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PRODUCTION OF α-AMYLASE AND *Saccharomyces cerevisiae* BIOMASS BY USING BY-PRODUCTS FROM FOOD INDUSTRY

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ABSTRACT

In this research the production of α-amylase enzyme using *Bacillus spp*. bacteria, and biomass production of *Saccharomyces cerevisiae* yeast using three types of media were assayed. The production of α-amylase enzyme is realized through two types of fermentations, liquid fermentation where bacterial culture *Bacillus spp*. is cultivated in LB medium, and solid-state fermentation where industrial wastes such as potato and banana peels are used as substrate. Cultivation of *Saccharomyces cerevis*iae yeast is done using three different media: Yeast-Peptone-Dextrose, Yeast-Peptone-Sucrose and molasses. Based on the obtained results, it is concluded that the process of enzyme production using industrial waste, as well as yeast biomass production has a potential, which should be further researched.

Keywords: α-amylases, SSF, SmF, *S. cerevisiae, Bacillus spp*., Bread production

INTRODUCTION

α-amylases belong to the group of endoamylases that catalyze the hydrolysis of the starch molecule to the shorter oligosaccharides. These enzymes catalyze the hydrolysis of internal α-1,4-glycosidic bonds of the starch molecule and in this case produce low molecular weight products, such as glucose, maltose and maltotriose units (**Souza and Magalhaes, 2010**). α-amylases can be produced by different species of microorganisms, but for commercial applications, they are mainly derived from bacteria of the genus *Bacillus* (**Tiwari *et al.*, 2015**). The production of α-amylase can be done through two fermentation processes, liquid fermentation, and solid-state fermentation. In this research, two types of fermentation for the production of α-amylase were analyzed.
Saccharomyces cerevisiae, also known as baker's yeast, is a eukaryotic unicellular microbe. More specifically, it is a globular-shaped, yellow-green yeast belonging to the fungi kingdom. Yeasts are best grown in a neutral or slightly acidic environment, aerobic, with an adequate supply of nutrients, at optimum temperatures of 28-30 °C. Usually, the strains reach the maximum density in yeast-peptone-dextrose media (YPD) (**Kovačević, 2015**). In this research is analyzed the cultivation of *S. cerevisiae* in YPD, yeast-peptone-sucrose media (YPS), and molasses medium. Molasses is an agro-industrial waste and a by-product of the sugar industry, as e such contains high amounts of monomeric and polymeric sugars and is used in the chemical industries to produce bread yeast and ethanol (**Shafaghat *et al.*, 2010**).

MATERIALS AND METHODS

Alpha amylase production through SSF fermentation

Substrate Preparation

Banana and potato peels were used as substrates, which were initially cut into small pieces and dried for 24 h at 70 °C. Dried peels were sieved in 4mm wire diameter sieve. The collected material was saved in tightly closed jar in a low humidity place (Kokab et al., 2003; Shukla and Kar, 2006).

Inoculum preparation

An isolated species S10-1 of agricultural soil used as a source of forage grass from the Bajgora region in Kosovo is used for inoculum preparation. This species, genetically identified as *Bacillus cereus* ATCC 14579 by the work of **Hyseni *et al.*, (2020)** was used in this research for the production of α-amylase. A colony of *B. cereus* was taken from the nutrient agar plate and placed in a 50 mL Falcon tube, to which a 5 mL LB medium was added. The tubes were placed in the incubator shaker for 18 hours at 37 °C (**Salman *et al.*, 2016**).

