Utilization of The Extract of Pulp of Carob for Lactic Acid Production by *Streptococcus thermophilus*

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Research Article

Abstract

Lactic acid is considered as a very important chemical compound with significant applications in pharmaceuticals, cosmetics and especially in the food industry. New applications, such as degradable plastics made from poly (lactic) acid, have the potential to greatly expand the market for lactic acid, if more economical processes could be developed. Industrial processes for the production of lactic acid typically use sucrose from cane and beet sugar, whey containing lactose and maltose and dextrose from hydrolyzed starch. The pulp of carob (*Ceratonia siliqua* L.) pods contains high contents of sugar (sucrose, fructose and glucose) and can be employed as a raw material for the production of lactic acid. The aim of the present study consists in the optimization of physicochemical parameters (temperature and pH) , and also the culture medium for producing lactic acid by *Streptococcus thermophilus* on the carob extract based medium. The optimum pH and temperature for bioactive metabolite production were 6.6 and 41°C respectively. Highest lactic acid production (29.95 g/L) was found when the strain was inoculated into the medium amended with Tween 80 at the concentration of 1%. Carob extract could serve as a good potential source of raw materials for the efficient production of lactic acid by *Streptococcus thermophilus*.

Keywords: Carob extract; Lactic acid production; S*treptococcus thermophilus;* Optimization.

1. Introduction

Lactic acid, also known as milk acid, is the most widely occurring carboxylic acid in nature [1]. It is considered to be one of the most useful chemicals, used in the food industry as a preservative, acidulant, and flavorings, in the textile and pharmaceutical industries, and in the chemical industry as a raw material for the production of lactate ester, propylene glycol, 2,3-pentanedione, propanoic acid, acrylic acid, and acetaldehyde [2]. In the cosmetic industry, lactic acid is used in the manufacture of hygiene and esthetic products, owing to its moisturizing, antimicrobial and rejuvenating effects on the skin, as well as of oral hygiene products [3]. It can be obtained either by the action of fermentative microorganisms or chemical synthesis [4]. Approximately 90% of the total lactic acid produced worldwide is by bacterial fermentation and the rest is produced synthetically by hydrolysis of lactonitrile [5]. Chemical synthesis from petrochemical resources always results in a racemic mixture of D/L-lactic acid, which is a major disadvantage of this approach, while microbial lactic acid fermentation offers an advantage in terms of utilization of renewable carbohydrate biomass, low production temperature, low energy consumption, and the production of optically high pure lactic acid [6].

The biotechnological production of lactic acid has received a significant amount of interest recently, since it offers an alternative way to prevent environmental pollution caused by the petrochemical industry and the limited supply of petrochemical resources [7]. In recent years research effort has focused on looking for new and effective nutritional sources and new progressive fermentation techniques enabling the achievement of both high substrate conversion rates and high production yields [8]. A number of different substrates have been used for the biotechnological production of lactic acid, including glucose, sucrose, lactose, maltose, mannose, xylose and galactose [9]. Raw material cost is one of the major factors in economic production of organic acids. Biological production offers significant advantages over chemical synthesis due to the fact that biological production can use cheap raw materials which mainly include plants [10]. Till date various agro industrial wastes such as whey, molasses, starch

waste, beet, cane sugar, cassava, bagasse, apple pomace, soybean, potato residue, pine apple waste, wheat bran, kiwi fruit peel, and citric pulp have been used for the production of organic acids [11].

The carob tree (*Ceratonia siliqua* L.) belonging to the family Cesalpiniaceae sub family of the family Leguminoseae, is widely used in the Mediterranean regions cultivated for ornamental and industrial purposes. It produces edible pods used as a fodder for breeding cattle; it has also a long history of application as a source of health products [12]. Carob pulp is high (40-56%) in total sugar content (mainly sucrose, glucose, fructose and maltose) [13], which can be low cost substrate for the production of lactic acid. The aim of the present study was to optimize lactic acid production by *Streptococcus thermophilus* grown in Carob extract.

