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Production of L(+) Lactic Acid using *Lactobacillus casei* from Whey

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ABSTRACT

The aim of this work was to study the fermentation of whey for the production of L(+) lactic acid using Lactobacillus casei. *The effect of different process parameters such as pH of the medium, temperature, inoculum size, age of inoculum, agitation and incubation time was monitored to enhance the lactose conversion in whey to L(+) lactic acid. Fermentations were performed without any pH control. The optimization of the fermentation conditions resulted in significant decrease in fermentation time, besides increase in lactose conversion to lactic acid. The optimized process conditions resulted in high lactose conversion (95.62%) to L(+) lactic acid production (33.73 g/L) after an incubation period of 36 h.*

Key words: Whey, lactic acid, lactose utilization, lactic acid bacteria, *L. casei*

INTRODUCTION

Whey, the greenish translucent liquid obtained from milk after precipitation of casein, has been viewed as one of the major disposal problems of the dairy industry, because of the high volumes produced and having a high biochemical oxygen demand (Marwaha and Kennedy, 1988; Mawson, 1994). As a general rule, about nine litres of whey is obtained for every kilogram of cheese produced. Thus, the volume of whey to be processed, originating from just one typical large scale cheese making operation can exceed 1 x 10^6 litres/day (Jelen, 2003). A dairy farm processing 100 t of milk per day produces approximately the same amount of organic products in its effluent, as would a town with 55000 residents (Sienkiewicz and Riedel, 1990).

The production of these products in large quantities leads to enormous quantities of whey as a byproduct in the dairy industries, which represents 85-95% of the milk volume and retains 55% of milk nutrients. Among the most abundant of these nutrients are lactose, soluble proteins, lipids and mineral salts (Marwaha and Kennedy, 1988; Mawson, 1994; Gonzalez-Siso, 1996). The availability of carbohydrate reservoir of lactose in whey and presence of other essential nutrients for the growth of microorganisms makes the whey one of the potential substrate for the production of different bio-products through biotechnological means. The production of lactic acid production

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through lactic acid bacteria could be an alternative processing route for whey lactose utilization.

Of the total lactic acid produced worldwide every year, about 90% are made by lactic acid bacterial fermentation and the rest is produced synthetically by the hydrolysis of lactonitrile (Hofvendahl and Hahn-Hagerdal, 2000). Microbial fermentation has the advantage that by choosing a strain of lactic acid bacteria (LAB) producing only one of the isomers, an optically pure product can be obtained, whereas synthetic production always results in a racemic mixture of lactic acid. The production of optically pure lactic acid is essential for the polymer synthesis in which lactic acid is used (Litchfield, 1996; Lunt, 1998). In addition, optically pure L(+) lactic acid is polymerized to a high crystal polymer suitable for fiber and oriented film production and is expected to be useful in the production of liquid crystal as well (Amass et al., 1998). Moreover, L(+) lactic acid is used by human metabolism due to the presence of L-lactate dehydrogenase and is preferred in foods as preservative as well as emulsifier (Litchfield, 1996; Jarvi's, 2001).

Presently, starch or sugar containing substances are used for the production of lactic acid. However, lactose rich dairy by-product whey can be low cost substrate for the production of lactic acid. The use of biotechnological techniques to find the suitability of whey for lactic acid production can serve dual purpose, i.e. production of valuable product, lactic acid and addressing to the whey disposal environmental pollution problem. Most of the work has been carried out on the production of D(-) and DL mixture of lactic acid. However, now a days, production of L(+) lactic acid has attracted more attention. In order to enhance the economics of the lactic acid fermentation process, it is necessary to increase the lactic acid concentration in the medium through optimization of fermentation conditions. The present work was, therefore, carried out to optimize the process conditions for efficient lactose conversion in whey to L(+) lactic acid.

MATERIALS AND METHODS

Micro-organism

Lactobacillus casei NBIMCC 1013 was procured from National Bank for Industrial Micro-organisms and Cell Cultures, Bulgaria.