Solid-state Fermentation

In 250 mL Erlenmeyer was placed 10 g of dried potato peel, dried banana peel, or a mixture of these two types of peels in a ratio 1: 1. Content was moistened with 7.5 mL of medium with mineral salts (3 g yeast extract, 5 g Peptone, 20 g Glucose, 15 g NaCl, 11 g Na_2HPO_4 x $2H_2O$, 6.1 g NaH_2PO_4 x $2H_2O$, 3 g KCl, 0.1 g $MgSO_4$ x$7H_2O$ in 1000 mL distilled water) and 7.5 mL of effluent from potato processing, reaching a total moisture content of 60 %. Prepared Erlenmeyer flasks are closed and placed in the autoclave for 15 minutes at 121 °C. After autoclaving, 1.5 mL of inoculation is added to each of them, and placed in the mixture for 72 hours at 37 °C (Shukla and Kar, 2006).

Extraction of the enzyme from solid-state fermentation

α-amylase is extracted by the contact method. After the incubation period, in each Erlenmeyer are added 50 mL of distilled water (5 mL per 1 g) and placed in the incubator shaker for 30 minutes. The content is filtered through Whatman filter paper and filtrate is centrifuged at 3300 rpm for 15 minutes. The supernatant is used as a raw enzyme (**Kokab *et al.*, 2003**).

Production of α-amylase through submerged fermentation

Preparation of the medium

LB medium is prepared based on the guidelines of produces. In 1000 mL of distilled water, 20 g of LB medium are dissolved and the content is autoclaved for 15 min. at 121 °C (**Salman *et al.*, 2016**).

Liquid fermentation

In 500 mL Erlenmeyer is placed 100 mL LB medium and 1.7 mL inoculum. The Erlenmeyer flasks are closed and placed in the incubator shaker for 72 hours at 37 °C. Each 24 h, 1% v/v sterilized effluent from the potato industry is added as inducer (Raul et al., 2014; Ul-Haq et al., 2002).

Cultivation of the yeast *Saccharomyces cerevisiae*

Inoculum preparation

A colony of *Saccharomyces cerevisiae* yeasts was taken from the YPD Agar plate and placed in a 50 mL tube, to which a 5 mL YPD medium was added. The tubes were placed in the incubator shaker for 24 hours at 26 °C (**Olivares-Marin *et al.*, 2018**).

Preparation of the medium and cultivation

The media used for yeast cultivation are YPD medium (10 g yeast extract, 20 g peptone, 10 g glucose, in 1000 mL distilled water), YPS medium (10 g yeast extract, 20 g peptone, 10 g sucrose, in 1000 mL distilled water) (**Olivares-Marin *et al.*, 2018**) and molasses medium (40 g molasses powder in 1000 mL distilled water). First, molasses is dissolved in water, then is hydrolyzed with HCl in the ratio of 1:400, and finally added 1 g yeast extract, 1.5 g NH_4Cl, and 0.1 g KH_2PO_4. The prepared media is autoclaved for 15 minutes at 121 °C (**Shafaghat *et al.*, 2010**).
Cultivation of *S. cerevisiae* yeast was done in 1 L Erlenmeyer with 200 mL medium, and in 6 L bioreactor with 1.5 L medium and 5 % inoculation. During the cultivation in Erlenmeyer three types of media were used (YPD medium, YPS medium and molasses medium). This type of cultivation was done at room temperature. Cultivation in the bioreactor was carried out using molasses and YPD medium. The pH of the medium was 5, the culture temperature was 30 °C, dissolved oxygen (pO_2) over 50.0 %, mixing 400 rpm/min, and airflow 3.00 L/min. Cultivation under these conditions was done for 72 hours.

Amylase activity

Amylase activity was determined by the spectrophotometric method. Initially, 0.8 mL of starch solution and 0.2 mL of the extracted supernatant are poured into Eppendorf tubes and incubated in a water bath at 50 °C for 30 min. Then 1 mL of 3,5-dinitrosalicylic acid (DNS) is added to each tube to stop the reaction and incubated in a water bath at 100 °C for 10 min. The contents are allowed to cool for a few minutes at room temperature and then the absorbance is set at 540 nm (**Madika *et al.*, 2017**).

