Journal of Food Science and Technology (Iran)

Homepage:www.fsct.modares.ir

Scientific Research

Isolation and identification of thermostable amylase producing bacteria from the wastewater of the canning factory

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ABSTRACT

The aim of this study was to isolate thermostable amylase producing bacteria from starch-rich wastewater of one of the canning factories and then molecular identification of these isolates. In addition, the thermostable amylase extracted from the bacterium was investigated for the optimum temperature and pH of enzyme activity. In this study, 14 heat-resistant microbial isolates were isolated from wastewater and only two isolates had amylase activity. Molecular identification of isolates based on amplification of 16S rDNA gene by B27F and U1492R primers and then sequencing of PCR product confirmed the presence of *Bacillus pumilus* and *Bacillus safensis*. The results showed that the optimum temperature and pH of amylase activity were 60°C and 7, respectively, and *Bacillus safensis* 7.67 (U/ml) had more amylase activity than of *Bacillus pumilus* 6.33 (U/ml).

ARTICLE INFO

Article History:

Received: 2023/4/30
Accepted: 2023/7/9

Keywords:

Thermostable amylase,

Bacillus,

Temperature,

Factory wastewater

DOI: 10.22034/FSCT.20.139.138
DOR: 20.1001.1.20088787.1402.20.139.9.3

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1. Introduction

The discovery of amylases actually depended on the extensive researches of Papen and Persoz. In 1833, they succeeded in discovering a compound called malt from green barley, which converts starch into sugar, and called this compound diastase, which is known today as amylase enzyme [1].Amylases have a protein structure and three subtypes of alpha-amylase(a-Amylase), beta-amylase(b-Amylase) and gamma-amylase (c-Amylase) are divided Amylases are one of the main enzymes used in industry. Many amylases of microbial origin are commercially available to meet the growing industrial needs. They have almost replaced chemical starch hydrolysers in the industrial processing of this product. Among the main advantages of using microbial enzymes over chemical methods for starch hydrolysis, we can point out their high economic production capacity, the tendency of microbes to genetic manipulation and the improvement of high pressure tolerance threshold [2]. Also, these enzymes are used in the pharmaceutical, medical, fermentation, food, textile and paper industries. In fact, amylases account for approximately 25% of all global enzyme sales. Properties of amylases such as thermal stability, stability against changespH, incubation period,Carbon sources as a stimulus, nitrogen sources, The particle size of the substrate to be fermented, the effect of stirring on its productionIt is important.Today, alpha-amylase is one of the most important and significant enzymes commercially, especially in the sweetener industry. For the process of sweeteners from starch, the temperature should be 50 degrees CelsiuspH equivalent7be This requires the use of heat-resistant alpha-amylase. The discovery of heat-resistant amylases not only solves the problem of stability, but also accelerates the speed of the reaction and reduces the possible contamination and viscosity of the environment, so it directly benefits starch processing industries at high temperature. Also, in addition to starch processing, it has wide applications in food, livestock, fermentation, health, paper production, detergents and preparation of carbohydrate products and has replaced the chemical hydrolysis method of starch in its industrial processing. Bacteria*Bacillus subtilis*، *Basilius Stearothermophilus* And *Bacillus amylofacins* They are known as good producers of amylase resistant to high temperature in the industry [3].In general, alpha-amylases obtained from bacterial sources have more thermal resistance than the types prepared from barley or sprouted wheat, and the latter are more resistant than fungal types. What are called maltogenic amylases today are the result of bacterial sources and in terms of composition and structure, they are very similar to the enzymes obtained from fungal sources. with the aim of achieving Bacteria with the potential to produce heat-resistant amylase,Effluent containing starch One of the canning factories in Hamedan province was used as a potential source of this bacterium, and after molecular identification of the microbial isolates, the production potential of this enzyme and the optimal conditions for amylase activity obtained from them were investigated.

