seviyesi %40 (v/w) optimum koşulları altında elde edilmiştir

Anahtar Kelimeler
Tarımsal atık, α-amilaz, katı hal fermantasyon (SSF), termofilik bakteri.

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INTRODUCTION

-Amylase (1,4-α-D-glucan glucanohydrolase, αEC 3.2.1.1) is a common secretory enzyme that catalyze the hydrolysis of internal α-D-(1,4) glycosidic bonds of starch at random points [1]. α -amylase is an important enzyme for industrial operations such as starch processing, pulp industries, textile, yeasting, baking, distillation industries and pharmaceutics [2,3]. This enzyme represents an industrial enzyme category which has a 25% share (approximately) in the enzyme sector [4,5]. α-amylase can be produced from various sources such as plants, animals and microorganisms. Enzymes originating from microbial sources generally meet industrial demand [4].

Solid state fermentation (SSF) is a process where microorganisms grow without any free water or in an environment containing a very small amount of free water. Due to its historical importance, it is being used for the production of basic foodstuff such as bread and cheese in the West and koji in the East for thousands of years [6]. Considering the last century and the past couple of decades, it is still being used for the production of biomolecules and products that are important for many industries, including food, pharmaceuticals, textile, biochemicals, bioenergy, and others [7,8]. As compared to conventional submerged fermentation (SmF), SSF offers important advantages such as low energy requirements, high productivity and less inhibitor effect for enzymatic production [9]. Its application at industrial scale, however, seems to be limited because of technological issues such as reactor design, heat transfer problems or cost of sterilization [10].

Thermophilic bacteria usually grow at temperatures as high as 50–80ºC [11]. Because thermophilic character is not associated with Gram identity (Gram + or -), spore formation state (spore forming or not) and respiratory type (aerobic or anaerobic) of the bacteria, thermophilic members can be found in each bacteria group. Thermophilic bacteria can produce DNA polymerases, lipases, amylases, proteases, xylanases and also exo-polysaccharides resistant to high temperature, salt and extreme pH conditions [12]. For these reasons, thermophilic bacteria have been great attention in biotechnology in recent years by many researchers.

The aim of this investigation was the optimization of α-amylase production by using thermophilic bacterium *Anoxybacillus* sp. under SSF. For this reason, the various process parameters were checked out such as various agriculture wastes, fermentation time, temperature and pH, particle size, initial moisture level, inoculum volume and influence of different metal ions.

MATERIALS and METHODS

Isolation and Identification of Thermophilic Bacterial Strain

In this study, bacterial strain was isolated from a thermal spring of Ömer, Afyonkarahisar in Turkey. Based on the quantities of α-amylase secreted by solid state media and the features of the enzyme, one strain was selected for following studies and identified as *Anoxybacillus* sp. on the basis of different biochemical and morphological tests and 16S rRNA gene sequence analysis. The phylogenetic tree was arranged with the neighbor joining process utilizing the Molecular Evolutionary Genetics Analysis (MEGA) [13].

Preparation of Inoculum

The thermophilic isolate was cultured in 50 mL of Nutrient Broth in a 250 mL glass bottle and inoculated with a loopfull of cells from one night old slant and kept at 55ºC in a shaker (120 rpm). After 12 h of incubation, 1000 μL of this medium was utilized for inoculation. By serial dilution and plating, the quantities of viable colonies in the inoculation medium was determined to be 7.4×10^7 CFU/mL.

Solid-State Fermentation (SSF)

Banana husk (BH), wheat bran (WB), rice husk (RH), apple bark (AB), orange bark (OB), lentil bran (LB), maize oil cake (MOC) and pistachio shell (PS) were provided from a regional market in Mersin, Turkey. One half of a gram of solid substrates which passed through sieve of 1.000 μm were put into 50 mL glass bottle. To adjust moisture contents (% by mass per volume), Tris HCl (0.1 M and pH 7.0) was added and then autoclaved at 121ºC for 15 min. The glass bottles were waited for cooling after autoclaving and then were inoculated with 1 mL spore suspension. After inoculation, the SSF mediums were incubated at 55ºC at 120 rpm.