3. Materials and Methods

3.1 Microorganism

Streptococcus thermophilus strains were obtained from GIPLAIT Dairy unit of Mascara city (North West of Algeria). It is an important starter for the dairy industry. It is used in combination with *Lactobacillus delbrueckii* subsp. *bulgaricus* for the production of yogurt. It is also used for the manufacture of cheeses in which high cooking temperatures are applied [14]. The strain of *Streptoccus thermophilus* is homofermentative and produces lactic acid from glucose fermentation [15].

3.2 Sample collection and extraction procedure

Samples of carob were collected during April-May, in 2014; from Mostaganem city North West of Algeria. Carob are washed and destoned and grounded. 2.5 liters of hot water were added to 1 kg of carob, homogenized and through a cloth. The extract obtained was centrifuged at 15.000 rpm for 10 minutes. The collected supernatant was used as culture medium. The extract is fixed in a pH between 6-6.5 and sterilized during 20 minutes at 120°C.

3.3 Growth experiments

Batch cultures of *Streptococcus thermophilus* were performed using 250 ml of the carob extract in Erlenmeyer flasks. To study the influence of different factors on bacterial growth and lactic acid production. The effect of the initial pH of the medium was studied at 41°C with pH 5.0, 5.3, 5.6, 5.8, 6.2, 6.6, 6.9 and 7.2, the pH being adjusted with 5M NaOH. The effect of temperature was studied at 36, 39, 41, 43, 45 and 48°C.

3.4 Analytical methods

Total sugars concentrations were analyzed by the Dubois method using phenol and sulphuric acid [16]. Lactic acid concentrations were evaluated by acidity titration with NaOH [17]. The measurement of the optical density (OD) at 620 nm was used to the biomass determination.

3.5 Kinetic of growth

We take off every two hours until 24 hours 10 ml of medium of fermentation. We make a reading in a spectrophotometer to a length of wave 620 nm.

3.6 Data processing

The calculation of the kinetic parameters of fermentations µmax (h^{-1}), Qsmax (g/g.h) and QALmax (g/g.h) has been performed using KALEIDAGRAPH software (Release 4.0.3 for windows, Synergy Software).

4. Results and Discussion

4.1 Effect of temperature on growth of *Streptococcus thermophilus* and lactic acid production

Table 1 and Figures 1 and 2 show the effects of temperature on the growth of *Streptococcus thermophilus* and lactic acid production. *Streptococcus thermophilus* was grown in M17 at 36, 39, 41, 43, 45 and 48°C, while the pH was kept constant at 6.5. As shown in the tab.1 and Figures 1 and 2 the optimum temperature for lactic acid production was 41°C, yielding 22.5 g/L of lactic acid at 15 h of fermentation. At 36, 43, 45, 48°C, lactic acid production was 6.45, 11.2, 21.6, 5.55 g/L respectively (Figure 1).

The follow up study of the biomass production of *Streptococcus thermophilus* by measuring the optical density at 620 nm, incubated at a temperature of 43°C, started at an initial OD of 0.31 after 20 hours fermentation, showed that biomass reached a maximum value of 4.56. This increase in biomass is stimulated by the consumption of an amount of sugar 36.54 g/L. However, the biomass production of *Streptococcus thermophilus*, incubated at temperatures of 35, 40, 43, 45 and 48°C gave 2.55, 2.67, 3.29 and 3.03 respectively (Figure 2).

On the basis of the obtained results, we can conclude that the maximum of biomass production of *Streptococcus thermophilus* is 2.1. Several authors like [18,19] showed that a strain is acidifying if the pH reduction equal at least 0.5 units within four hours [20]. Reported that the optimal temperature for the growth of *Streptococcus thermophilus* is 42°C. The maximal growth rate and the lactic acid production obtained after 15 hours of incubation at 36°C are 0.19 h^{-1} and 3.65 g/g.h, respectively. The values

of μ_{max} and Qs_{max} decrease when the temperature increase to 41°C (μ_{max}=13.56 h^{-1} and ALQ_{max}=6.58
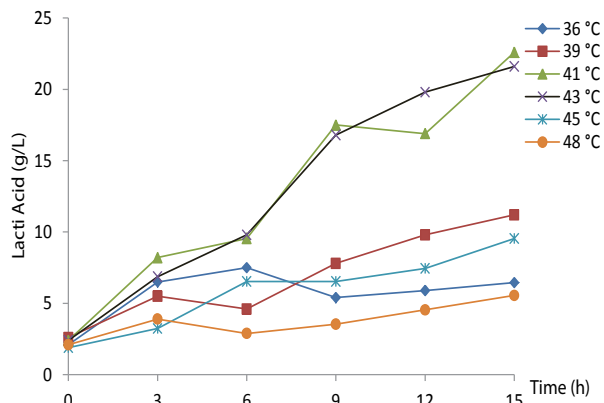

