Investigating the possibility of Microbial Production of Mannitol from Waste Bread

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ABSRACT

According to the significant role of sugar alcohols (Polyols) in food industries, in the present study the possibility of microbial production of mannitol from bread waste was studied.

Microbial growth and amylase production were investigated by five Iranian native strains of Bacillus spp in starch agar and broth. The best strain was selected, and its growth curve was determined. Leuconostoc mesentroides PTCC 1059 was used as a control strain to convert fructose to mannitol. In order to determine the ability of selected strains in converting waste breads into mannitol sugar, a culture medium was prepared from waste of Lavash and Baguette breads. Afterward, the ability to convert starch into fructose by Chemical analysis glucose test was used, and then bio-conversion analysis of fructose to mannitol by HPLC analysis was investigated.

HPLC results showed that the Bacillus subtilis and Leuconostoc mesentroides PTCC 1059 had the ability of producing mannitol at a rate of 4.8g/L from fructose 5%, 0.15 g/L from Lavash bread 5%, and 0.2g/L from Baguette bread.

Key words: Waste Breads, Polyol, Mannitol, *Bacillus* spp., *Leuconostoc mesentroides* HPLC

INTRODUCTION

In nature, polyols are found in fruits. They are produced by fungi, yeast and bacteria. Polyols play several roles in carbon storage and protection during osmotic and oxidative stresses. They are hydrogenated carbohydrates widely used in the food industry as sugar replaces. They display a taste and sweetness similar to sucrose but with reduced calories. Furthermore, several health-promoting benefits have also been attributed to polyols such as low-glycemic, low- insulinemic , and anticariogenic properties [1]. Mannitol is a common six-carbon sugar alcohol. It is one of the most abundant carbohydrates in nature, occurringin bacteria, yeasts, fungi, algae and some plants and fruits. Mannitol is widely used in the food, pharmaceutical, medical and chemical industries. Production of mannitol by extraction of plant raw material is no longer economically relevant. Industrial production of most sugar alcohols is performed by catalytic reduction of sugars with hydrogen gas and nickel at high temperature and pressure for which highly pure sugar substrates and costly chromatographic purification steps are needed. Regardless of the limitations of this chemical method, until now it is the only process that is able to assume the high market demand for sugar alcohols as sorbitol, mannitol, or xylitol, estimated to be thousands of tons per year. However, processes using bacteria

and yeasts have proved which biotechnological production may represent an efficient and cost-effective alternative to the chemical production. In most heterofermentative species as leoconostoc spp and lactobacillus spp, fructose and glucose can act as an external electron acceptor in a reaction mannitol dehydrogenase (MDH) [2].

Starchy substances constitute the major part of the human diet for most of the people in the world. Some plant examples with high starch content are corn, potato, rice and wheat. Starch is polysaccharide compound with glycosidic bonds. Among the starch hydrolyzing enzymes, α–Amylases have considerable commercial value, especially in the sweetener industry. α–Amylases are extracellular enzymes that cleave α-1 linkages between adjacent glucose units in the linear amylase chain and ultimately generate glucose units. Microorganisms like fungi and bacteria have been used for amylase production. Bacillus subtilis is widely used for production of α–Amylases which are used in various industries like starch industry to produce glucose and fructose by liquefaction of starch [3]. Experts considered waste bread as the greatest national extravagance and therefore in addition to emphasis on consumption

optimization, they have demanded prevent the wasting subsidies allocated to bread [4]. Given the importance of polyols in low calorie diet and also to reduce environmental losses, the aim of the present study was using waste bread which it could be used as a low- cost substrate in the production of mannitol by microbial fermentation.

MATERIALS AND METHODS

Bacterial strains

Five iranian native Bacillus strain consisted of Bacillus subtilis, Bacillus megaterium ,Bacillus cereus, Bacillus anthracisa and Bacillus mycoides were obtained from Islamic Azad university-Tehran, North Branch Microbiology laboratory. Standard strain Leuconostoc mesentroides PTCC 1059 was prepared from the Iranian Research Organization for Science and Technology. These strains were cultured in Nutrient agar (Merck, Germany) and were incubated for 24 h at 37°C. After growth, in accordance with reference book, verification of strains was evaluated by microbiological methods included examination of microscopic, macroscopic and biochemistry characteristics [5].