Bread preparation

The main ingredients for bread production are flour, yeast, salt, and water. In a laboratory container, 100 g of wheat flour, 60 mL of water,1 g NaCl, and 1 g of yeast were placed and mixed until a dough mass is formed. The obtained dough is placed in a scaled cylindrical flask of 250 mL to observe its fermentation after 2 hours and after 18 hours. After shaping, the bread is baked and then analyzed. The quality of bread produced was determined through analysis of quality parameters. The porosity and specific volume of bread were mainly analyzed as quality parameters.

Determination of bread porosity

From the middle of the bread is cut pieces in the shape of a cube with dimensions of 3 cm^3, with a volume of 27 cm^3. Then in a 50 mL scaled cylindrical flask 30 ml of oil are added. Inside the cylinder is placed a piece of bread, earlier compressed like a ball destroying the porous structure. Measured volume is used to subtract the volume of bread at the beginning, which results in the bread porosity volume (**Xhabiri and Sinani, 2011**).

Determination of bread volume

Bread volume was determined using the seed displacement method. Flax seeds were placed in the scaled cylindrical flask until the bottom was covered. In the scaled cylindrical flask, 30 gr of bread are placed and the scaled cylindrical flask was filled until the top with flax seeds. Flax seeds were measured with another graduated cylinder and the difference was considered as bread volume. The specific volume is calculated by equation (**Al-Saleh and Brennan, 2012**):
Specific volume (mL/g) = volume of bread (mL) / weight of bread (g) [1]

RESULTS AND DISCUSSIONS

Growth of Bacillus subtilis bacteria

In Figure 1 the growth curve of *Bacillus cereus* is shown. The growth of these bacteria was accomplished using an LB medium at 37 °C for 72 hours. Results showed a very quick adoption of bacteria to the conditions offered and consequently exhibit a short lag phase. Contrary, a rapid growth rate during the exponential phase was observed. While the maximum production of biomass of 1.22 g/L was reached after 36 hours of fermentation.

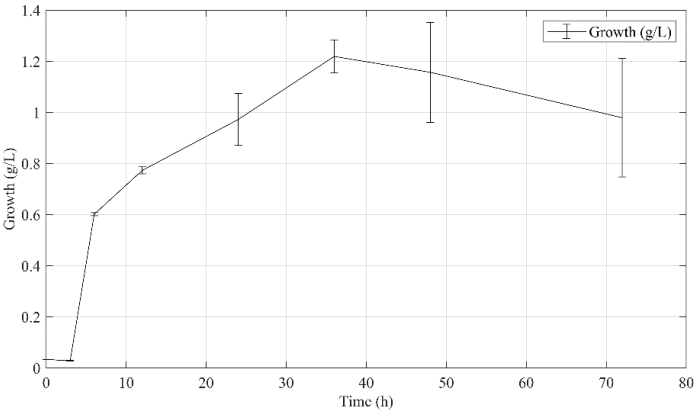

Figure 1 The growth g/L of *Bacillus spp.* in LB medium

α-amylase enzyme activity through liquid fermentation

Results of α-amylase activity from *Bacillus cereus* in liquid fermentation are shown in Figure 2. Raw supernatant from liquid fermentation was used as an enzyme. Based on the obtained results for activity, it is observed that at zero hours of fermentation the activity was zero. While the maximum enzymatic activity is achieved after 36 hours of fermentation, and the value of the activity in this time interval was 1.05 U/mL. It is observed that after 36 h of fermentation the activity decreased with the lowest point at 72 h of fermentation with 0.88 U/mL α-amylase activity. The reason for this decrease may be the simultaneous production of other enzymes, which affects the reduction of amylase activity, and maybe the absence of nitrogen sources in the medium. Moreover, the reduction of biomass affects the activity of the enzyme too. Compared with the results of **Božić *et al.*, (2011**), lower amylase activity was observed. The reason for the low activity maybe the absence of nitrogen sources in the medium. Through the fermentation only effluent from potato processing was added which was used as inducer and as carbon source. However, no nitrogen source was added through fermentation (**Božić *et al.*, 2011)**.