2- Materials and methods

In this research, 3 different areas of the effluent of a canning factory located in Hamadan, Iran were used to isolate amylase-producing microorganisms. All the isolates were initially screened by biochemical tests such as gram staining, catalase test and starch hydrolysis.

1-2- Sampling of factory effluent

In this research, the effluent from the production of low-fat mayonnaise in Sahar

Hamadan Food Industry Factory, which contains starch, was selected as a possible source of amylase-producing bacteria, and samples were taken from the first, middle, and end of the effluent path in sterile containers, and in order to isolate bacteria with Amylase activity at 4°C under sterile conditionstaken to the laboratory.

2-2- Cultivation and purification of heat-resistant bacterial isolates from wastewater

Effluent samples to pity^{7-}10 They were diluted by Ringer Sterile (Merck, Germany). After diluting the tubes for 10 minutes in the incubatorPersian medicine، Iran) were placed at 80°C to remove non-sporing bacteria. Then the samples onNutritagar culture medium was cultured superficially and kept at 50 degrees Celsius for 48 hours. Different colonies were selected in terms of size, color and shape and were cultured linearly on nutrient agar culture medium containing 1% starch and kept in a greenhouse at 50 degrees Celsius for 24 hours. Finally, separate colonies as pure isolates inNutrient broth culture medium (Merck, Germany) containing 15% glycerol (Merck, Germany) were kept at -20°C.

2-3- Initial screening of heat resistant amylase producing isolates andExtraction Heat resistant amylase from selected isolates

In order to check the morphology of the isolates, each one was taken under warm staining and then microscopic observation and catalase activity of isolates by3% hydrogen peroxide was evaluated. ThenFor the primary screening of amylase-producing isolates, gram-positive bacilli isolates with positive catalase activity were cultured on nutrient agar medium containing 1% starch and kept in a greenhouse at 50 degrees Celsius for 24 hours. Contains starchfor 1 minutewas spilled The presence of a clear and brown area of hydrolysis around the colony and growth areas was recorded as a positive result of amylase activity of each of the microbial isolates. After the activation of the isolates and the initial screening of each of the amylase positive isolates in nutrient broth (Merck, Germany) containing 1٪ Starch was inoculated and kept in a greenhouse at 50°C for 24 hours. Then, pour 1 ml of the medium into sterile microtubes and swirl vigorouslyg12,000 for 10 minutes at 4 degrees centigradeSigma, Germany) became. Then, their supernatant, after filtration with 0.45 micrometer filters, was used to estimate amylase activity (cell-free soup containing amylase) [4, 5].

2-4- Measuring amylase activity of isolates

To calculate amylase activity of the isolates, glucose standard solutions (Merck, Germany) with concentrations of 10 to 100 micromol/ml were used.. To measure absorbance,First, 1 ml of standard solutions with 1 ml of 1% phosphate buffer.(Arya Tab, Iran)and 2 ml of 3,5-dinitrosalicylic acid reagent(DNS) mixed And then 4 milliliters of water was added to it and it was kept in a hot house at a temperature of 50 degrees Celsius for 10 minutes. Then the amount

absorbance at 540 nm using a spectrophotometerUV/VIS (Aquaytic, Germany)was measured Also, for control and microbial isolates, the same standard test was done, but instead of 1 ml of standard solution, 1 ml of cell-free soup containing amylase containing 1% starch was used for microbial isolates. an enzyme unit (U/ml) was considered equivalent to the amount of enzyme needed to release 1 micromol of reducing sugar per minute [5-7].