Figure 1. Evolution of lactic acid concentration with time at different temperature.
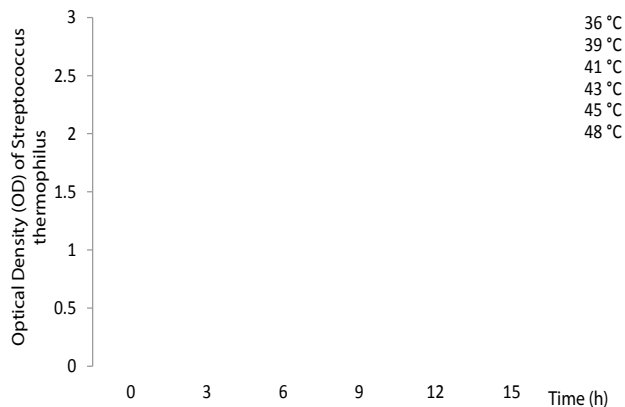

Figure 2. Biomass evolution at different temperatures.

Table 1. The specific maximal growth rate, lactic acid production and sugar consumption rate at different temperatures in *Streptococcus thermophilus.*

T°C	μ_{max} (h^{-1})	Qs_{max} (g/g.h)	Qs_{max} (g/g.h)
36°C	0.19	3.65	4.54
39°C	1.54	3.13	3.54
41°C	2.09	11.56	5.89
43°C	1.49	13.56	6.58
45°C	0.65	9.65	3.47
48°C	0.98	5.29	2.54

g/g.h) (Figures 1 and 2) (Table 1).

4.2 Effects of initial pH on lactic acid and biomass production and sugar consumption by *Streptococcus thermophiles*

The results obtained (Figure 3 and 4) showed that the bacterial biomass and lactic acid production and the sugar consumption by *Streptococcus thermophilus* are inhibited in the culture medium at a pH value of 5, 5.3 and 5.8. However, there is a considerable increase in the biomass and lactic acid levels with the elevation of the pH value. Homofermentative Streptococcus ferment sugars by the Embden-Meyerhoff-Parnas (EMP) pathway to pyruvate, which is converted into lactic acid and ATP. During the exponential phase of growth, bacteria require a lot of ATP to ensure the synthesis and production, but it this requirement decreases in the stationary phase.

At pH 6.6, *Streptococcus thermophilus* culture gave the best yield of the lactic acid and biomass productions 25.3 g/L and 2.9 respectively. This explains the optimum fermentation conditions when we add the carob extract to the culture medium.

On the basis on the obtained results, we can conclude that the lactic acid production inhibits the growth of lactic acid bacteria. However, lactic acid bacteria maintain its alkaline intracellular pH than the extracellular medium, since the cell membrane is impermeable to extracellular protons (lactate molecule) and cells secrete quickly dissociated lactic acid in the extracellular medium. The difference in pH values between the cytoplasm and the extracellular medium causes a pH gradient (ΔpH), which permit the protons excretion to the external medium. At the end of the exponential phase of growth, the amount of available ATP becomes insufficient. The pH gradient disappears and the hexokinasic activity decreases which inhibit the bacterial growth [21] (Figures 3-5) (Table 2).

The comparison of the kinetic profiles revealed that the sugar consumption rate varies with different pH. For example, the sugar consumption rates are higher in pH 6.5 than to pH6.2, the values range from 9.58 g/g.h (at pH 5.2 at 4.65) to 11.23 g/g.h for the other
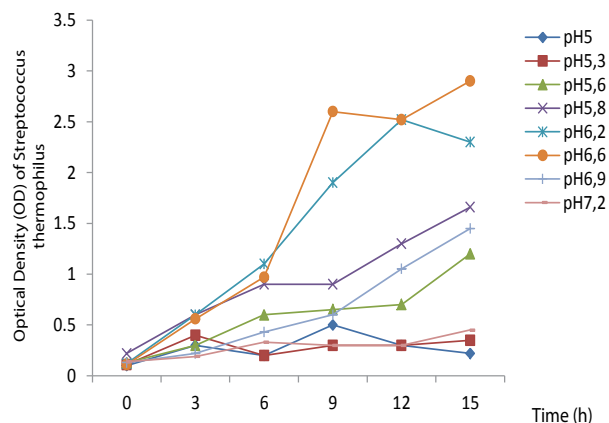

Figure 3. Biomass evolution at different pH and in 41°C.

pH (6.9 and 7.2).