Optimization of Process Parameters

Investigation of the influence of different physico-chemical factors and cultural conditions was necessary for the optimization of enzyme production of *Anoxybacillus* sp. used in SSF. The strategy was to optimize every factor independently and we studied the optimal conditions after in each experiments. Fermentation time (24–144 h), temperature (40-75°C), pH (citrate buffer 0.1 M, pH 3.0, 4.0, 5.0, and 6.0, Tris-HCl buffer 0.1 M, pH 7.0, 8.0, and 9.0, carbonate/bicarbonate buffer 0.1 M, pH 10), particle size (500–2.000 μm), inoculum size (10–60% by mass per volume), initial moisture level of the SSF substrate (40–70% by mass per volume) and different metal ions (Co^{2+},Ca^{2+}, Mn^{2+},Cu^{2+}, Pb^{2+}, Cd^{2+}) were optimized.

Enzyme Extraction and Assay

The fermented SSF substrates were mixed properly with water and then shaked by shaker at 120 rpm for 60 min. The fermented extracts were compressed using muslin cloth. The extracts were centrifuged at 10.000 g for 8 min. The upper solution used as the crude enzyme after centrifugation. α-Amylase assay was determined by Bernfeld method [14].

RESULTS and DISCUSSION

Morphological, Physiological, Biochemical Tests and 16S rRNA Gene Sequence Analysis

It was observed that isolated bacteria (SO-6) were in the form of bacilli, gram positive, creates spores, mobile and thermophilic (Table 1). It was determined that this isolate was close to *Anoxybacillus* sp. according to the results of morphological, physiological and biochemical tests and 16 rRNA analysis (Accession no. KJ434783). Figure 1 demonstrates the phylogenetic tree analysis. 16rRNA sequence is as follows:

GCTTTTGGATCGTTAGCGGCGGACGGGTGAG-
TAACACGTGGGCAACCTGCCCTGTAGACGGGG
ATAACACCGAGAAATCGGT-GCTAATACCGGAT
AACACGAAAGGCCGCATGGTCTTTCGTTGAA-
AGGCGGCGCAAGCTGTCGCTACAGGATGGGCCCG-
CGGC-GCATTAGCTAGTTGGTGAGGTAACGGC-
TCACCAAGGCGACGATGCGTAGCCGACCTGAG
AGGGTGATCGGCCACACTGGGACTGAGACACG
GCCCAGACTCCTACGGGAGGCAGCAGTAGGGA-
ATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC
GCCGCGTGAGCGAAGAAGGCCTTCGGGTCGTAAA
GCTCTGTTGTTAGGGAAGAACAAGTACCGCAGT-
CACTGGCGGTACCTTGACG-GTACCTAACGAGGA-
AGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA-
ATACGTAGGTGGCAAGCGTTGTCCGGAATTATTG-
GGCGTAAAGCGCGCGCAGGCGGTTCCTTAAGT
CTGATGTGAAAGCCCACGGCTCAACCGTGGAGG
GTCATTGGAAACTGGG-GGACTTGAGTGCAGA-
AGAGGAGAGCGGAATTCCACGTGTAGCGGTGA-
AATGCGTAGAGATGTGGAGGAACACCAGTG-
GCGAAGGCGGCTCTCTGGTCTGTAACTGACG
CTGAGGCGCGAAAGCGTGGGGAGCAAACAG
GATTAGATACCCTGGTAGTCCACGCCGTAAAC-
GATGAGTGCTAAGTGTTAGAGGGTATCCACCC-
TTTAGTGCTGTAGCTAACGCATTAAGCACTCCGC
CTGGGGAGTACGCTCGCAAGAGTGAAACTCAA-
AGGAATTGACGGGGGCCCGCACAAGCGGTGGA-
ACCTTGTGGTTTAATTCGAAGCAACGCGAAGA-
ACCTTACCAGGTCTTGACATCCCCTGACAACCCGA-
GAAATCGGGCGTCCCCCTTCG...

