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Effect of Various Parameters of Carbon and Nitrogen Sources and Environmental Conditions on the Growth of *Lactobacillus Casei* in the Production of Lactic Acid

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ABSTRACT
Lactic acid is widely used in food and pharmaceutical industries and is produced by two biological (fermentative processes) and synthetic methods. Due to the production capacity of isomer L(+), the highest global production of lactic acid is done by fermentation. Lactobacillus casei strain was used in this study that is the homofermentative bacteria producing L(+)-lactic acid. Two nitrogen sources of yeast extract and corn steep liquor and two sugar sources of sucrose and molasses at temperatures of 37 Cº, 40 Cº and 42 Cº as well as two pHs 5 and 7 were used in the study. The results showed that higher lactic acid is produced by increasing the amount of yeast extract compared to the use of corn steep liquor. Moreover, Lactobacillus casei using sucrose produced more lactic acid than the molasses. The optimal temperature for lactic acid produced by the bacterium was 37 Cº in these experiments. In addition, it was found that Lactobacillus casei could produce more lactic acid at pH = 5 than at pH = 7, and the production of the acid reached the maximum value at pH = 5. In optimum conditions, 60/90g/L Lactic acid was obtained after 24 hours incubation of samples (pH = 5 and 37 Cº).
Keywords: (+) L-lactic acid, Lactobacillus casei, corn steep liquor, yeast extract, molasses.

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INTRODUCTION

Lactobacillus microorganisms are known as the favorite residents of digestive tract. Lactic acid is the only or main product of the microorganisms, by which they can reinforce an acidic environment. These conditions are not suitable for many pathogenic bacteria in digestive tract and thus destroy the bacteria [1]. The bacteria obtain their required energy through metabolism of sugars during the fermentation process, and produce lactic acid as the main and final product [2]. Lactic acid is widely used as a flavoring and preservative in food, pharmaceutical, leather and textile industries. The acid is also polymerized into a biodegradable poly lactic acid (PLA) that is used in the manufacture of sutures to close wounds as well as prostheses in the pharmaceutical industry. In addition, it is applied to make products with chemical base [3]. In today's industry, this acid is produced both as fermentative and chemical form. With today's improvement in fermentative processes, fermentative production of lactic acid is 90% and its chemical production is only 10% [4].

Lactobacillus casei is a strain of lactic acid bacteria with a remarkable phenotype and variabile genotype having a variety of bacterial colonies, some of which are present in the human digestive duct. Homofermentative L. casei can produce L(+)-lactic acid. Lactic acid, which is found in both optically active form of D(-) and L(+), is produced by a chemical reaction through hydrolysis of lactonitriles and microbial fermentation processes [5]. One of the features of the industrial microorganisms to produce lactic acid is their ability of quick and complete fermentation of cheap raw materials, the need for a minimum amount of nitrogen material and high yield of production [6].

Nabi Bidhendi and Bani Ardalan [7] studied continuous and discontinuous production of lactic acid from whey using immobilized lactobacillus, compared the use of wooden laminates, the use of brick and glass with adsorption and eggshell with glutaraldehyde with covalent bonding and observed that wood can have the maximum amount of adsorption and is the best stabilizer for production. Parmjit *et al*. [8] explored whey fermentation and production of L(+)-lactic acid by *Lactobacillus casei*. The effects of various parameters such as pH, medium, temperature, inoculation, age of inoculation, mixing and incubation time were examined on the increase of lactose into lactic acid. Optimization of vaporization conditions resulted in the reduced fermentation time and increased conversion rate of lactose into lactic acid.

Chooklin *et al*. [4] investigated the palm syrup fermentation by *Lactobacillus casei* TISTR 1500 as the microorganism producing lactic acid. They observed that physical and chemical properties determine the amount of lactic acid production, and total palm syrup concentrate, dry cell weight and lactic acid increase by increasing sugar. Lactobacillus has complex food requirements because they are microorganisms that can have a limited bioproductivity and cannot make the growth factors needed by their body. They cannot just grow on the carbon source and mineral salts of nitrogen.

