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Isolation and Characterization of Amylase Producing Yeasts and Improvement of Amylase Production

[Amilaz Üreticisi Mayaların İzolasyonu ve Karakterizasyonu ve Amilaz Üretiminin Arttırılması]

H. Tansel Yalçın,
Cengiz Çorbacı

Ege University, Faculty of Science, Biology Department, Basic and Industrial Microbiology Section, 35100, Bornova-Izmir, Turkey

Yazışma Adresi
[Correspondence Address]

H. Tansel Yalçın

Ege University, Faculty of Science, Biology Department, Basic and Industrial Microbiology Section, 35100, Bornova-Izmir, Turkey
Tel. +90 232 311 24 36
Fax. +90 232 311 17 99
E-mail. tansel.yalcin@ege.edu.tr

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ABSTRACT

Objectives: Because most of the yeasts from nature are not harmful as compared to bacteria, interest in yeasts with potential use in biotechnological processes has increased in recent years. Therefore, in this study, we aimed to isolate and characterize amylase producing yeasts and investigate the effects of different parameters on the production of amylase.

Methods: Five different sources were used for the isolation of the yeasts. For screening of the amylase production, the isolates were incubated in Amylase Activity Medium at 30°C for 3 days. The amylase activity was determined by dinitrosalicylic acid method. Molecular characterization of the amylase producing yeasts was performed with restriction fragment length polymorphism (RFLP) of the 5.8S and 18S rRNA regions, and sequence analysis of D1/D2 domain of the 26S rRNA region.

Results: In total, twenty five yeast isolates were obtained from five different sources. Following the incubation in medium containing starch for the screening of amylase production, it was found that 12 yeast isolates produce amylase, and among the isolates, three of them showed the highest amylase activity. The isolates (19-3, 19-6 and 19-7) having the highest activity were identified as *S. fibuligera*. The strains showed the highest amylase production at 30°C and pH 5.5. Among additives, sodium chloride, polyethylene glycol, sodium dodecyl sulfate, Triton X-100 and Tween 80 stimulated the production of amylase. Other additives showed negative influence on the production of enzyme.

Conclusion: The study revealed that favorable fermentation conditions and the selection of suitable growth parameters played key roles in the production of amylase by *S. fibuligera*.

Keywords: *Saccharomycopsis fibuligera*, yeast, molecular characterization, improvement of amylase production

Conflict of Interest: There is no conflict of interest between the authors.

ÖZET

Amaç: Doğadan elde edilen pek çok mayanın bakterilere nazaran daha zararsız olmasından dolayı, son yıllarda biyoteknolojik işlemlerde kullanılma potansiyeli olan mayalara karşı ilgi artmıştır. Bu nedenle çalışmada, amilaz üretici mayaların izolasyonu, karakterizasyonu ve amilaz enzimi üretimi üzerine çeşitli parametrelerin etkisinin araştırılması amaçlanmıştır.

Gereçler ve Yöntemler: Mayaların izolasyonu için 5 farklı kaynak kullanılmıştır. Amilaz üretiminin taranması için izolatlar, Amilaz Aktivite Ortamında 30°C'de 3 gün inkübe edilmiştir. Amilaz aktivitesi, dinitrosalisilik asit yöntemine göre saptanmıştır. Amilaz üretici maya izolatlarının moleküler karakterizasyonu, 5.8S ve 18S rRNA bölgelerinin restriksiyon fragment uzunluk polimorfizmi (RFLP) ve 26 rDNA bölgesi D1/D2 domaini sekans analizi ile gerçekleştirilmiştir.