Effect of Different Agriculture Wastes on α-amylase Production

Selection of a suitable solid substrate for fermentation process in SSF method is an important factor [15]. Various agroindustrial materials, particularly BH, WB, RH, AB, OB, MOC, LB and PS were tested for the selection of the most convenient substrate material to optimize bacterial growth and enzyme production. As can be seen in Figure 2, maximum amylase production (2.532 U/mg) was obtained in a medium containing RH alone as the substrate. The order of production of α -amylase from maximum to minimum was found to be RH>WB>MOC>BH>OB>AB>LB>PS. Therefore, RH was used as a substrate in all subsequent studies.

Effect of Fermentation Time on α -amylase Production

Incubation period required to reach maximum enzyme level depends on culture characteristics and it is based on growth speed and enzyme production [16]. To determine the best fermentation period, tests were conducted from 24 h to 144 h. A gradual increase was seen in enzyme production from 24 to 48 h and maximum enzyme activity was determined to be 2.820 U/mg in 48 h, after

Table 1. Morphological, physiological and biochemical tests.

Properties	Isolate SO-6
Gram Staining	+
Spore	+
Cell Shape	Bacilli
Pigmentation	Yellow
Aerobic respiration	+
Growth Temperature	25-80°C
Growth Ph	3.0-11.0
Mobility	+
Casein Hydrolysis	+
Starch Hydrolysis	+
Gelatin hydrolysis	-
Lipase Activity	+
Catalase Activity	+
Urease Activity	-

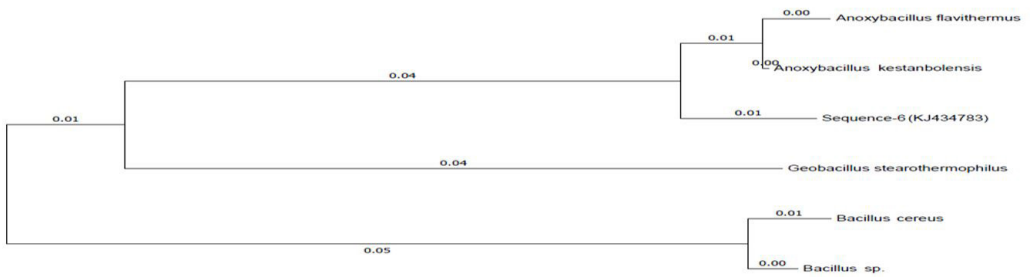

Figure 1. Evolutionary relationships of taxa.

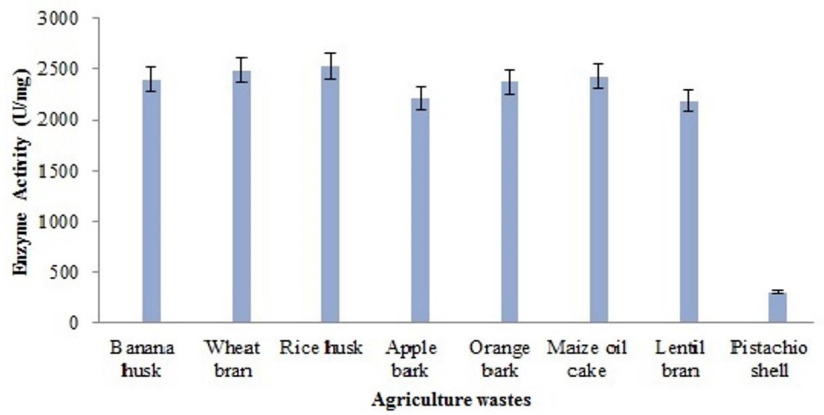

Figure 2. Effect of different substrates on α-amylase production by *Anoxybacillus* sp. using SSF. Process conditions: initial moisture content 50% (% by volume per mass), inoculum size 30% (% by volume per mass), particle size 1.000 μm, fermentation time 24 h, pH 7.0 and temperature 55°C.