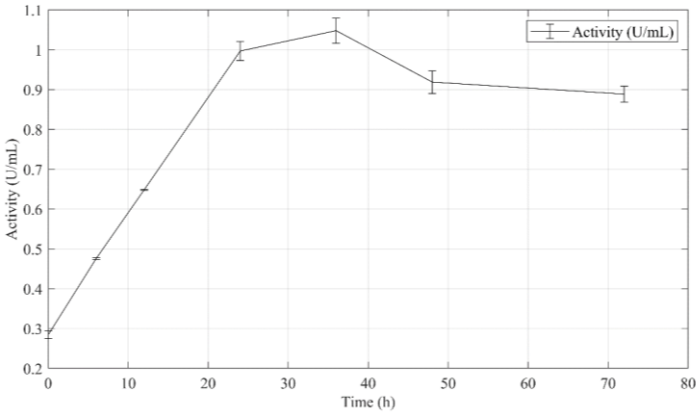

Figure 2 Amylase activity produced by *Bacillus cereus* in U/ mL.

α-amylase enzyme activity from solid-state fermentation

Agricultural waste is used as a substrate to carry out both liquid and solid-state fermentation in order to reduce the cost of the fermenting medium. These wastes contain sources of carbon and nitrogen necessary for the growth and metabolism of organisms. These nutrient sources are found in many agricultural wastes such as potato, banana, corn, wheat, rice and are mainly used for the production of α-amylase. To carry out solid-state fermentation, are used two types of industrial waste (potato and banana peels), and a combination of them in 1:1 ratio. From the results obtained (Figure 3.), it can be seen that at 0 h, the amylase activity is zero and during fermentation the maximum enzymatic activity is reached after 72 hours of fermentation in combined substrate with 5.5 U/mL. It is also observed that when only potato peels were used as a substrate, the enzymatic activity gradually increased and the maximum value of 1.53 U/mL was reached after 72 hours of fermentation. The opposite happened when banana residues were used as a substrate. In this case, the maximum activity was reached after 24 hours of fermentation (3.19 U/mL), and this activity has been decreased throughout the fermentation. The reason maybe that the moisture-holding capacity of banana peels was lower than that of potatoes, which has led to a decrease in enzyme production. Our results of enzymatic activity, when banana residues were used as substrate are in line with the results of **Shaista *et al.*, (2003)**, where even in this case it was found that maximum enzymatic activity is achieved after 24 hours of fermentation (**Kokab *et al.*, 2003**).

Whereas compared with the results of **Shukla *et al.,*** (**2006**) it is observed that lower enzymatic activity was produced in this research. However, the moisture content of the substrate is a major factor in enzyme production, hence lower moisture content in the substrate affected the activity (**Shukla *et al.,* 2006**).