Bulgular: 5 farklı kaynaktan toplamda 25 adet maya elde edilmiştir. Amilaz üretiminin taranması için nişasta içeren ortamda inkübasyondan sonra, 12 maya izolatı amilaz üretmiş ve bunlardan 3 tanesi en yüksek amilaz aktivitesi göstermiştir. En yüksek aktivite gösteren izolatlar (19-3, 19-6 ve 19-7), *S. fibuligera* olarak tanılanmıştır. Strainler en yüksek amilaz üretimini 30°C ve pH 5.5'de göstermiştir. Katkı maddelerinden sodyum klorür, polietilen glikol, sodyum dodesil sülfat, Triton X-100 ve Tween 80 amilaz üretimini artırmıştır. Diğerleri, enzim üretimi üzerine negatif etki göstermiştir.

Sonuç: Çalışma, uygun fermentasyon koşullarının ve uygun büyüme parametrelerinin seçiminin, *S. fibuligera* tarafından amilaz üretiminde anahtar roller oynadığını göstermiştir.

Anahtar Kelimeler: *Saccharomycopsis fibuligera*, maya, moleküler karakterizasyon, amilaz üretiminin artırılması

Çıkar Çatışması: Yazarların çıkar çatışması bulunmamaktadır.

Introduction

Amylase are enzymes which hydrolyze starch molecules to give diverse products including dextrin and progressively smaller polymers composed of glucose units [1, 2]. These enzymes are among the most important enzymes for biotechnology with great significance, constitute a class of industrial enzymes having approximately 25% of the world enzyme market [3]. Amylases have many applications in bread and baking industry, starch liquefaction and saccharification, textile desizing, paper industry, detergent industry, analyses in medical and clinical chemistry, food and pharmaceutical industries [4, 5].

Amylases can be obtained from several sources, such as plants, animals and microorganisms. Today a large number of microbial amylases are available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry. The amylases of microorganisms have a broad spectrum of industrial applications as they are more stable than when prepared with plant and animal amylases [4]. The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity and the fact that they are easily manipulated to obtain enzymes of desired characteristics [6]. Amylases are derived from several fungi, yeasts and bacteria [5, 7-9].

Starch is the best substrate for production of yeast cells in a large scale due to its low price and easily available raw material in most regions of the world. Because most of yeasts from environments are safe (GRAS) compared to bacteria, interest in amylolytic yeasts has increased in recent years as their potential value for conversion of starchy biomass to single-cell protein and ethanol has been recognized [1]. To date, it has been noticed that the terrestrial yeasts which can produce extracellular amylolytic enzymes include *Arxula adeninivorans*, *Candida japonica*, *Filobasidium capsuligenum*, *Lipomyces*, *Saccharomycopsis*, *Schwanniomyces* [10, 11].

Considering the industrial importance of amylase, in this study, we aimed to isolate and characterize amylase producing yeasts from different sources and to determine the optimum conditions for the production of amylase.

Materials and Methods

Sampling and isolation

A total of five different sources (soil, bread, rice, rice flour and flour) were investigated for the isolation of yeasts. Yeast isolation was performed on Yeast Malt (YM) agar composed of (g/L); 3 g yeast extract, 3 g malt extract, 5 g peptone, 10 g glucose and 15 g agar. Inoculated plates were incubated at 27°C for 5-7 days. Colonies with distinct morphological differences such as color, shape and size were picked and purified by streaking at least three times on YM agar plates [12, 13].

Amylase screening

Amylases producing yeasts were screened on Amylase Activity Medium (AAM) [soluble starch 5g/L; peptone 5g/L; yeast extract 5 g/L; $MgSO_4.7H_2O$ 0.5 g/L; $FeSO_4.7H_2O$ 0.01 g/L; NaCl 0.01 g/L; agar 15g/L] plates. Incubation at 30°C was carried out for 3 days, after which the plates were stained with lugol solution (Gram iodine solution: 0.1% I_2 and 1% KI). The colonies forming the largest halo zone were selected for further investigation [14].

DNA sequencing and RFLP analysis of ribosomal RNA regions

DNA isolation was performed according to Liu et al. [15]. Oligonucleotide primers for amplification of gene sequences and sequencing of the genes are given in the following sections. The amplification reactions were performed with Corbett Cool Gradient Palm Cycler CGI-96, under the conditions as described elsewhere [13]. Both strands of the DNAs were sequenced on an ABI 3130XL automated sequencer (Applied Biosystems, USA), following the manufacturer's instructions.