5-2- Determination of temperature andpH Optimizing amylase activity and investigating thermal stability amylase

To determine the optimal temperature of amylase enzyme activityFirst, 1 milliliter of cell-free amylase soup containing 1% starch along with1 ml of 1% phosphate buffer and 2 ml of reagentDNS mixed And then 4 milliliters of water was added to it, then it was kept in a greenhouse at different temperatures of 30 to 90 degrees Celsius for 10 minutes, and then the amount of amylase activity was measured to determine the optimal temperature by the methodDNS was measured To determine the thermal stability of amylase, 1 ml of cell-free broth containing amylase with1 ml of 1% phosphate buffer and 2 ml of reagentDNS mixed And then 4 milliliters of water was added to it and it was heated at temperatures of 60 to 90 degrees Celsius for 60 minutes. Then starch was added to a concentration of 1% and kept in a greenhouse at the optimal temperature obtained from the previous step, and amylase activity was measured at 540 nm using a spectrophotometer.UV/VIS was measuredTo check the effect of changespH on enzyme activity Amylase was done in such a way that first, 4 ml of 1% phosphate buffer was added to 1 ml of the amylase soup prepared from the previous steps for the selected bacterial isolates, and thenpHit using 0.1 normal hydrochloric acid (Merck, Germany) and 0.1 normal soda (Merck, Germany) equivalent to 6, 7, 8 and 9 bypHMeter(Metrom, Switzerland)was set. and amylase activity after regulationpHdesired by the reagentDNS It was measured according to the previous method [5-7].

6-2- Molecular identification of the isolateI see

1-6-2- ExtractionDNA from selected isolates

In order to extract the bacterial genome, first each of the pure isolates was cultured in Nutrient Agar medium, then several bacterial colonies were suspended in phosphate buffer and incubated for 10 minutes in g7500 was centrifuged to extract bacteria from the precipitateDNA by extraction kitDNA (Sinacloon, Iran) was used and qualityDNA It was investigated by gel electrophoresis (1% agarose gel).

2-6-2- Chain reactionPCR

*reaction solution*PCR *involved in*16 µl of sterile deionized water, 2 µl of primersB27F AndU1492R(Bayonir, Korea) with concentrationpicomol/µl10 ‹ml25 Mr. Mix (single equipment, Iran) andml5 DNA Was. The steps to perform this reaction after activation at 95°C for 5 minutes, 35 cycles of the temperature-time program, including annealing at 94°C for 30 seconds, annealing at 50°C for 45 seconds, extension at 72°C to The duration was 2 minutes and then the final development was at 72°C for 10 minutes and the final cooling stage was at 4°C for 5 minutes. The PCR product of about 1200 bp was sent for sequencing (Macrogen, Korea) [8, 9].

Universal primers[1] For gene reproduction16S rRNA involved inB27F AndU1492R (Bayonir,

[1] -Universal

Korea) with the following sequence with concentrationpicomol/µl 10 were prepared:
Forward: B27F (5´-AGAGTTTGATCCTGGCTCAG-3´)
Reverse: U1492R (5´-ACGTGGTTTGAAGAGATTTTCG-3´)

3-6-2- Electrophoresis gel

First, to prepare 1% agarose gel, agarose powder (Sina Clone, Iran) in bufferTBE 0.5x(DNA Biotech, Iran) was dissolved and heated until the liquid became clear. Then before cooling the gel to itµl 1 Cybersif (Cinacloon, Iran) was added and after mixing, it was poured into a glued and combed tray. The closed gel was transferred to the electrophoresis tank (Nogen Pars, Iran) [9]. To load the productPCR Amount per wellµl 7 reaction productsPCR It was transferred into one of the wells as wellµl 3 ladderskb 1 (Cynaklon, Iran) was injected into one well as a negative control of the reaction productPCR LackingDNA considered, electrophoresis was performed for 30 minutes with a voltage of 70 to 75 volts. Finally, the formation of the product bandPCR The equivalent of bp 1500 was checked by matching with the marker by transeliminator device [9].

2-6-4- product sequencingPCR and analysis of racial evolution of isolates

After product purificationPCR with a purification kit (Intron, Korea) according to the manufacturer's instructionsµl40-50 of the corresponding purified product was sent to Macrogen Korea for sequencing, sequencing using primersB27FAndU1492R It was done by this company. Each sequence was subjected to double-sided reads. on averagebp850 to 1200 nucleotides per sequence were read on each side and aligned with the sequences in the gene database with the help of BLAST program on the site.http://www.NCBI.nlm.nih.gov was compared Isolates with 99% to 100% similarity were identified as the same bacterial species. By entering the read sequences in the Mega software, a kinship tree was prepared from the sequences for the isolates.