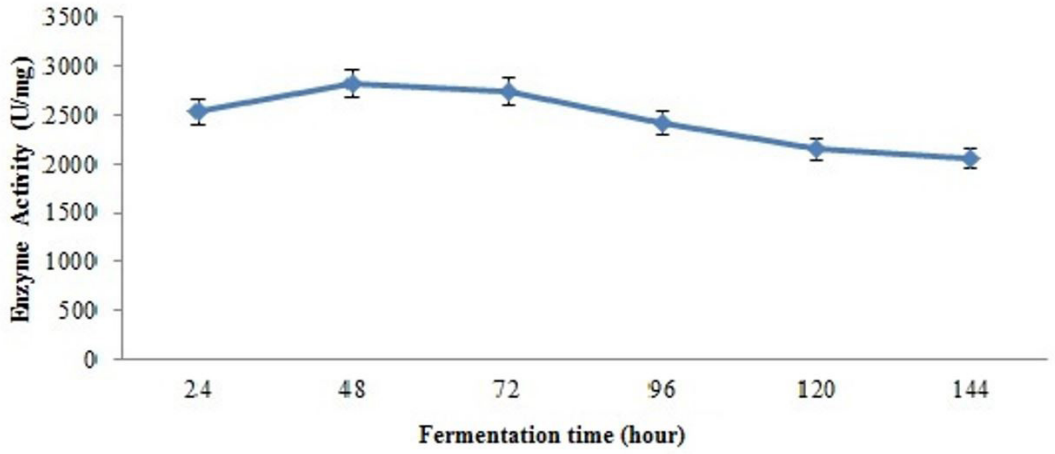

Figure 3. Effect of fermentation time on α-amylase production by *Anoxybacillus* sp. under SSF using RH as substrate. Process conditions: initial moisture content 50% (% by volume per mass), inoculum size 30% (% by volume per mass), particle size 1.000 μm, temperature 55°C and pH 7.0.

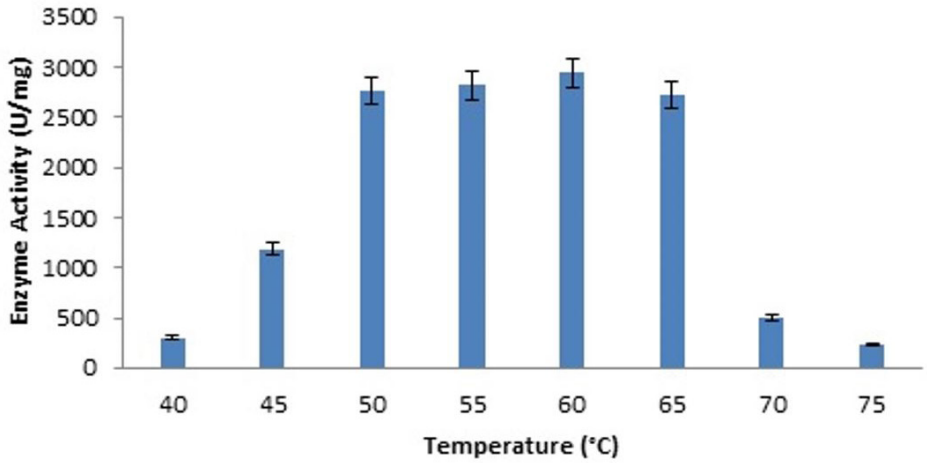

Figure 4. Effect of temperature on α-amylase production by *Anoxybacillus* sp. under SSF using RH as substrate. Process conditions: initial moisture content 50% (% by volume per mass), inoculum size 30% (% by volume per mass), particle size 1.000 μm, fermentation time 48 h and pH 7.0.

which a gradual decrease was observed (Figure 3). Decrease in enzyme efficiency after that hour may depend on the depletion of media or the denaturation of enzyme resulting from its interaction with other components in the medium or a variation in pH of the medium [17]. Fermentation time was applied as 48 h in all subsequent experimental works.