Restriction fragment length polymorphism (RFLP) of ITS1-5.8S-ITS2 rRNA and 18S rRNA region. PCR amplicons of ITS1-5.8S-ITS2 region were produced using primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC CC GCT TAT TGA TAT GC-3'). Amplification was performed for 40 PCR cycles with denaturation at 95°C for 2 min, annealing at 58°C for 2 min, and extension at 72°C for 2 min, with the final extension for 10 min.

Primers for amplification of 18S rDNA were P108 (5'-ACC TGG TTG ATC CTG CCA GT-3') and M3976 (5'-CTA CGG AAA CCT CTA CGG AAA CCT TGT TAC GAC T-3'). Amplification was performed for 40 PCR cycles with denaturation at 95°C for 40 s, annealing at 59°C for 40 s, and extension at 72°C for 2 min, with the final extension for 10 min.

*Hae*III, *Hinf*I, *Rsa*I and *Taq*I FastDigest restriction endonucleases (Fermentas) were used separately to digest the amplification products. PCR products (10 µl) were digested in 30 µl final volume of digestion mixture consisting of 1 µl restriction enzyme (Fermentas), 2 µl 10x restriction enzyme buffer (Fermentas) and 17 µl deionized H_2O. The mixtures were incubated for 5-10 min at 37°C (65°C for *Taq*I) according to the manufacturer's instruction. Nebcutter V2.0 program, available at http://tools.neb.com/NEBcutter2, was used to predict and verify the size of each restriction fragment.

Sequencing of D1/D2 domain of 26S rRNA region. The following primers were used for amplifications: NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG–3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3'). Amplification was performed for 35 PCR cycles with denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 2 min, with the final extension for 10 min. The sequences were compared pairwise using

a BLASTN search and were aligned with the sequences of related species retrieved from GenBank using the multiple alignment program CLUSTAL W version 2.0 software. A phylogenetic tree was constructed using the Tamura-Nei neighbor joining method by MEGA software version 5.0 [16]. Confidence levels of the clades were estimated from bootstrap analysis (1,000 replicates) [17].

Amylase production and assay

Extracellular amylases were produced in submerged fermentation; this production was carried out in 250 ml Erlenmeyer flasks containing 100 ml of liquid AAM buffered with acetate buffer (pH 5.5). The media were then incubated at 30°C in an orbital shaker set at 150 rpm for 48 h. Three replicate fermentations were carried out for each culture medium.

The enzyme assay was performed according to the method by Rick and Stegbauer [18]. The enzyme extract (0.5 ml) was transferred to a test tube containing 0.5 ml of 1.0% soluble starch solution. The mixture was incubated at 60°C for 10 min. Then 1.0 ml of dinitrosalicylic acid reagent (DNS) was added to each test tube. The tubes were placed in boiling water for 5 min and cooled at room temperature. The contents of tubes were diluted up to 10 ml with distilled water. The absorbance was determined at 546 nm using a spectrophotometer and converted to mg of maltose from the standard. One unit is equivalent to that amount of enzyme, which catalyze the hydrolysis of soluble starch into 1.0 mg maltose hydrate per minute under standard assay conditions. Total protein content was estimated by the Bradford method using bovine serum albumin as the standard [19].

Improvement of amylase production

Effect of initial pH. The fermentation medium was prepared with varying the pH values (4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0) to investigate the effect of initial pH on the production of amylase. The media are buffered with acetate buffer (for pH 3-6) and Na-phosphate buffer (for pH 6-7).

Effect of temperature. To study the effect of temperature on amylase production in the submerged fermentation, fermentations were carried out at different temperatures (25, 30, 35 and 40°C).