2-7- Statistical method

Effects of treatments including temperaturein seven levels (30, 40, 50, 60, 70, 80, 90 degrees Celsius)AndpH in four levels 6, 7, 8 and 9 on amylase activityBased on factorial statistical method and completely random statistical design, it was investigated in three repetitions. Data variance analysis with softwareSPSS done.

3. Results and Discussion

3-1- Microbial tests

Total bacteria count results(CFU/ml) It showed that the primary area of the effluent has10^3×26 ofMore microbial flora than the middle areas10^3×8 and the end of the effluent10^3×3 had.From the wastewater sample, 14 isolates of heat-resistant, gram-positive, bacilli-shaped and catalase-positive bacteria were obtained.In the present study, amylase producing bacteria,belonging to the genus Bacillus, It was isolated from the effluent of one of the factories producing food products.

2-3- Investigation of amylase production by bacterial isolates

Among 14 thermophilic microbial isolates, only isolates of 19 andB They were able to break down the starch in the nutrient agar medium, which was a positive sign of their amylase activity.The results of amylase activity of microbial isolates with the help of glucose standard are shown in Table 1.

Table 1. Amylase activity of microbial isolates after 15 and 30 minutes

Enzyme activity (U/ml)		Microbial isolates
30min	**15min**	
6.33	3.39	**B**
7.67	6.20	**19**

3-3- Determining the optimal conditions of amylase activity of the isolates

In order to compare the effect of temperature on amylase enzyme activity, the activity of two isolates (B and 19) was measured at different temperatures. The pattern of temperature effect on two isolates was almost the same.Amylase activity increased in the temperature range of 30 to 60 degrees CelsiusSo that the maximum activity was observed at 60 degrees CelsiusAnd then from 60 to 90 degrees Celsius, the enzyme activity showed a decreasing trend (Figure 1), which can be due to the denaturation of the enzyme at high temperature. Enzyme activity of isolate 19 is higher thanB It was shown at a temperature of 60 degrees Celsius. The results of statistical analysis showed that temperature has a significant effect on amylase activity($p<0.05$) has it.

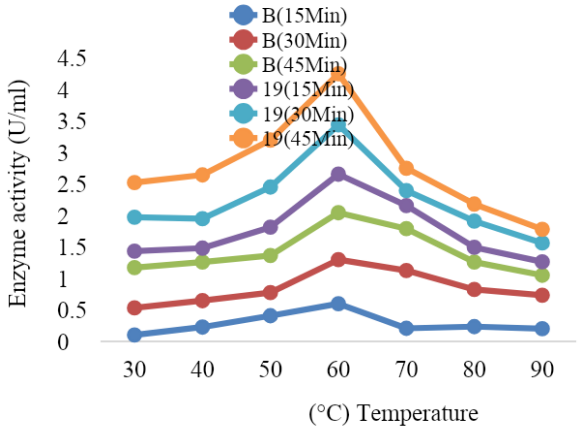

Fig 1. Optimum temperature of amylase activity of isolates in 15, 30 and 45 min

To investigate the thermal stability of amylase, enzyme soup without starch obtained from twoSeparated (19 and B) for 60 minutes in the oven placed in the range of 60 to 90°C, then starch was added to them and placed at 60°C, which was determined as the optimal temperature in the previous step, but after heat treatment, the enzyme apparently lost its active protein structure and its activity decreased (Figure 2).