Therefore, they need a different set of amino acids and vitamins. The growth factors are usually provided by nitrogen sources such as yeast extract, peptone and ammonium sulfate. In particular, yeast extract has the greatest effect due to the presence of purines, pyrimidine and vitamins B [9, 10, 6]. According to all the scientific resources available, this study tried to optimize suitable culture medium to produce a high concentration of the bacterium and the resultant metabolites (Typically, lactic acid) by considering the minimum facilities at a laboratory scale so that the results could be used in the food and pharmaceutical systems.

MATERIALS AND METHODS

Microstructures: *Lactobacillus casei* donated by the Laboratory of Pharmacy faculty, Tehran University was used in this study.

Medium: in order to select a suitable sugar source, 100cc ingredients of the medium MRS (except for the sugar source) were first prepared in a 250cc Erlenmeyer flask in six separate flasks, then the studied sugars (sucrose, and then sugar beet molasses) were added to the two mixes. Three flasks were set to pH 7, the remaining flasks were set to pH 5 by adding some hydrochloric acid (HCl), and then an equal amount of the intended bacterium (*Lactobacillus casei*) was added to each. Flasks were incubated at temperatures 37 Cº, 40 Cº and 42 Cº with the stirring speed of 150 rpm. To determine the effect of different nitrogen sources, 100cc media were prepared in the 250cc flask with the exception that the two nitrogen sources of yeast extract (YE) and corn steep liquor (CSL) (0.2% yeast extract and the remaining corn steep liquor were added to the medium) replaced with nitrogen sources of MRS medium. The provided media were sampled at intervals of 8, 12 and 24 hours.

Measurement of final lactic acid: the exact amount of lactic acid was measured based on the chromatography methods using HPLC. Thus, it was necessary to obtain a standard curve of lactic acid after defining the method properties. Therefore, various concentration samples were prepared from pure lactic acid and 20 microliters of it were injected into the device. Of course, three injections of each concentration were conducted on the intraday and between three different days in order to consider the coefficient of intraday and between days variations. After obtaining the standard curve of lactic acid, test samples were injected that included injecting 20 ml of the freshly prepared supernatant (normal test), the supernatant plus volumetric 1% of lactic acid (test 1%) and the supernatant plus volumetric 2% of lactic acid (test 2%). Each of the test samples were injected three times into the device. Lactic acid was added to the supernatant with the aim of the preparation of internal standard so that the existence of lactic acid could be ensured by increasing the height and area under the intended peak curve in the test sample. The concentration of lactic acid in the supernatant was obtained after obtaining areas under the curve and height of the test sample and data compliance with the standard curve.

Experimental design: repeated measures design was used to evaluate the effects of time, source, temperature and pH on lactic acid with the within group factor of time and between group factors of source, temperature and pH in this study. Bonferroni post hoc test was used to detect significant differences for mutually between different levels of time. As well as to detect significant differences between different levels. Moreover, Tukey post hoc test was used to determine significant differences between different levels of source and temperature. SPSS 16 was used to analyze data, and statistical significance was considered at 0.05.

RESULTS AND DISCUSSION

Source of nitrogen

The nitrogen source is the most important factor affecting for Lactobacillus growth [11]. However, high levels of nitrogen in the extract can cause cell death [12]. Examining Figures 1, 2 and 3, it can be seen that maximum production of lactic acid in three samplings was obtained by adding raw material of yeast extract. The results indicate that the maximum production of lactic acid is 60/90g/L in the comparison between the two nitrogen sources of yeast extract and corn steep liquor that is related to yeast extract at a temperature of 37 °C and pH = 5. As seen, yeast extract had the highest production of lactic acid after 8 hours, 12 hours and 24 hours of incubation of media. Due to the high yield production of lactic acid by *Lactobacillus casei* using the raw material of the yeast extract compared to corn steep liquor, in all media conditions studied and with regard to the fact that the better treatment with the production of 60.90g/L lactic acid was associated with an increase in the raw material, it could be found that since yeast extract is one of the raw materials necessary for the growth of the microorganism and the best source of nitrogen for growth and lactic acid production, decrease in the use of the yeast extract and replacement of the nitrogen source with corn steep liquor would result in a significant reduction in lactic acid production. Hujanen *et al*. [9] also provided yeast extract and corn steep liquor as nitrogen sources with *Lactobacillus casei* and observed that the bacterium could use both nitrogen sources and produce lactic acid. However, evaluation of the results reported in these researchers research indicated that production of lactic acid by *Lactobacillus casei* in the same medium conditions using the nitrogen source of yeast extract was more than that of the condition when corn steep liquor was provided as nitrogen source with the bacterium. Our results is consistent with these findings. Furthermore, *Lactobacillus casei* production was increased from the beginning of the fermentation to 24 afterward by both nitrogen sources of corn steep liquor and yeast extract that is compatible with the results obtained in our study [9].