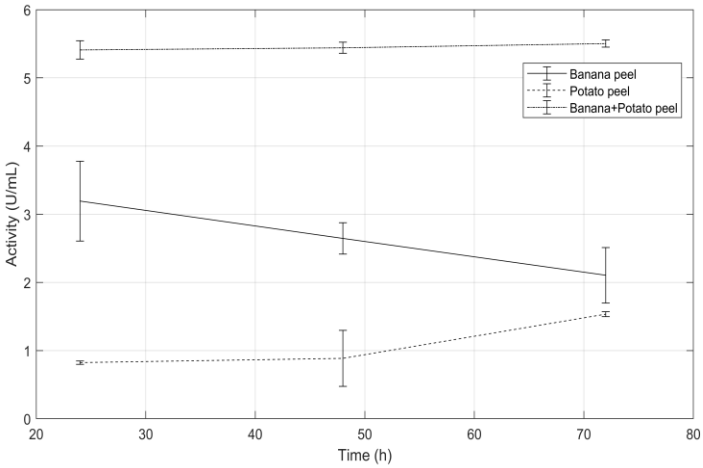

Figure 3 Amylase activity in U/L from Solid-state fermentation

Cultivation of Saccharomyces cerevisiae in YPD and YPS medium in Erlenmeyer

Figure 4 shows the *S. cerevisiae* yeast growth curve in YPD and YPS medium and utilization of sugars during 72 hours of fermentation. With the analysis of this curve, it is observed that during the lag phase the *S. cerevisiae* are mainly acclimated to the provided conditions and have not shown an increase in biomass or sugars consumption, while during the exponential phase the yeast have consumed the sugar present in the medium and have shown the maximum increase in biomass.

Carbon resources are known to be essential for yeast growth, multiplication and production. According to previous research by various authors, it is shown that the best biomass growth was when glucose and sucrose were used as carbon sources. Based on this fact, the same carbon sources were analyzed in this research as well. Our results show that in the case when glucose was used as a carbon source, *S. cerevisiae* reached maximum biomass of 7,132 g/L at 72 hours of fermentation with an average specific growth rate of 0.00388 h^{-1}. However, when sucrose was used as a carbon source, it is observed that the final biomass during 72 hours of fermentation was lower of 6,688 g/L, with specific growth rate of 0.0036 h^{-1}. The reason is the necessity for sucrose to break down first into glucose and fructose and then the released glucose is consumed by the yeast, which is a step backward for the growth process.

If the biomass growth results are compared with the results of **Siedlarz *el al.,*** (**2015**), it is observed that in our case the yeast cells have shown a slower adaptation to the conditions offered, and as a consequence an extension of the lag phase appeared (from 0-12 hours), then continuing at a rapid growth rate during the exponential phase. This prolongation of the lag phase can be caused as a result of pour adaptation of microorganisms to the offered conditions, taking into account that the optimal temperature of yeast growth is 30 °C, while in our case the offered temperature was 20 °C (**Siedlarz *et al.,* 2015**).

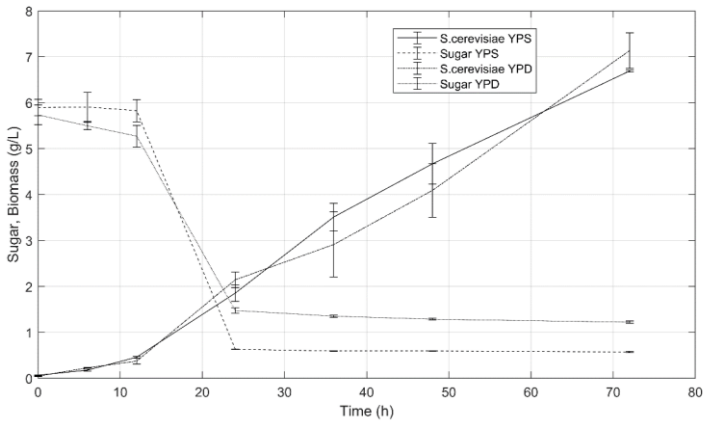

Figure 4 Biomass growth of *S. cerevisiae* in YPD and YPS medium, and sugar reduction

Cultivation of Saccharomyces cerevisiae in YPD medium in bioreactor

YPD medium was used to produce *S. cerevisiae* biomass using the 6 L bioreactor. Key growth parameters were monitored and maintained such as pH 6, pO_2 > 30 %, cultivation temperature 30 °C, mixing 400 rpm/min, total flow 3.00 L/min, and gas mixture 21 % O_2. Figure 5 shows the increase of *S. cerevisiae* biomass in the YPD medium in the 6 L bioreactor with a 1.5 L working volume. As seen in the figure, the highest biomass growth is achieved after 48 hours of fermentation with a biomass of 8.12 g/L and a specific growth rate of 0.008 h^{-1}. Comparing the results with the fermentation in Erlenmeyer with the YPD medium, the highest amount of biomass has been produced in the bioreactor with 1 g/L more. Moreower, the highest biomass produced in the bioreactor was reached 24 h earlier than in erlenmeyer and this may be due to better aeration of the substrate.