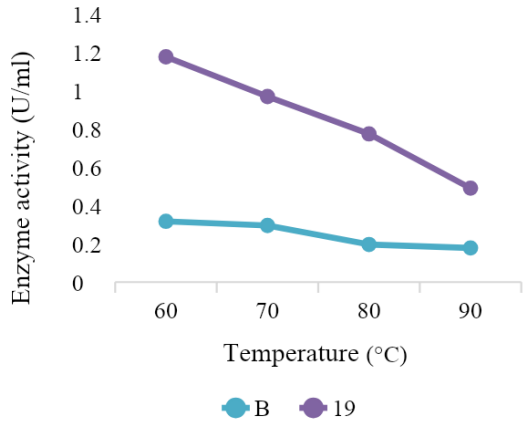

Fig 2. Thermal stability of amylase activity of isolates at 60 °C to 90 °C

In reviewing the workpH On the activity of amylase enzyme, it was determined that this enzyme has maximum activity inpH Equal to 7is andThe results of statistical analysis showed thatpH Significant effect on amylase activity($p<0.05$). Also, the results showed that the relative activity of amylase in the domain pH 6 to 7 was increasing while this activity in the domainpH 8 to 9ProcessThere was a decrease.Comparing the optimal conditions obtained for the activity of the studied enzyme with other studies, the results showed that the obtained amylase similarly has optimal activity at a temperature of 50-60 degrees Celsius andpH The optimum is approximately equal to 7. Similar to our study, Thipswamy et al. (2006) showed that temperature andpH The optimum activity of bacillus amylase that was isolated from industrial wastewater was 60 degrees Celsius

and 6.5 degrees respectively [10]. Also, Sagsena and Sink (2011) showed that temperature andpH The optimum amylase activity of Bacillus studied by them is 50 degrees Celsius and 6 degrees respectively [11]. Das et al. (2004), temperature andpH Optimum activity of alkaline and heat-resistant amylase extracted from*Bacillus subtilis* reported 52-55 degrees Celsius and 9 respectively. They showed that the thermal stability of this enzyme is 95°C for ten minutes, but in this condition, it lost 60% of its activity [12]. But there have been studies that have reported a higher optimum temperature for amylase. In this context, Cordeiro et al. stated that the amylase activity of the heat-resistant bacillus under their study had an optimal temperature of 70 °C, but similar to our study, it had a low thermal resistance at high temperature. This bacterium could tolerate a temperature of 50 °C for 2 hours. But at temperatures of 60, 70 and 90 °C it lost 4%, 13% and 38% of its activity, respectively, they reported thatpH The optimum of this bacteria was 7.5 [13]. Also, Echmidt et al. (2010), optimal conditions for amylase activity extracted from*Bacillus mejavensis* a high temperature equal to 80 degrees Celsius andpH6.5 [14] and Srivastava and Baroah (1986) high temperature equal to 82 degrees Celsius andpH reported 6.9 as the optimal activity conditions of this enzyme [15]. In relation to amylase activity level extracted from microorganisms, especially bacilli, different values have been reported, which can be attributed to various factors such as the type of microbial strain (wild or mutant), cultivation conditions, scale of production (industrial or laboratory) and so on. Al-Khalil and Gaffer (2011), the optimum temperature of 50-70 degrees Celsius for amylase extracted from*Bacillus Stearothermophilus* reported, but significantly at 70 degrees, it had a thermal stability of 60 minutes. By modifying the conditions and composition of the cultivation environment, they succeeded in producingU/ml) were 57.7-63.3 [16]. In relation to other amylase-producing microorganisms, we can mention a type of Streptomyces that has equivalent amylase activity (U/mg) 11.7 at a temperature of 50-60 degrees andpH It was equal to 6 [17]. Considering that there are many reports in the field of optimizing the production conditions of heat-resistant amylase on an industrial scale and increasing the high efficiency of this enzyme obtained from heat-resistant bacilli, but based on the results of our study, the amount of amylase activity obtained (U/ml) 6.2-7.67 is relatively favorable compared to other studies that have only been conducted on a laboratory scale on a wild microbial strain isolated from nature. For example, Ajaei and Faged (2003), amylase activity (U/ml) 4.2 for*Bacillus lychniformis*And (U/ml) 6.24 for*Bacillus subtilis* [18] and Nemasivavam and Nirmala (2013) amylase activity (U/ml) reported 6-8.5 for Aspergillus [19]. Of course, the possibility that in our study, the bacterial growth temperature of 50 degrees Celsius was effective in reducing amylase activity is not far from expected. Regarding the relationship between amylase activity and bacterial growth temperature, we can refer to the study of Wind et al. (1994). They failed to identify in the research*Bacillus stearothermophilus* with the high potential of heat-resistant amylase production, they reported that the amylase studied by them has an optimal activity temperature of 70 degrees and 5.5-6 =pH and at the optimal temperature for bacterial growth of 65 degrees, it acts as a growth-dependent enzyme, but when the growth temperature decreases from 65 to 40 degrees, the production of amylase decreases.(U/ml) It increases from 3.9 to 143 [20]. Therefore, lowering the temperature is effective in increasing its production and vice versa. In our study, the decrease in amylase activity with the increase in temperature above 60 degrees Celsius can be caused by the denaturation of the protein structure. The stability of the protein structure depends on the stabilizing forces resulting from a large number of relatively weak molecular interactions. in this