Effect of Temperature on Bacterial Growth and α -amylase Production

Temperature control in substrate bed has crucial importance to SSF because bacterial growth and the production of enzymes or metabolites is generally sensitive to temperature [15,18,19]. As shown in Figure 4, the enzyme activity increased 9.7 times with an increasing in the temperature from 40 to 60°C and the optimum enzyme production temperature of *Anoxybacillus* sp. was determined as 60°C (2.942 U/mg). The production of thermophiles at high temperatures is technically and economically important that it minimizes the risk of contamination, facilitates mixture by reducing adhesiveness, and causes a high level of substrate solubility [20]. The enzyme activity decreased 13 times when the temperature increased from 60 to 75°C. This reduction can be attributed to the decreased of bacterial growth

above the optimum fermentation temperature (data not shown). Thus, temperature in the substrate bed of 60°C was used for further studies.

Effect of pH on α -amylase Production

pH is one of the important factors for each fermentation process and depends on the type of moistening agent used in media. Each microorganism has an optimum pH range so that it can grow and become active. A substrate formation taking account of the buffering capacity of different components used or a buffer formulation containing components which do not have a harmful effect on biological effectiveness should be used for overcoming pH variation problem during SSF process [21]. As inferred from Figure 5, optimum pH value was determined to be 6.0 (3.060 U/mg) by using different buffers for various pH values for the production of α-amylase. Subsequent works were carried out by using this value as a basis.

Effect of Substrate Particle Size on α-amylase Production

The size of substrate particle is the most critical factor for microbial growth and enzyme activity [22]. The enzyme activity rised up 8.2 times with an increasing in the particle size from 500 to 1.500 μm. The highest enzyme production (3.060 U/mg) was found in the medium with a particle size of 1500 μm (Figure 6). It is preferred for microbial growth as surface are might grow due to smaller particle size, but growth may be inhibited as substrate may be agglomerated in smaller sizes. At 2.000 μm, activity of enzyme dropped down up to 57.3% as compared to the optimum enzyme activity at 1.500 μm. In larger sizes, it is not preferred as an insufficient surftace area will be formed for microbial attack [23]. Substrate with 1.500 μm particle size was preferred for subsequent works.

Effect of Initial Moisture Content of Substrate on α-amylase Production

The critical importance of humidity level in a SSF setting and its effects on the biosynthesis and oscillation of enzymes may be attributed to the effect of humidity on the physical characteristics of solid particles in the medium [16]. A low humidity content will cause a decrease in the solubility of substrate nutrients and a lower inflation level [24]. However, it is believed that an increase in humidity level reduces the porosity of wheat bran and thus, limits oxygen transfer [18]. As shown in Figure 7, maximum enzyme efficiency i.e. 3.175 U/mg was obtained in 60% substrate humidity content in trials conducted for determining the optimum humidity content because of its importance. Subsequent works were carried out based on this humidity content.