Evaluation of growth and starch analysis

From the samples of bacterial strains, streaking on starch agar medium (Nutrient agar 28g, starch 20g and distilled water 1L) was prepared. Samples incubated at 37°C for 24h. In order to determine the ability of digesting the starch enzymatic by the strains, iodine reagent was used. The clear zone around the colony of bacterial strains was evaluated and the best strain was selected [5].

Preparation of the bacterial inoculation culture

Bacterial inoculation was prepared from the optimum bacterial strain producing amylase enzyme in culture medium Nutrient agar (Merck, Germany), and it was incubated at 37°C for 24h. Then opacity of that was adjusted through determined spectrophotometrical (model UV 2pc, SHIMADZU) at a wavelength nm 600 on the 0.8-1(equivalent to CFU / ml 108 × 5) [5].

Determination of maximum enzyme production

From the selected strain inoculation culture, starch broth in the ratio of 10% was added on (Nutrient agar 8g, Starch 20g and distilled water 1L) and it was incubated at 37°C for 4 days. To determine the growth curve and subsequently amylase production at two h intervals, absorption rate in samples before and after centrifugation (4,000rpm, 10min), were measured at 600 and 620nm, respectively. After adding the iodine reagent 5% (Iodine 3g, potassium iodine 6g, distilled water 1L), the evaluation of amylase production by bacteria in 620nm was conducted [3].

Chemical identification of sugars formed in culture medium

To identify and evaluate the type of sugars formed in the starch broth culture, after inoculation of selected strain, amylase enzyme production in the ratio of 10% was incubated at 37°C for 52 h in 180 rpm. After centrifuging for 10min at 4000 rpm, chemical tests including Mulish, Tollen, Seliwanoff and Benedict on samples were performed [3]. As a standard sample, these tests were provided on the starch, fructose, glucose and mannitol.

Study of the biohydrolysis of starch in lavash and baguette wastes

A culture medium was prepared from the waste bread at the ratio of 5% in distilled water and sterilized by autoclaving. Then inoculation culture from the optimum bacterial strain producer amylase enzyme and Leuconostoc mesentroides PTCC 1059 was added separately at the ratio of 10% and the samples were incubated at 37°C for 3 days in 180 rpm. During the incubation time, at intervals of two hours, a part of the culture medium was centrifuged in 4,000rpm, and chemical tests were conducted to identify sugar on them [5]. Starch 5% also was used as a control.

Ability to produce monnitol from fructose

After preparing the medium fructose 5% (g/cc) in distilled water and filtration by filter paper 0.22 micron (Chmlab Co, Spain), inoculated culture of selected bacterial strain and Leuconostoc mesentroides PTCC 1059 at a rate of 10% was added separately. The samples were incubated at 37°C for 24h in 180rpm. After centrifugation for 15minutes in the 4000rpm, supernatant was moved at 4°C for a week for monnitol deposits [6].

Ability of bacterial synergism in mannitol production from bread wastes

Inoculated culture of 10% from bacterial selected strains was added to the 5% media of lavash and baguette waste bread. After that, it was incubated at 37°C for 52hours with shaking at 180rpm. Then, samples were centrifugated (4000rpm, 15min) Inoculation culture of Leuconostoc mesentroides PTCC 1059 was added to the supernatant and then, culture was incubated at 37°C for 24h with shaking at 180rpm [7].

After the second centrifugation(4,000 rpm, 15 min), of the culture medium with the above conditions, the sample in order to mannitol deposits were transferred at 4°C for 7days [5]. Fructose 5% and inoculation culture Leuconostoc mesentroides PTCC 1059 were used as control culture.

Determination of sugars

To determine the type of sugars and the amount of mannitol produced by two strains of Leuconostoc mesentroides PTCC 1059 from waste of bread and fructose broth culture medium, the samples were examined by High performance liquid

chromatography system manufactured by Agilent (Model 1200). Calibration curve was used based on the concentration of 0.01, 0.05, 0.1, 0.5, and 1g of mannitol in 100 ml. Fructose and mannitol 5% were used as the control samples.

RESULTS

Among bacterial strains tested in this study, only Bacillus subtilis showed to have the ability to hydrolyze starch. Maximum amylase enzyme production by this strain according to its growth curve was determinate at 50 h, and it was considered as a selected strain for continuing this research (Fig 1).