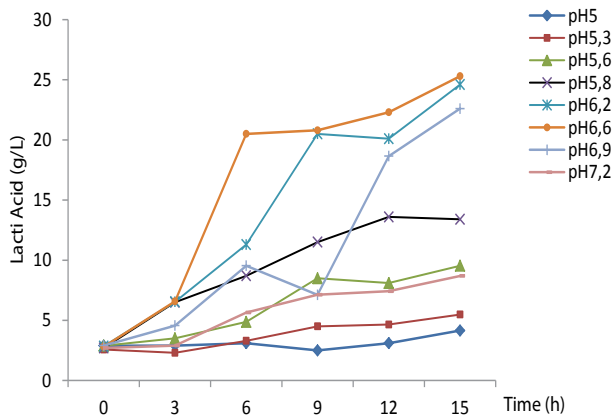### 4.3 Optimization of the culture medium parameters

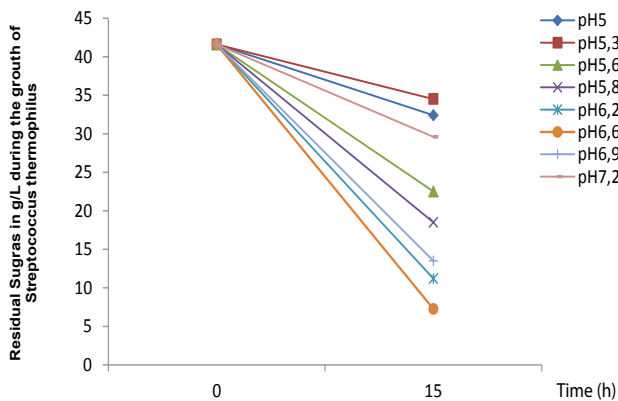**Figure 4.** Evolution of lactic acid concentration with time at different pH and at T=41°C.

Figure 5. Evolution of total sugars concentration (g/L) with time at different pH.

Table 2. Maximum specific growth speeds (μ_{max}), lactic acid production (QAL_{max}) and sugar consumption (Qs_{max}) of *Streptococcus thermophilus* at different pH values.

pH	μ_{max} (h^{-1})	Qs_{max} (g/g.h)	QAL_{max} (g/g.h)
5	0.43	11.2	1.54
5,3	0.98	12.5	1.86
5,6	1.04	6.58	2.47
5,8	0.54	5.48	3.65
6,2	1.2	9.58	4.58
6,6	0.98	4.13	6.26
6,9	0.65	4.56	5.87
7,2	0.88	11.23	3.56

Effect of Tween 80: The effect of Tween 80 on the production of the bacterial biomass and lactic acid by *Streptococcus thermophilus*, cultivated on a culture medium based on carob extract was studied. The obtained results Figures 6 and 7 revealed that the culture of *Streptococcus thermophilus* in the presence of a concentration of 0.5 g/L of Tween 80 increase significantly the lactic acid production with a rate of 16.32 g/L. After addition of 1 g/L Tween 80 and incubation of 15 hours a high level of lactic acid 27.5

g/L is obtained, but the 2 g/L of Tween 80 showed a negative effect on the biomass and lactic acid production. According to the literature, the addition of Tween 80 allows the excretion of lactic acid in the culture supernatant after the creation of pores in the cell membrane [22].