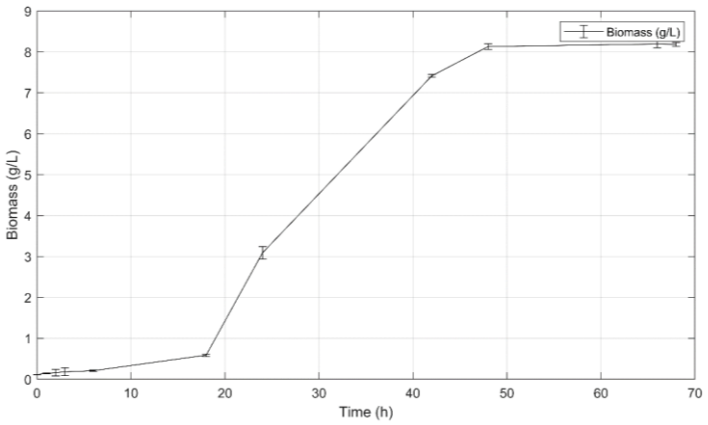

Figure 5 The growth g/L of *S. cerevisiae* in YPD medium in bioreactor

Saccharomyces cerevisiae in the molasses medium in erlenmeyer and in the bioreactor

Figure 6 shows the growth curves of *S. cerevisiae* yeast during fermentation in bioreactor and erlenmeyer, as well as the utilization of sugar during 72 hours of fermentation. Fermentation is carried out using as medium molasses powder, which is initially pre-treated, enriched with other nutrients and then placed in bioreactor and erlenmeyer to carry out the fermentation process.

From the results obtained, it is observed that the yeast grown in bioreactor have quite quickly acclimated to the offered conditions, by showing a short lag phase, and immediate sugar consumption. After the end of the lag phase (after 6 hours) it is observed an exponential growth phase. Though, maximum biomass achieved was only 1,376 g/L at 72 hours of fermentation, with an average specific growth rate of 0.0006 h^{-1} and an average biomass yield per substrate $Y_{x/s}$= 0.64 g/g. Whereas if the yeast growth is analyzed during the fermentation in Erlenmeyer, then it is observed that the yeasts have shown very small growth and the maximum biomass only 0.185 g/L was reached after 48 hours of fermentation. The reason for such a small increase of biomass in Erlenmeyer process may be the lack of optimal growing conditions as the temperature offered during this fermentation has been less than 20 °C and also the aeration of the substrate was based only in mixing the substrate.

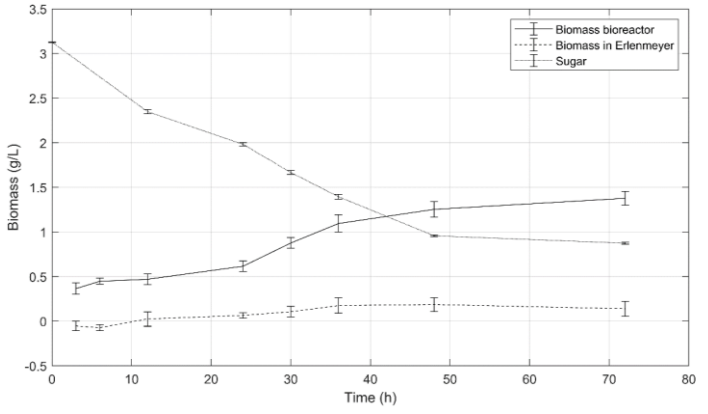

Figure 6 Biomass growth of *S. cerevisiae* in bioreactor and Erlenmeyer and sugar reduction in molasses

Oxygen saturation of the substrate was monitored throughout the fermentation. Graphically, the results are presented in figure 7, where it is clear that the exponential increase of the yeast has been accompanied by a decrease of the dissolved oxygen in the substrate as a result of the utilization of the oxygen by yeast. After 20 hours of fermentation, the cascade system was activated where as

independent parameters aeration and mixing were used to keep the dissolved oxygen in the substrate above 30 %. After 56 hours of fermentation as shown in the figure, the aeration system failed due to blockage of the filter, which also pushed the cells to enter the death phase.