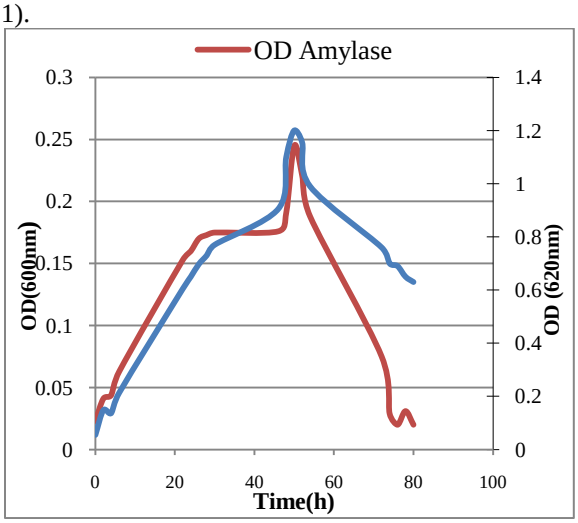

Fig.1. Growth curve and amylase enzyme production by *Bacillus subtilis* in starch broth medium

In this study, Benedict test was evaluated as the best chemical test to assess the bio-conversion of sugars in bacteria based on the color separation [5]. The results of this test, showed that the bacteria were able to convert starch into simpler sugars such as monosaccharides and disaccharides during 50 h in starch broth, lavash and baguette culture mediums, while it was negative for Leuconostoc mesentroides PTCC 1059. For this purpose, the Bacillus subtilis was used for converting starch to fructose and then Leuconostoc mesentroides PTCC 1059 was used for converting fructose to mannitol. The ultimate ability to produce mannitol by two bacterial strain Bacillus subtilis and Leuconostoc mesentroides PTCC 1059 was confirmed by the formation of small white crystals and HPLC analysis (Figs. 2-4).

Based on the HPLC chromatogram, the amounts of mannitol in three sediment samples obtained from the fructose, lavash and baguette culture mediums inoculated with Leuconostoc mesentroides PTCC 1059 were 4.8, 0.15 and 0.2 g/l, respectively. (Table 1)

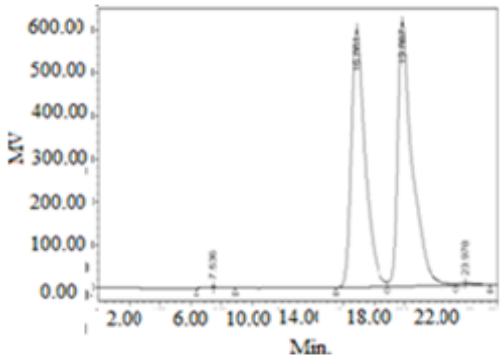

Fig. 2. HPLC chromatogram of mannitol in Fructose 5% inoculated sample

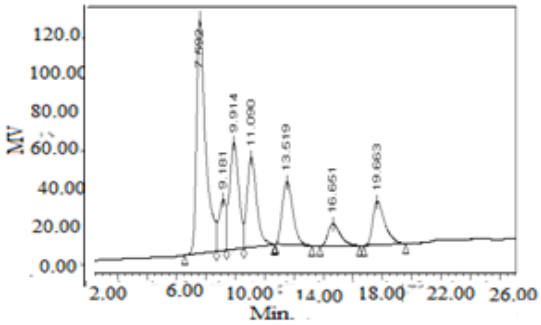

Fig.3. HPLC chromatogram of mannitol in lawash inoculated sample

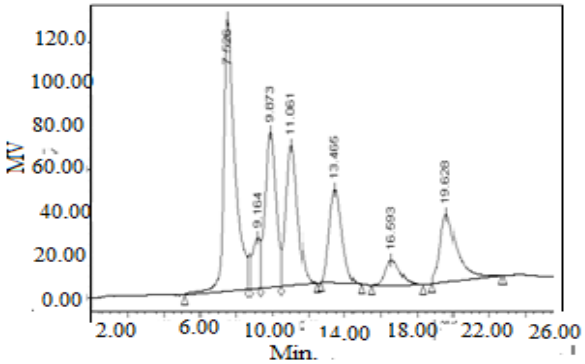

Fig.4. HPLC chromatogram of mannitol in baguette inoculated sample

Table1. Analysis of HPLC curve

	Mannitol	
Analysis of HPLC	area(µV*sec)	gr/lit
Fructose 5%	39,486,485	4.8
Lawash Bread 5%	1,758,998	0.15
Baguette Bread 5%	2,056,137	0.2