Reported that the addition of Tween 80 showed a positive effect on the biomass and lactic acid production [23]. Suggested that the addition of Tween 80 in the culture medium stimulated considerably the transfer of carbohydrates through the cell membrane and consequently the increasing of the lactic acid production (Figures 6 and 7) (Table 3). The addition
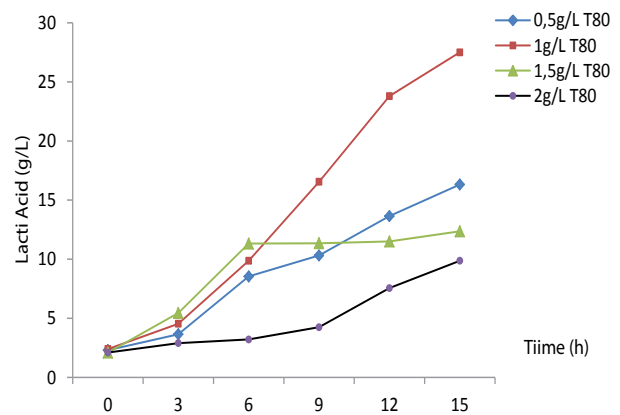

Figure 6. Evolution of lactic acid concentrations with time at different Tween 80 concentrations (T=41°C).
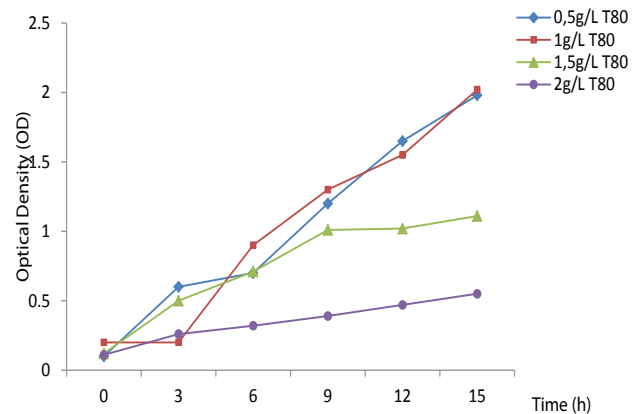

Figure 7. Evolution of biomass with time at different Tween 80 concentrations (T=41°C).

Table 3. Maximum specific growth speeds (μ_{max}), lactic acid production (QAL_{max}) and sugar consumption (Qs_{max}) of *S. thermophilus* at different Tween 80 concentrations.

Tween80	μ_{max} (h^{-1})	Qs_{max} (g/g.h)	QAL_{max} (g/g.h)
0.5 g/L	1.29	3.65	3.11
1 g/L	2.41	6.35	4.16
1.5 g/L	1.69	5.19	2.15
2 g/l	2.65	2.98	0.97

of 1g/L of was found to be most interesting to the lactic acid production which was 4.16 g/g.h for QAL max and 2.41 h^{-1} for µmax (Table 3).

Lactic acid production in the optimal conditions: The Figure 8 shows the patterns of growth (OD$_{620}$), pH, carbohydrate consumption and lactate production in the optimized medium. The *Streptococcus thermophilus* strain grows immediately without latency phase and reached the stationary phase after 14 hours of culture in the optimized environment. According to the kinetic profiles, the maximum biomass plate coincides with the consumption of the majority of the carbon substrate (sugars=7.25 g/L to 15 hours of fermentation). It also appears that the growth and lactic acid production are inhibited when the pH becomes 3.9 in the optimized medium. This is due to the strong acidity resulting from the accumulation of lactic acid [24,25], concluded that inhibition may be due to carbon source exhaustion.
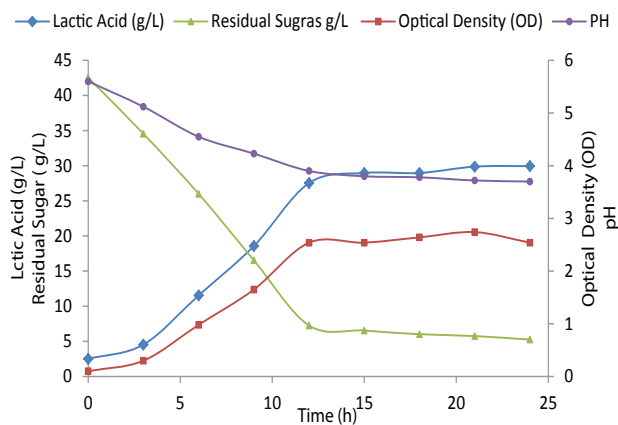

Figure 8. Evolution of the optical density, pH, lactic acid concentrations and total sugars with time in optimal conditions (pH 6.6, T80=1g/L and T=41°C).