Effects of different additives. NaCl [0.1 and 1.0%, w/v] and in concentrations at 100 and 1000 ppm different additives [dimethyl sulfoxide (DMSO), polyethylene glycol (PEG), phenylmethylsulfonyl fluoride (PMSF), sodium dodecyl sulfate (SDS), Triton X-100 and Tween 80] were added to fermentation media and their effects on enzyme production were studied by carrying out fermentation process.

Results and Discussion

In the study, a total of twenty five yeast isolates were obtained. Then, these yeast isolates were screened for amylase production on AAM plates. Following the incubation, the plates were stained with lugol solution and clear zones were appeared around growing yeast colonies indicating starch hydrolysis. Among all tested yeast isolates; twelve (numbered as 10-1, 12-3, 13-2, 15-5, 16-7, 16-9, 17-2, 17-5, 18-3, 19-3, 19-6 and 19-7) showed amylase activity. The three yeasts (19-3, 19-6 and 19-7) having the largest clear zones were selected for identification and amylase production in submerged system (Table 1 and Fig. 1).

Identification of the yeasts

In the study, the amylase producing isolates, 19-3, 19-6 and 19-7, were identified by using sequencing of D1/D2 domain of the LSU rRNA gene and the RFLP analysis of ITS1-5.8S-ITS2 and the small subunit ribosomal RNA gene.

For the three isolates, PCR amplification of the ITS1-5.8S-ITS2 region and the small subunit rRNA gene rendered amplicons size in 560 and 1800 bp, respectively (Table 2). PCR products of the yeasts were digested with four FastDigest restriction endonucleases mentioned earlier. The size and number of the fragments obtained by digestion were compared with type species' patterns (*Saccharomycopsis fibuligera* NRRL Y-2388). According to the results, we found that RFLP patterns of the yeast isolates were similar to those of the type species. The approximate length of the amplified products and the restriction fragments of the yeast isolates and type species observed after digestion with the restriction endonucleases are summarized in Table 2.

The isolates, 19-3 (JQ277248.1), 19-6 (JQ277249.1) and 19-7 (JQ277250.1), have identical nucleotide sequences in the D1/D2 domain of 26S rRNA and are closely similar to *S. fibuligera* NRRL Y-2388 (EU057552.1). Consequently, according to the RFLP analysis of the rRNA genes and sequencing of the LSU gene, the isolates were identified as *S. fibuligera*. Phylogenetic placement of *S. fibuligera* strains are shown in Fig. 2.

Physiologically, yeasts are said to have biotechnological importance. Therefore, accurate identification of local yeasts isolated from different environments is necessary. Progress in molecular biology has led to the development of new techniques for yeast identification based on similarity or dissimilarity of DNA, RNA and proteins [20]. The ribosomal genes are popular targets for PCR based systems for detection and identification of yeast strains. A number of techniques using PCR amplification of the ribosomal genes have been suggested for identification of yeast species. Among these methods, those based on the RFLP analysis of the DNA that encodes the ribosomal RNA genes (5S, 5.8S, 18S and 26S) and the non-coding ITS (internal transcribed spacers) and IGS (intergenic spacer) regions appear to be useful for the detection of many yeast and fungal species. Also, the D1/D2 region of the large subunit (LSU) ribosomal RNA gene (26S rRNA) of all currently recognized ascomycetous yeasts have been sequenced and these studies have shown that strains belonging to the same species gene-

Table 1. Starch hydrolysis abilities by twelve yeasts using plate assay method

Strain no.	Diameter of hydrolysis zone (mm)	Strain no.	Diameter of hydrolysis zone (mm)
10-1	8	17-2	6
12-3	7	17-5	4
13-2	8	18-3	5
15-5	5	19-3	18
16-7	6	19-6	16
16-9	4	19-7	17