DISCUSSION

considering the microbial enzymatic capabilities, our study has tried to use bread wastes for producing valuable materials such as polyols. Therefore, converting starch to mannitol was performed by two bacteria in two steps which in the previous study, it has not been checked. In recent years, for different

reasons including poor quality and low price of traditional bread, the country has encountered with a lot of bread wastes that sometimes up to 30% of bread converted into wastage. According to a lot of bread consumes in Iran, this amount is considerable[8]. Iran is the second Largest consumer and the third abuser of bread in world [9]. In this study, the ability of five strains of bacillus spp to break down starch and convert it to mannitol was studied. The results of this study were indicated that Bacillus subtilis was the optimum bacterial strain producer amylase enzyme after 50h of incubation. Fungal amylases have been widely used for the preparation of oriental foods. In spite of the wide distribution of amylases, microbial sources namely fungal and bacterial amylases are used for the industrial production due to cost effectiveness, consistency, less time and space required for production and ease of process modification and optimization. Among bacteria, Bacillus spp is widely used for α–amylase production to meet the industrial needs and In food industry, the production of glucose from starch has been replaced by acid hydrolysis method [3]. According to research carried out by Saha in 2003, Bacillus subtilis is one of the most important bacterium producing enzyme amylase [10]. In the next stage, Leuconostoc mesentroides PTCC 1059 was used to study the production of mannitol from glucose and fructose. Studies showed that in a culture medium that there were glucose and fructose simultaneously for producing monnitol leuconostoc spp and lactobacillus spp species have the highest maximum cell growth [11]. Due to the fact that fructose is more expensive than glucose using glucose as a source of sugar in the production of mannitol, will be more economic [7]. Several heterofermentative lactic acid bacteria belonging to the genera leuconostoc and lactobacillus produce mannitol from glucose and fructose [2]. Leuconostoc mesentroides PTCC 1059 spp has shown the ability of producing 4.8g/l mannitol from 50g fructose [1]. In the other words, this bacteria converted 0.1g fructose present in the culture medium to mannitol at $37^{\circ C}$ after 50 h of incubation Studies indicated that Lactobacillus intermedius NRRL B-3693 spp by using 300g of fructose in PH- controlled and at $37^{\circ C}$ can produce 198g mannitol [5]. Saha (2006) investigated the production of mannitol by using molasses as a carbon source. L.intermedius NRRL B-3693 specie produced mannitol (104 gl−1) from a mixture of molasses and fructose syrup (1:1; total sugars, $150gl^{-1}$; fructose/glucose, 4:1) [4].

In the presence of fructose, Leuconostoc mesentroides are able to produce mannitol and also ferment carbohydrates to equimolar amounts of lactate, carbon dioxide and ethanol. Leuconostoc is one of the most abundant lactic acid bacteris found in plants and vegetables and the natural inhabitant of this acid is on the surface of plants [7].

Leuconostoc mesenteroides NRRL B-1149 spp during fermentation at $28^{\circ C}$, 5% fructose was used in batch culture fermentation; the yield of mannitol was 78%.

Leu. mesenteroides ATCC-9135 and Leu. pseudomesenteroides ATCC-12291 used fructose and produced sequencely 86% and 91% mannitol [1].

The ability to produce mannitol from complex carbohydrates such as starch that composed of the main sugar of bread wastes was very lower than fructose. These amounts, 0.15 and 0.2 g/l were obtained from lavash and baguette bread, respectively.

CONCLUSION

Due to the presence of bread wastes in Iran, production of mannitol from bread wastes by microbial fermentation is not only having economic value, but also can reduce environmental losses.

ETHICAL ISSUES

Ethical issues including plagiarism double publication and/or submission, redundancy, etc. have been completely observed by the authors.

COMPETING OF INTEREST

The authors have declared that no competing interest exists.

AUTHORS' CONTRIBUTIONS

All the authors made an equal participation.

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