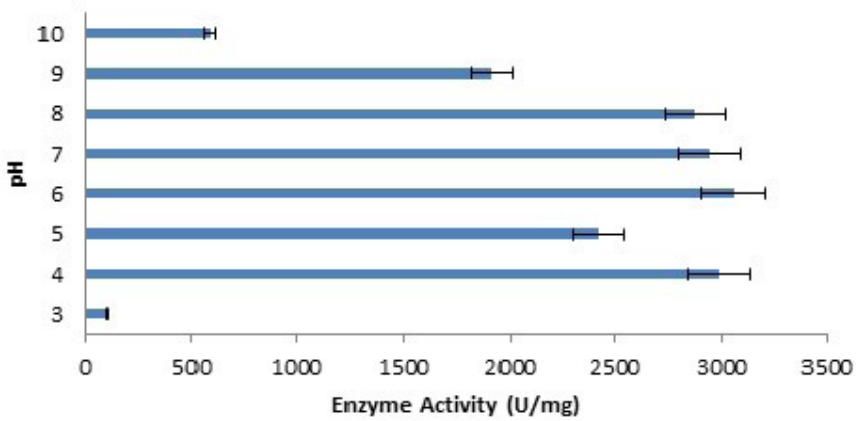

Figure 5. Effect of pH on α–amylase production by *Anoxybacillus* sp. under SSF using RH as substrate. Process conditions: initial moisture content 50% (% by volume per mass), inoculum size 30% (% by volume per mass), particle size 1.000 μm, fermentation time 48 h and temperature 60°C.

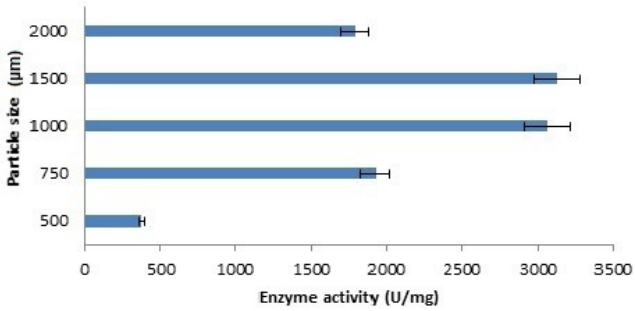

Figure 6. Effect of substrate particle size (µm) on α-amylase production by *Anoxybacillus* sp. under SSF using RH as substrate. Process conditions: initial moisture content 50% (% by volume per mass), inoculum size 30% (% by volume per mass), fermentation time 48 h, pH 6.0 and temperature 60°C.

Effect of inoculum Size on α-amylase Production

Inoculum level is one of the other important parameters for the production of α-amylase [25]. As presented in Figure 8, the lowest enzyme yield was obtained at the lowest value of 10% inoculum size, whereas the maximum enzyme yield (3.274 U/mg) was obtained at 40% inoculum size. At 60% inoculum size activity of enzyme dropped down up to 55.5% as compared to the optimum enzyme activity at 40% inoculum size. This results agreed with that of Ozdemir et al., [25]. Thus, 40% was used for further studies.

Effect of Metal Ions on α-amylase Production

Different metals exhibit different behavior in terms of their ability to act as an effector [26]. Metallic co-factors are important for enzymatic reaction because the existence or absence of metal regulates enzyme activity. The presence of a specific metallic ion in addition to the source of basic nutrient may inhibit or reinforce enzyme production. Co^{2+}, Ca^{2+}, Mn^{2+} ,Cu^{2+}, Pb^{2+} and Cd^{2+} metal ions in a 5 mg/L concentration and a control group without any metal ion were tested under the optimum fermentation conditions. As shown in Figure 9, α-amylase production increased in presence of Ca^{2+}, Co^{2+} and Mn^{2+} ions, but slightly decreased in presence of Cu^{2+}. Michelin et al., (2010) reported that amylase was activated by calcium (34%), cobalt (41%), and manganese chlorates (47%). Saboury (2002) also determined Co^{2+} as activator of α-amylase from *Bacillus amyloliquefaciens*.

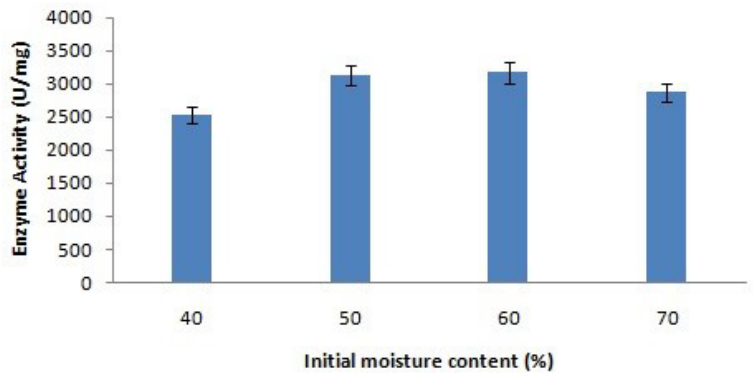

Figure 7. Effect of initial moisture content on α-amylase production by *Anoxybacillus* sp. under SSF using RH as substrate. Process conditions: inoculum size 30% (% by volume per mass), particle size 1.500 µm, fermentation time 48 h, pH 6.0 and temperature 60°C.