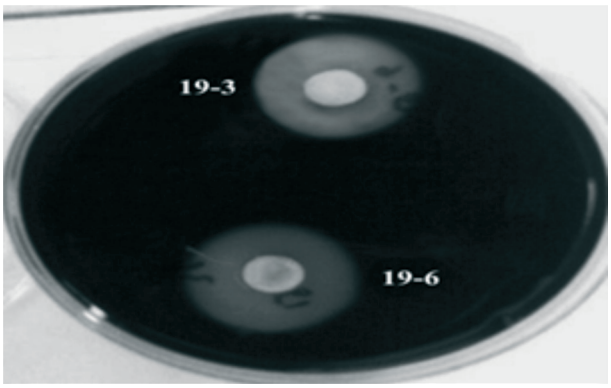

Fig. 1. Representation of the yeast strains showing amylase activity in petri dish

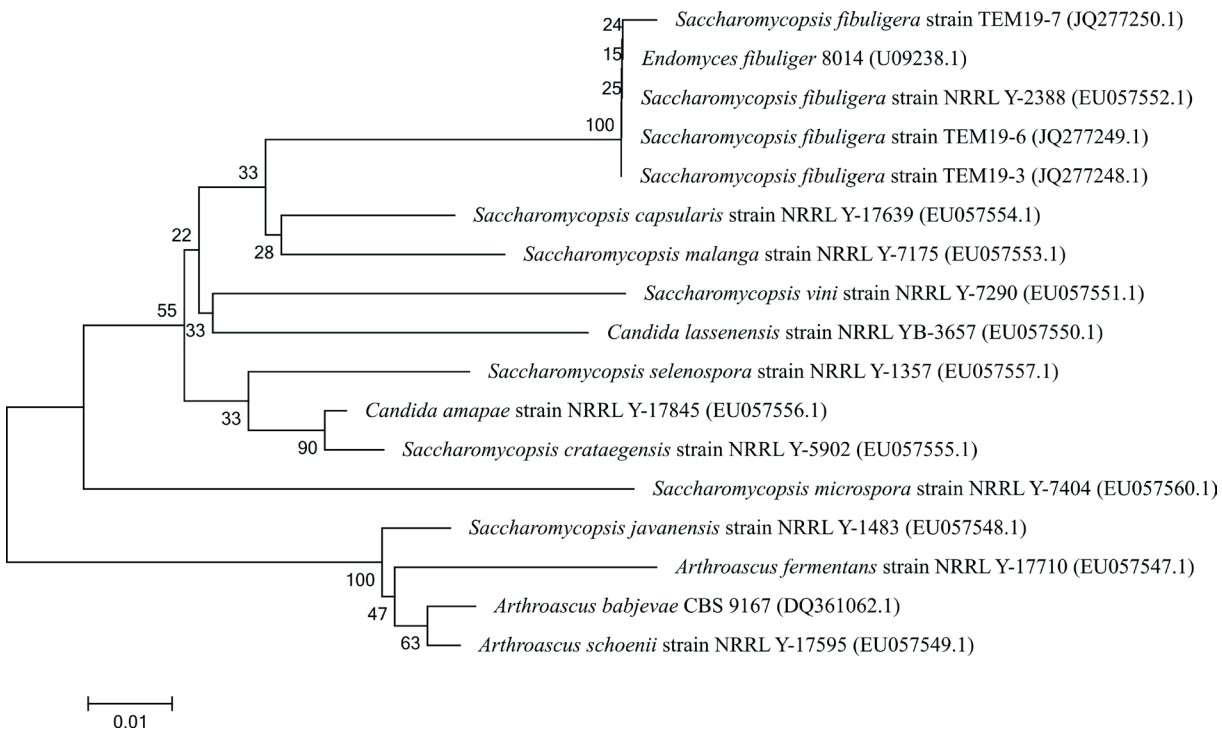

Fig. 2. Phylogenetic tree showing the placement of *S. fibuligera* strains constructed by the neighbor joining method based on the D1/D2 domain of the LSU rRNA. The sequences of the related species were obtained from the GenBank

Table 2. Results of molecular identification of the amylase producing yeasts showing the highest starch hydrolysis activity