Carbohydrate source

As a sugar source, molasses was compared with sucrose and the effect of each on the lactic acid produced by *Lactobacillus casei* was studied. Table 1 shows the results. As seen, maximum production of lactic acid is 35/53g/L that is related to the sugar sucrose in the medium after 24 hours of incubation at 37 C° and initial pH 5. The process of increasing the amount of lactic acid produced by sucrose compared to molasses was observed in three samplings (8 hours, 12 hours and 24 hours) and in all conditions ranging from pH = 7 and pH = 5 as well as at temperatures of 37, 40, 42 C°.

After studying and observing the results obtained in the test medium conditions, it can be found that lactic acid production in media containing sucrose was significantly higher than that of when molasses was used by *Lactobacillus casei*. Since sucrose is simpler than molasses, it could be concluded that the more simpler the sugar available the bacteria, the more ability the microorganism will have to use it and produce lactic acid.

Chooklin *et al*. [4] studied the level of lactic acid using the raw material of palm produced by *Lactobacillus casei* and observed that lactic acid production increases within 24 hours of the onset of the activity of bacteria. Moreover, they could observe that *Lactobacillus casei* using glucose had the maximum production of lactic acid. The researchers concluded that maximum production of lactic acid is related to the use of glucose, fructose and sucrose, respectively. The results indicated that the microorganism used simple sugars easier and produced more lactic acid. This result is compatible with our results [4].

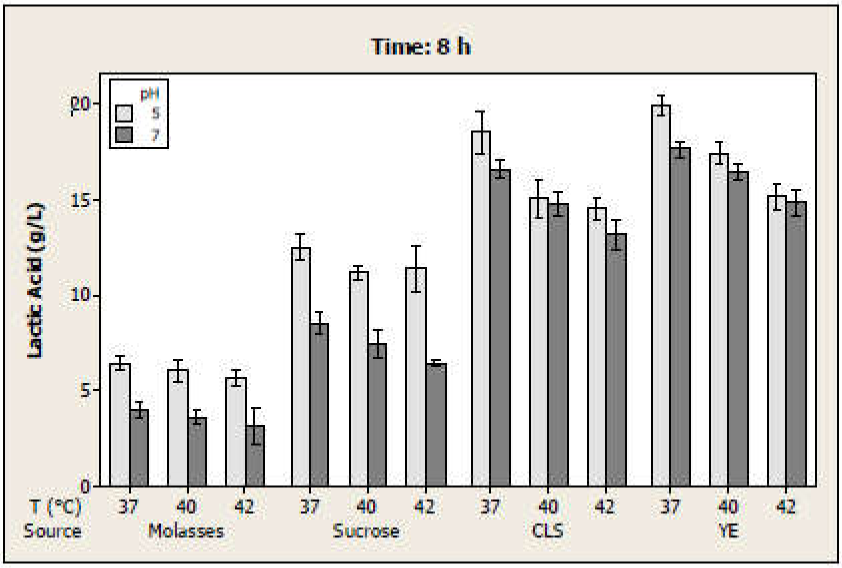

Figure 1. Comparison of lactic acid produced by *Lactobacillus casei* in nitrogen sources of yeast extract and corn steep liquor and sugar sources of sucrose and molasses after 8 hours of bacterial growth