Hydrophobic interactions and hydrogen bonds between atoms and molecules play a major role in increasing thermal resistance. The main mechanisms of decomposition in the stable protein structure are related to the deamination of asparagine and glutamine, and the formation of succinamide in aspartate and glutamate, which leads to the hydrolysis of the peptide bond [21].

3-4- Molecular identification of microbial isolates

For molecular identification of microbial isolates, first the productPCR District 16S rRNA isolates It was taken on an electrophoresis gel, and Figure 3 shows the confirmation of band formationbp 1500 and perform the reactionPCR Gives.From Geneious softwareprime used to edit nucleotide sequences and two speciesbacteria*Bacillus pumilus* And*Bacillus safensis* by blast-edited sequence on sitehttp://www.NCBI.nlm.nih.gov with 99%identity was identified. According to Figure 3, the evolutionary tree of the kinship relationship of these isolates is displayed.

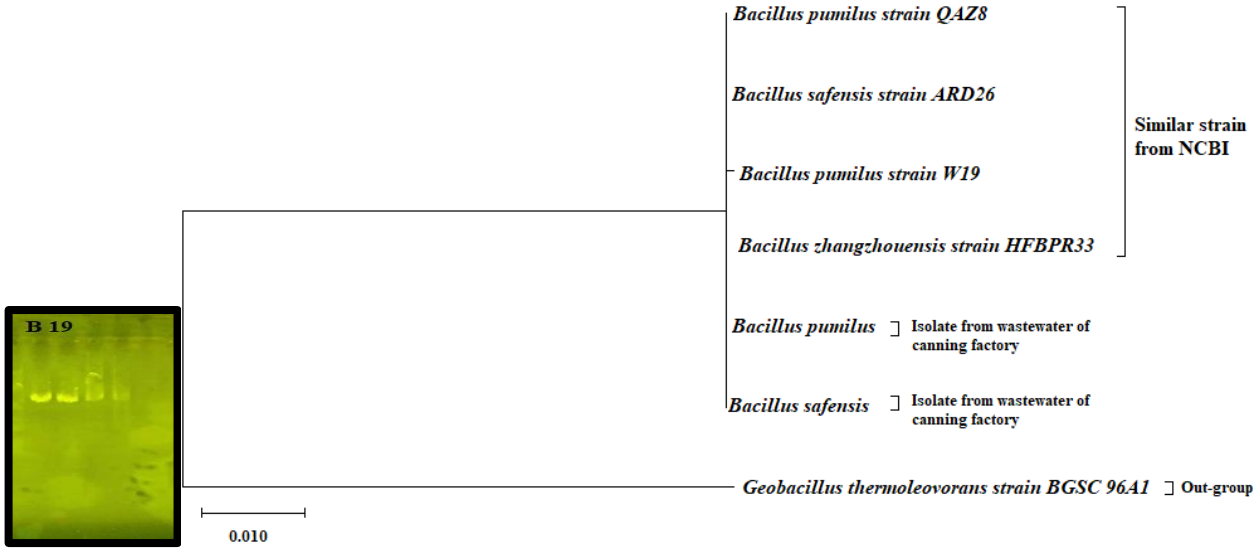

Fig 3. Phylogenetic tree of thermostable amylase producing isolates obtained from wastewater of canning factory