Strain No.	ITS1-5.8S-ITS2 rDNA (bp)			18S rDNA (bp)			Sequence homology of D1/D2 domain of 26S rDNA (%)	Species
	HaeIII	**HinfI**	**RsaI**	**HaeIII**	**RsaI**	**TaqI**		
19-3	540+20	300+250	560	630+320+220+180 +170+150+120	670+390+280+160 +150+110	800+420+250+200 +60+60	99	*S. fibuligera*
19-6	540+20	300+250	560	630+320+220+180 +170+150+120	670+390+280+160 +150+110	800+420+250+200 +60+60	99	*S. fibuligera*
19-7	540+20	300+250	560	630+320+220+180 +170+150+120	670+390+280+160 +150+110	800+420+250+200 +60+60	99	*S. fibuligera*

rally exhibit nearly 99% sequence identity. In recent years, many researchers have suggested that a polyphasic approach may be the best way to achieve proper microbial identification. So, in the present study, accurate identification of yeast strains was performed using different molecular techniques [21-23].

Amylase production in submerged fermentation

In the present research, total amylase activities in the liquid media after incubation at 30°C for 24 h were reached maximum levels and then, the activities were gradually decreased in subsequent days (Fig. 3). The reason for the situation may be the production of extracellular proteases. Therefore, in the present study, the results were evaluated by 24 h incubation in the further experiments. Among the three strains; 19-7 gave better yield as shown in Fig. 3.

In our study, the yeast strains were investigated for amylase production in submerged fermentation (SmF) process using a suitable medium. SmF has been traditionally used for the production of industrially important enzymes because of the ease of control of different parameters such as pH, temperature, aeration and oxygen transfer and moisture [5, 24].

Improvement of cultural and nutritional conditions for amylase production

Effect of pH. The effect of initial pH on the production of amylase was carried out in the study. The optimum pH was determined by measuring the activity at 30°C over a range of pH from 3 to 7 using the following buffer: acetate buffer (pH 3-6) and Na-phosphate buffer (pH 6-7). The production of amylase by the strains was found to be the best at pH 5.5 (Fig. 4). Below and above this pH, amylase production decreased gradually. Moreira et al. [25] found that the amylase production by *Aspergillus tamarii* was higher at pH 6 while Nahas and Waldemarin [26] observed the maximum amylase production by *Aspergillus ochraceus* at initial pH 5.0. Also, the result is in agreement with the result of Hostinova [27], who found that the optimal pH for glucoamylase and α-amylase of *S. fibuligera* was 5.0-6.2.

Among the physical parameters, the pH of the growth medium plays an important role by inducing morphological change in the organism and in enzyme secretion. The pH change observed during the growth of the organism also affects product stability in the medium [1]. Amylase is a pH sensitive enzyme and the selection of optimal pH is essential for the production of amylase [28].

Effect of temperature. The impact of the cultivation temperature on the amylase production by *S. fibuligera* strains was conducted in liquid medium at 25, 30, 35 and 40°C. The enzyme activities increased progressively with increase in temperature from 25°C reaching a maximum at 30°C. Above 35°C, there was a reduction in the amylase activities. The influence of temperature