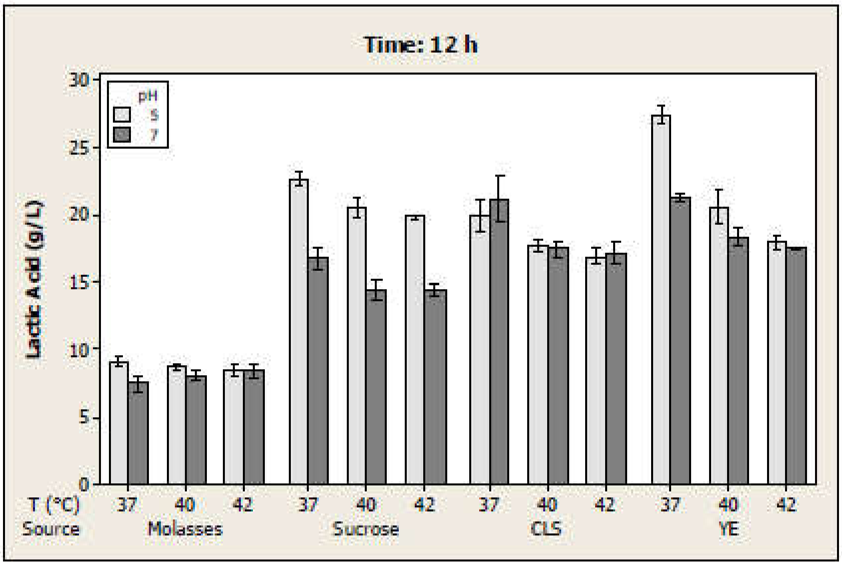

Figure 2. Comparison of lactic acid produced by *Lactobacillus casei* in nitrogen sources of yeast extract and corn steep liquor and sugar sources of sucrose and molasses after 12 hours of bacterial growth

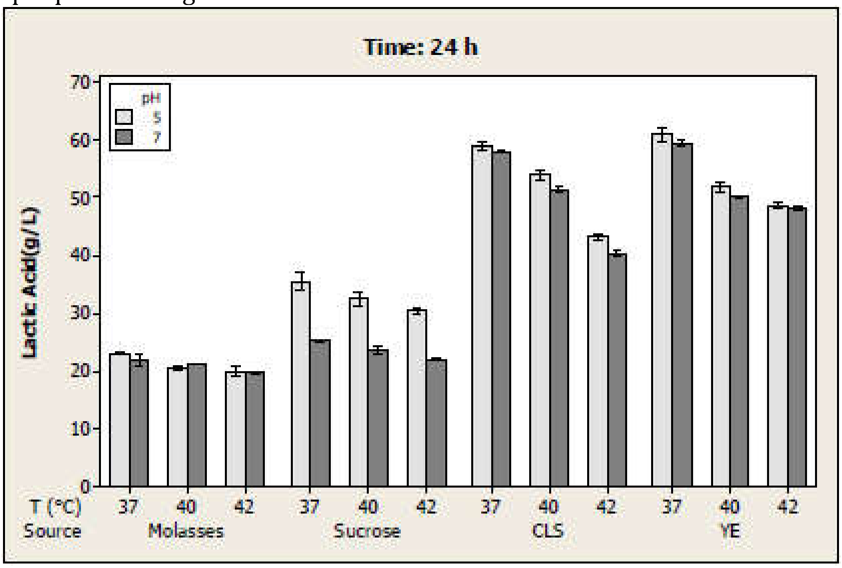

Figure 1. Comparison of lactic acid produced by *Lactobacillus casei* in nitrogen sources of yeast extract and corn steep liquor and sugar sources of sucrose and molasses after 24 hours of bacterial growth

Temperature and pH

After analyzing the results from the lactic acid produced by *Lactobacillus casei* at temperatures of 37, 40 and 42 C°, it was seen that the maximum production of lactic acid was at 37 C° and acid production decreases with increasing temperature. As seen in Figures 4, 5 and 6, and this decreased process with the raw material of corn steep liquor is 58/93g/L, 54/03g/L and 43/36g/L after 24 hours at pH = 5 and temperatures of 37, 40 and 42 C°. Among the two pHs used in this study, maximum production of lactic acid was at pH = 5. For example, the production of lactic acid using the nitrogen source of yeast extract at 42 C°, pH = 7 and pH = 5 and equals 48/16g/L and 48/70g/L, respectively.