Gene sequence of two speciesbacteria*Bacillus pumilus* And*Bacillus safensis* With*Accession number* Respectively OL774705 And OL774706 AtNCBI Was recorded. Based on the results of molecular identification of the isolates and also the evaluation of amylase activity resultsThey, therefore*Bacillus safensis* as bacteria with the potential to produce amylase with greater activity than*Bacillus pumilus* Confirmed.

4 - Conclusion

Since most of the food processes are usually performed at high temperatures, the use of thermophilic alpha-amylases of bacterial origin, which have the ability to function and maintain their structure at these temperatures, can be helpful to advance these processes as much as possible.. With the increasing demand of amylase in the industry, the present study considered low-fat mayonnaise factory effluent as a possible source for the bacteria producing this enzyme, with the aim of obtaining amylase resistant to high temperature.Since the field of application of amylase enzyme is very wide according to their characteristics,

determining the characteristics and characteristics of this group of enzymes can be very effective in choosing and using them correctly. The results of our study showed that this enzyme at 60 degrees Celsius andpH equal to 7 has an optimal activity which decreases with the passage of time at this temperatureThe temperature below 60 degrees Celsius and around 55 degrees Celsius can be defined as the optimal temperature for this enzyme. In order to identify heat-resistant amylase-producing microbial isolates, two screened microbial isolates were molecularly identified and two species*Bacillus pumilus* And*Bacillus safensis* Confirmed. In terms of enzyme activity, ((U/ml) 7.67)*Bacillus safensis* has more amylase activity than ((U/ml) 6.33) *Bacillus pumilus* was, therefore, it is suggested as the selected bacteria for further studies to use amylase in the food industry.

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سایت مجله: www.fsct.modares.ac.ir

مقاله علمی_پژوهشی

جداسازی و شناسایی باکتری‌های تولید کننده آمیلاز مقاوم به حرارت از فاضلاب کارخانه کنسروسازی

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اطلاعات مقاله

تاریخ های مقاله :

تاریخ دریافت: ۱۴۰۲/۲/۱۰

تاریخ پذیرش: ۱۴۰۲/۴/۱۸

کلمات کلیدی:

آمیلاز مقاوم به حرارت،

باسیلوس،

دما،

پساب کارخانه

DOI: 10.22034/FSCT.20.139.138

DOR:20.1001.1.20088787.1402.20.139.9.3

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چکیده

هدف این مطالعه جداسازی سویه‌های میکروبی مولد آمیلاز مقاوم به حرارت از فاضلاب غنی از نشاسته یکی از کارخانه‌های کنسروسازی و سپس شناسایی مولکولی این جدایه‌ها بود. به‌علاوه آمیلاز مقاوم به حرارت استخراج شده از باکتری به لحاظ دما و pH بهینه فعالیت آنزیمی مورد بررسی قرار گرفت. در این مطالعه ۱۴ جدایه میکروبی مقاوم به حرارت از پساب جدا گردید، که تنها دو جدایه دارای فعالیت آمیلازی بود. شناسایی مولکولی این دو جدایه براساس تکثیر ژن 16S rDNA توسط پرایمرهای B27F و U1492R و سپس توالی‌یابی محصول PCR ، حضور *Bacillus pumilus* و *Bacillus safensis* را تایید کرد. نتایج نشان داد که دما و pH بهینه فعالیت آمیلازی جدایه‌ها به ترتیب ۶۰ درجه سانتی‌گراد و ۷ بود و *Bacillus safensis* با عملکرد (U/ml) ۷,۶۷ دارای فعالیت آمیلاز بیشتری نسبت به *Bacillus pumilus* با عملکرد (U/ml) ۶,۳۳ بود.