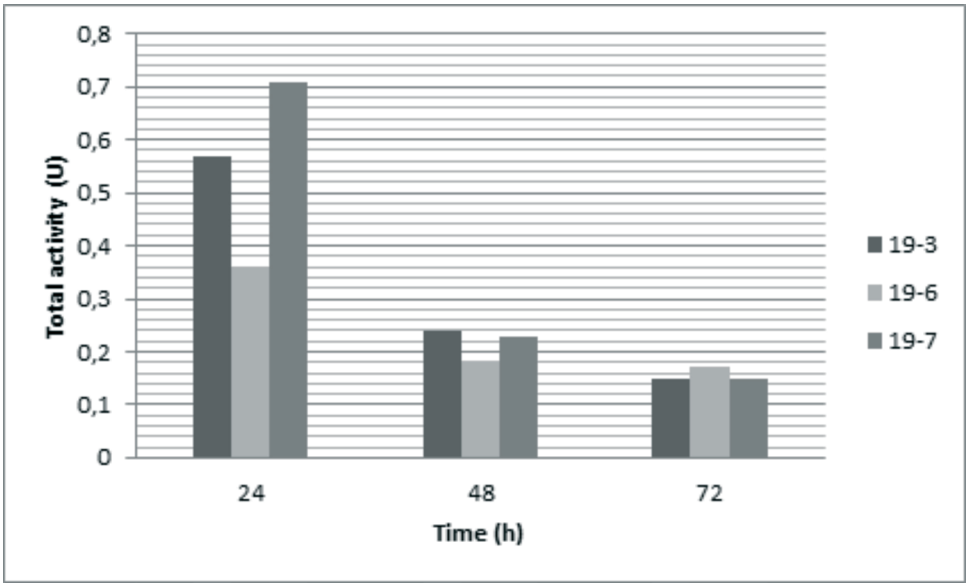

Fig. 3. Effect of incubation period on amylase production

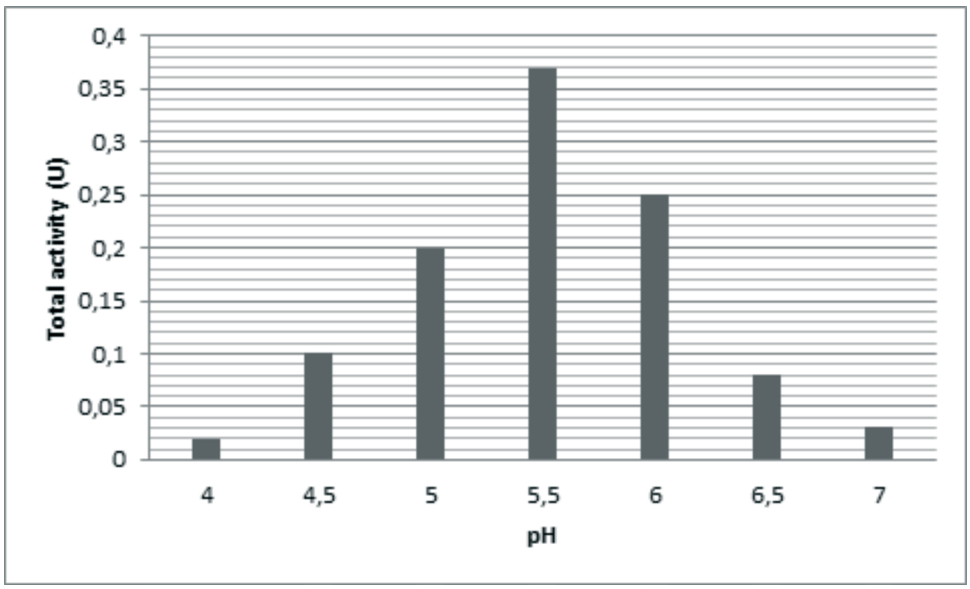

Fig. 4. Effect of initial pH on amylase production by the strain 19-6

on amylase production is related to the growth of the organism. Among the fungi, most amylase production studies have been done with mesophilic fungi within the temperature range of 25-37°C. Optimum yields of α-amylase were achieved at 30-37°C for *Aspergillus oryzae* [1]. Similar results were also reported by Rene and Hubert [29] with *Filobasidium capsuligenum*.

Effects of different additives. Among additives, 1% NaCl, SDS [at 100 ppm], PEG [at 100 and 1000 ppm], Triton X-100 [at 100 and 1000 ppm] and Tween 80 [at 100 and 1000 ppm] stimulated the production of amylase by 1.37, 1.15, 1.42, 1.67, 1.76, 1.91, 1.75 and 1.58 folds, respectively. Other additives that were investigated showed negative influence on the production of enzyme (Fig. 5).