In the case of better treatments related to the use of the raw material of yeast extract and temperature of 37 C°, the production of lactic acid with pH = 7 and pH = 5 equals 59/43g/L and 60/90g/L, respectively. Hujanen *et al*. [9] also provided yeast extract and corn steep liquor as nitrogen sources with bacteria. and observed that the bacterium could use both nitrogen sources and produce lactic acid. Using statistical method of surface response, they found optimum temperature around 35 C° as the optimum temperature to produce the greatest amount of acid produced by the above-mentioned bacteria. Moreover, Qi and Yao [13] reported the optimum temperature of 37 C° [9, 13]. Rincon *et al*. [14] also reported the optimum pH of 4.5 that indicates acidophility of the microorganism. This is very close to the optimized pH obtained in our study.

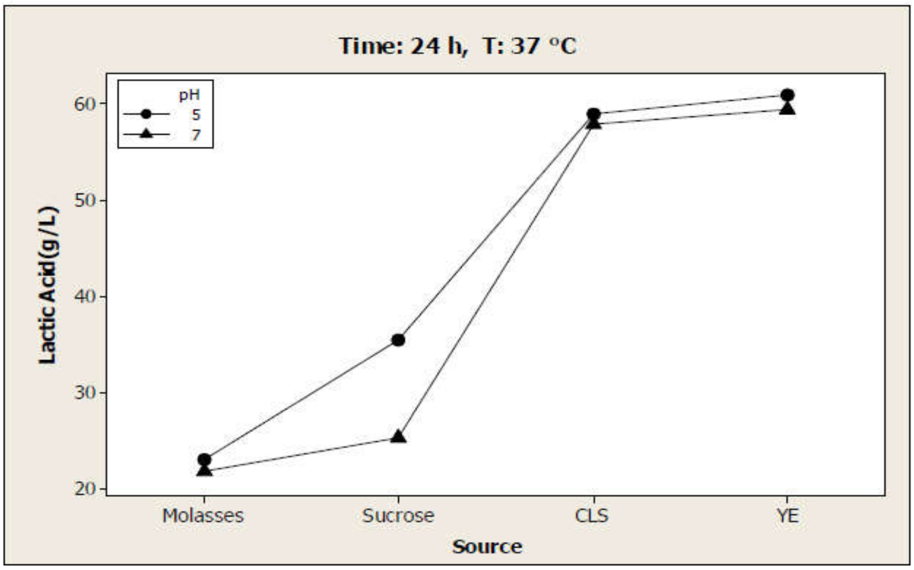

Table 4. The lactic acid produced by *Lactobacillus casei* after 24 hours of incubation at 37 C˚ and pH 5 and 7