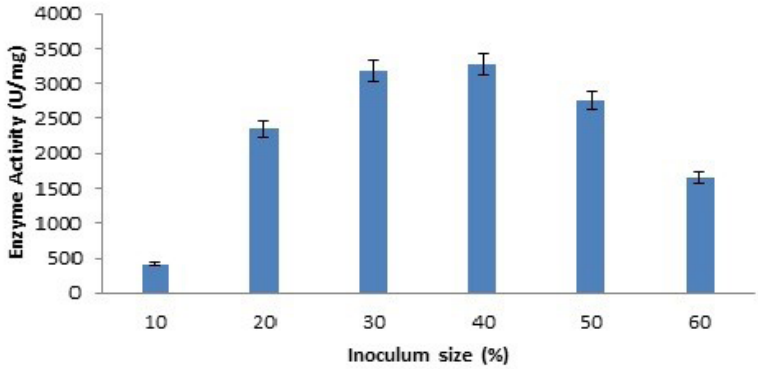

Figure 8. Effect of inoculum size (% by volume per mass) on α-amylase production by *Anoxybacillus* sp. under SSF using RH as substrate. Process conditions: initial moisture content 60% (% by volume per mass), particle size 1.500 μm, fermentation time 48 h, pH 6.0 and temperature 60°C.

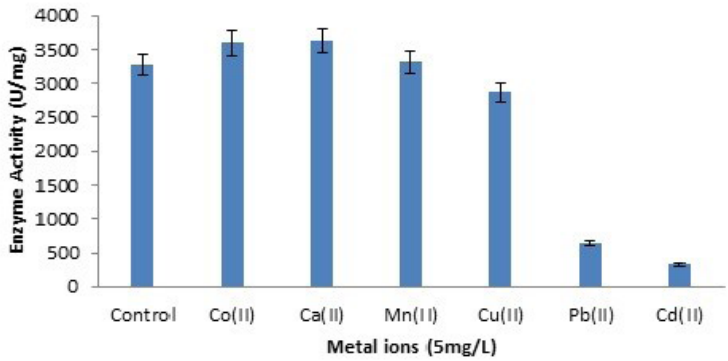

Figure 9. Effect of metal ions on α-amylase production by *Anoxybacillus* sp. under SSF using RH as substrate. Process conditions: initial moisture content 60% (% by volume per mass), inoculum size 40% (% by volume per mass), particle size 1.500 μm, fermentation time 48 h, pH 6.0 and temperature 60°C.

Our results showed good similarity with their findings. Enzyme production sharply decreased in presence of Pb^{2+} and Cd^{2+} ions. It is well known that Pb^{2+} and Cd^{2+} ions are not essential for living organisms. This reduction can be attributed to the inhibition of bacterial growth because of toxic effect of Pb^{2+} and Cd^{2+} ions (data not shown).

CONCLUSIONS

This study determined optimum conditions for the production of thermostable α -amylase through SSF technique by using thermophilic *Anoxybacillus* sp. The effects on enzyme production of different agricultural wastes, fermentation period, temperature, pH, particle size, humidity, inoculum level, and metal ions were explored and enzyme production was optimized. The highest enzyme activity was obtained as 3.628 U/mg at 48th hour, 60°C, pH: 6.0, 1500 μm particle size, 60% initial humidity and 40% inoculation volume and in presence of Ca^{2+} ion by using RH as substrate.

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