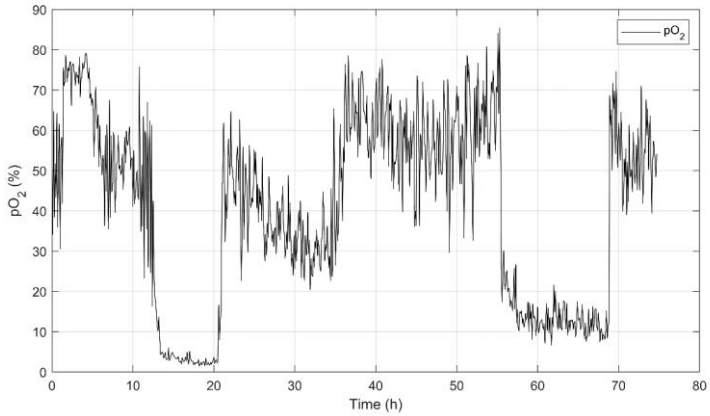

Figure 7 Oxygen saturation of the substrate

Bread analysis

From the results presented in table 1 and table 2, it was observed that the porosity and the specific volume were higher in the case where for the production of bread yeast grown in laboratories were used, compared to the bread produced using commercial yeast. The reason for achieving these results was that laboratory-cultivated yeasts were fresh, alive and quite active compared to commercial yeasts. Results of a specific volume of bread from this research are compared with the results of **Al-Saleh *et al.*, (2012)**. In our case, produced bread shown a lower specific volume. The volume of bread varies depending on the quality, type and composition of the flour. Therefore, it can be concluded that the reason for the appearance of the lowest volume maybe the type and composition of flour which are used or the length of time of sample analysis, as the volume of bread was analyzed 5 days after baking.

Table 1 Porosity of bread produced with yeast from different hours of cultivation and commercial yeast

BREAD PRODUCED USING	POROSITY (%)
Yeast grown in YPD medium for 24 hours	66,67
Yeast grown in YPD medium for 48 hours	59,25
Yeast grown in YPD medium for 72 hours	70,37
Yeast grown in YPS medium for 24 hours	62,96
Yeast grown in YPS medium for 48 hours	59,25
Yeast grown in YPS medium for 72 hours	66,67
Commercial yeast	62,96
Yeast + medium with molasses	62,96
Yeast cells cultured in molasses medium	59,25

Table 2 Specific volume of bread produced with yeast from different hours of cultivation and commercial yeast.

PRODUCED BREAD USING:	VOLUME (mL/g)
Yeast grown in YPD medium for 24 hours	2,36667
Yeast grown in YPD medium for 48 hours	2,36667
Yeast grown in YPD medium for 72 hours	2,06667
Yeast grown in YPS medium for 24 hours	2,36667
Yeast grown in YPS medium for 48 hours	2,06667
Yeast grown in YPS medium for 72 hours	2,06667
Commercial yeast	2,06667
Yeast + medium with molasses	2.1
Yeast cells cultured in molasses medium	2.13333

CONCLUSION

This study shows that the production of α-amylase enzyme is possible by using *Bacillus subtilis* bacteria as fermenting organisms during liquid and solid fermentation. This study also shows that the cultivation of *Saccharomyces cerevisiae* yeast is possible when the medium contains powdered molasses, in addition to cultivation in YPD, YPS media.

Particular attention should be paid to the use of industrial waste as alternative sources of carbon and nitrogen for the production of α-amylase and the cultivation of yeast. Once such a thing is possible, it is economical and profitable.

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