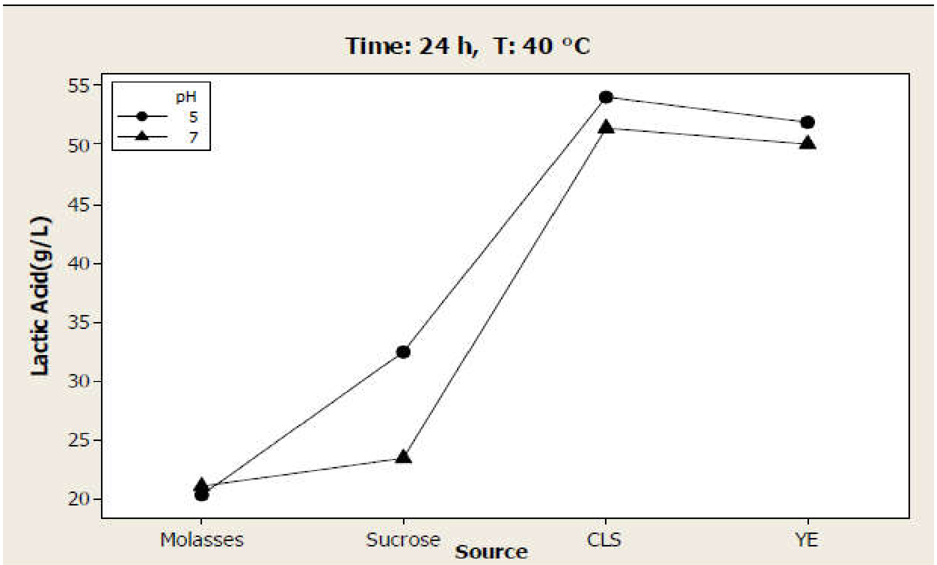

Table 5. The lactic acid produced by *Lactobacillus casei* after 24 hours of incubation at 40C˚ and pH 5 and 7

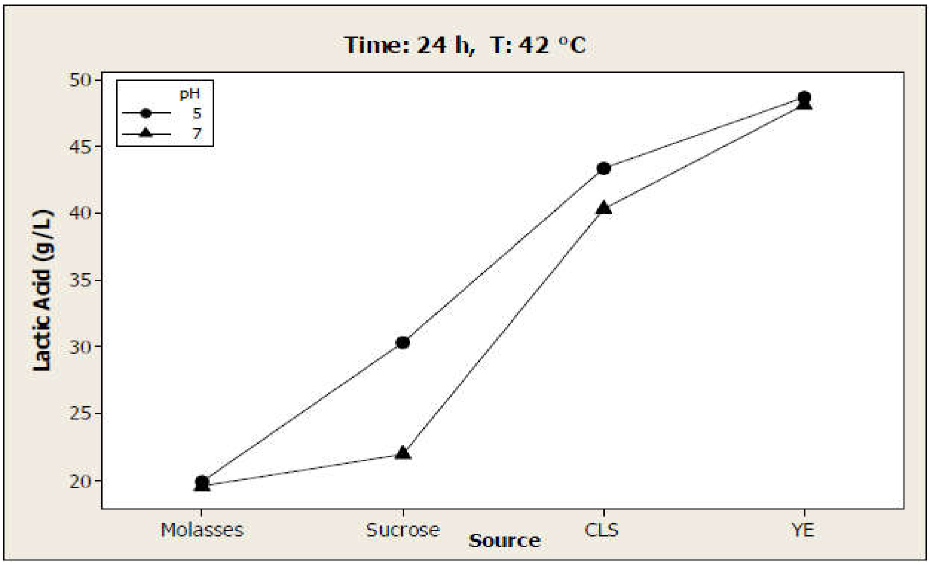

Table 6. Lactic acid produced by *Lactobacillus casei* after 24 hours of incubation at 42C˚ and pH 5 and 7

CONCLUSION

These research findings showed that the best source of sugar from the two sources tested for the growth of the bacterium *Lactobacillus casei* and lactic acid produced by the bacterium is the sugar source of sucrose. Since other studies in this area also represent a better use of sugar sources as our study, it could be concluded that *Lactobacillus casei* consumed the simple sugars better. The bacteria using yeast extract could produce more Lactic acid compared to the existence of nitrogen source of corn steep liquor in the medium that results from the raw materials necessary for the growth of microorganism in yeast extract. *Lactobacillus casei* at temperature 37C° and pH = 5 could produce the highest lactic acid. It should be noted that although there was no significant difference in the amount of lactic acid produced by *Lactobacillus casei* when using high levels of yeast extract compared to its decrease and replacement with corn steep liquor, with regard to the significant price difference between the two nitrogen sources and given that more lactic acid was produced by corn steep liquor, economic production could be expected by increasing the amount of corn steep liquor and declining the amount of yeast extract. Furthermore, in the case of sugar sources used in the study, although there was a significant difference in the amount of lactic acid produced by *Lactobacillus casei* after using sucrose and molasses, replacement of molasses with sucrose could be justified with regard to appropriate production of lactic acid by molasses and a lower price than sucrose.

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