Enzymes, which are highly effective and specific biocatalysts, are more efficient than chemical catalysts used in organic reactions due to their wide range of pH and temperature stability. Most of the substrates used in industrial processes being artificial require continuous investigation of new enzymes capable of performing these reactions. Throughout the world, there is an increasing interest in finding new potential microbial enzyme producers.

One of the most effective and successful methods for the discovery of new enzymes is the isolation of microorganisms from natural habitats. Production of industrial

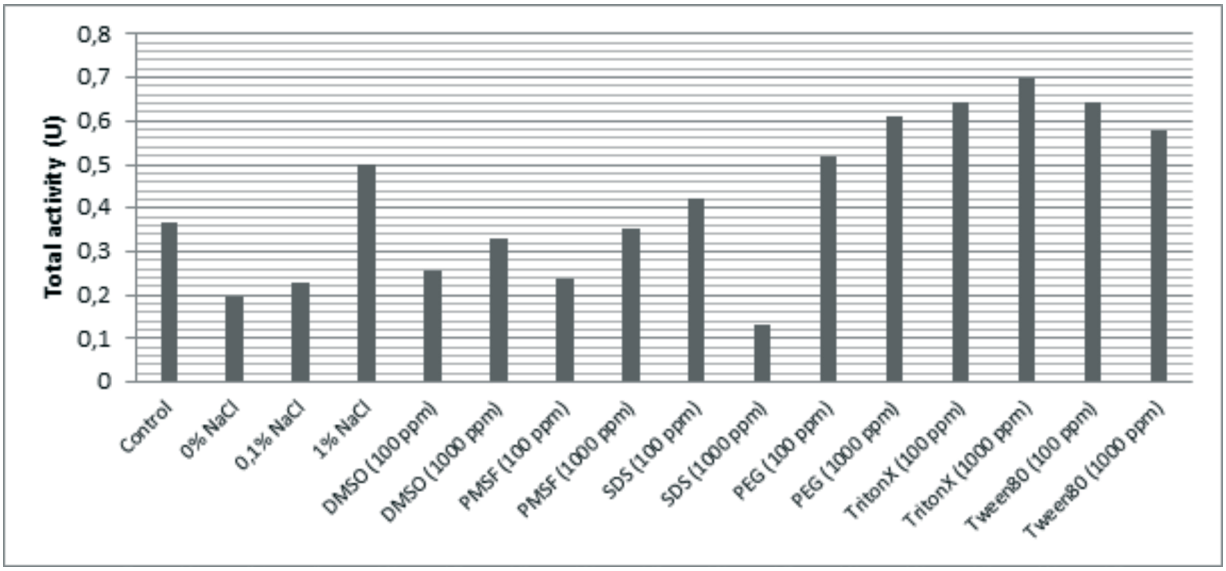

Fig. 5. Effect of different additives on amylase production by the strain 19-7

enzymes by indigenous microorganisms has contributed to the domestic economy. Thus, screening of microorganisms for the selection of appropriate strains is a key preliminary step in the production of desired metabolites. Non-pathogenic yeasts capable of producing industrially useful enzymes are gaining importance. As this group of microorganisms can be cultured easily and produces industrially useful extracellular enzymes with high levels. One of the most important products secreted by the yeasts is amylase, that appeals the interest of scientists and industrial researchers since it can be exploited for several applications in the detergent, food, pharmaceutical, and beverage industries. Taking our country's versatile biodiversity into account, in this research, we aimed to screen the yeast isolates producing carbohydrates degrading enzymes due to their biotechnological importance. The results indicated that the present strains of *S. fibuligera* are viable source of amylase due to their production capability in a wide range of pH and temperatures. It should also be noted that amylase producer strains with increasing enzyme activities at high temperature are promising candidates for the industrial applications.

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Author contribution: Both authors participated in the isolation of samples, identification and enzyme experiments.

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