But in our work, it is not the case, as the concentration of sugars consumed equal 37.29 g/L, so it remains in the optimized culture medium 5.25 g/L of the sugars not consumed by the bacteria (Figure 8).

Growth kinetics and lactic acid production: In order to understand and interpret properly biological phenomena and specifically the growth kinetics and production of lactic acid, the obtained experimental results are expressed with a mathematical model using "Kaleidagraph" software, which can indicate the growth or acidification variables by equations depending on time and comprising a variable number of parameters with no biological significance.

Growth kinetics: The growth kinetics of *Streptococcus thermophilus* (Figure 9) showed that the obtained curves adjusted by the optimization program and test concentrations are very close,

which manifest a square error well minimized by the optimization function with models describing although the dynamic behavior of microorganisms. Therefore, the adjustment of the bacterial growth is expressed by the following quartic function: $Y=0.089-0.043+0.048x^2-0.003x^3+5.741x^4$. With regard to the percentage of explanation of the model (R), we can see in the figure that we have obtained very good values of R=0.994. This means that the polynomial models identified from experiments allow predicting the variable values of responses in the explored area (Figure 9).

Kinetics of lactic acid production: The kinetics of the production of lactic acid illustrated in Figure 10. Shows that the adjustment has stimulated the production during all phases of growth with a very high correlation coefficient R (R=0.997). This explains that
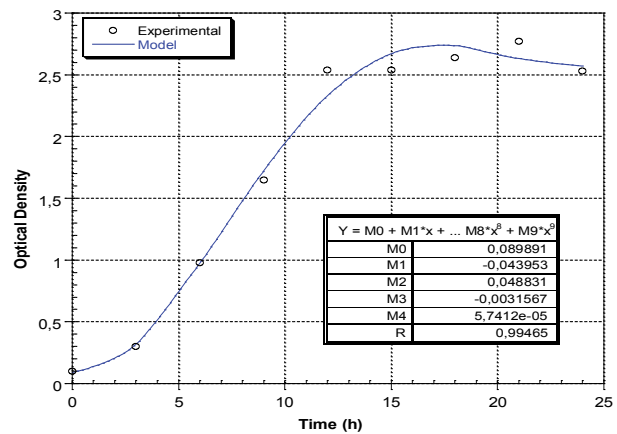

Figure 9. Comparison between the experimental values of the optical density (symbols) and the calculated values (lines) in different times.
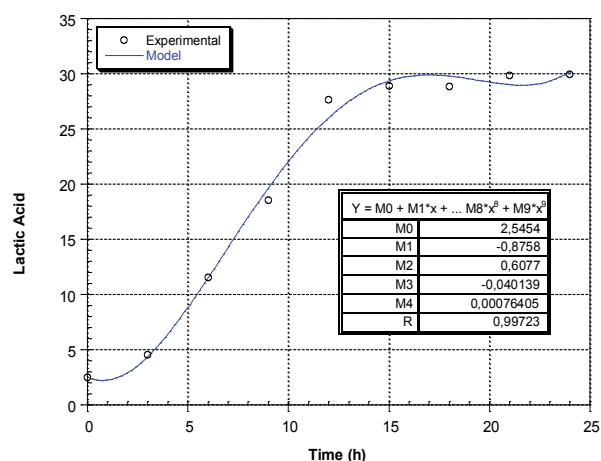

Figure 10. Comparison of experimental values of lactic acid (symbols) with the calculated values (lines) in different times.

the experimental points are in very good correlation with those calculated. Therefore, the amount of lactic acid is expressed by the cubic equation below:

$Y=2.545-0.875x+0.607x^2-0.0401x^3+0.00076x^4$ (Figure 10).

5. Conclusion

This study has shown the potential use of *Streptococuus thermophilus* for the bioconversion of Carob extract into lactic acid. Under optimized conditions, the best result for lactic acid production (29.95 g/L) was obtained after 15 hours of fermentation in optimal conditions, 41°C, pH of 6,6 and 1% of Tween80. Carob extract was proven to be an economically feasible and promising raw material for industrial production of lactic acid.

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