Maintenance and cultivation of the culture

The bacterial culture was revived on MRS (de Mann Rogosa Sharpe) broth with pH 6.2±0.2. The process of activation of the freeze dried culture was carried out on a regular basis by transferring them after every 48 h up to three generations. The culture was maintained on MRS slopes (MRS medium supplemented with 15.0 g/L agar) by subculturing, aseptically at fortnight intervals and stored at 4°C, until further use.

Preparation of starter culture

The bacterial culture was grown in 50 mL of MRS medium in 250 mL Erlenmeyer flask. After sterilization, the medium was inoculated with a loopful of cells from agar slant and incubated at 37°C for 24 h under stationary conditions.

Fermentation medium

Whey powder was procurred from Sigma-Chemicals Company (USA) and was reconstituted (6%, w/v) with water to prepare liquid whey having lactose concentration of 4% (w/v). Whey clarification was carried through protein precipitation induced by heating the whey at 90°C for 20 min. Precipitated proteins were removed by centrifugation at 4,000 rpm for 15 min. The treated whey was supplemented with yeast extract (0.75%, w/v), manganese sulphate (20 mg/L), and calcium carbonate (1.5%, w/v). The whey medium was sterilized at 121°C for 20 min. The fermentation medium prepared in this way was used for the production of lactic acid using *Lactobacillus* cells.

Optimization of process parameters

Different process parameters such as pH, inoculum age, inoculum size, temperature, agitation, and incubation period were optimized by varying the respective parameters to enhance lactose utilization and lactic acid production from whey medium.

Analytical techniques

The fermented broth was used for the determination of lactic acid and residual lactose. Lactic acid estimation was accomplished using high performance liquid chromatograph (HPLC) system following the method of Marsili et al. (1981) with little modifications. Samples were filtered through 0.20 µm membrane filters. A Bio-Rad Aminex HPX-87H column (300 x 7.8 mm)

packed with a sulphonated divinyl benzene-styrene copolymer was used for the separation of compounds. The mobile phase (0.005 M H_2SO_4), was fed at a flow rate of 0.6 mL/min and temperature was kept 50°C. The concentration of L(+)-lactate was estimated using an enzymatic kit (Boehringer Mannheim, Germany). The residual lactose concentration was estimated following the procedure of White and Kennedy (1981). All analyses were performed in triplicate and the mean values are reported.

RESULTS AND DISCUSSION

Effect of pH

The effect of pH on lactic acid production was evaluated by using fermentation medium having a pH range of 5.0-6.8 (Figs. 1 and 2). The maximum lactose conversion (95%, w/v) and lactic acid production (33.48 g/L) was observed at pH 6.5.

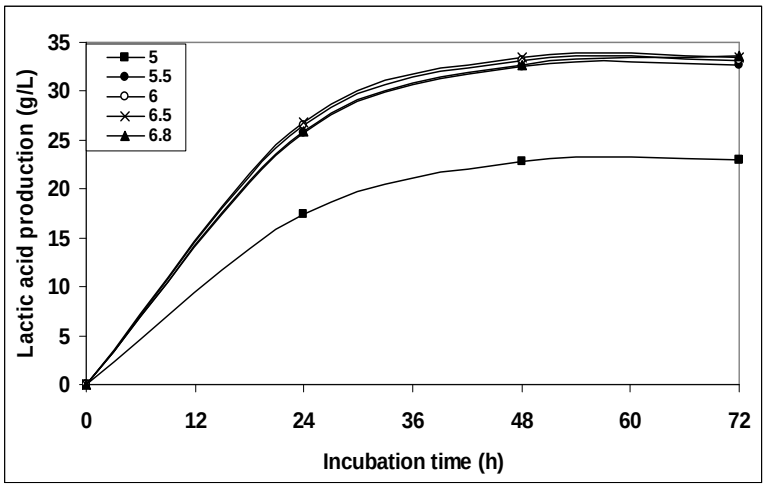

Figure 1 - Lactic acid production in whey by *L. casei* with pH as a function

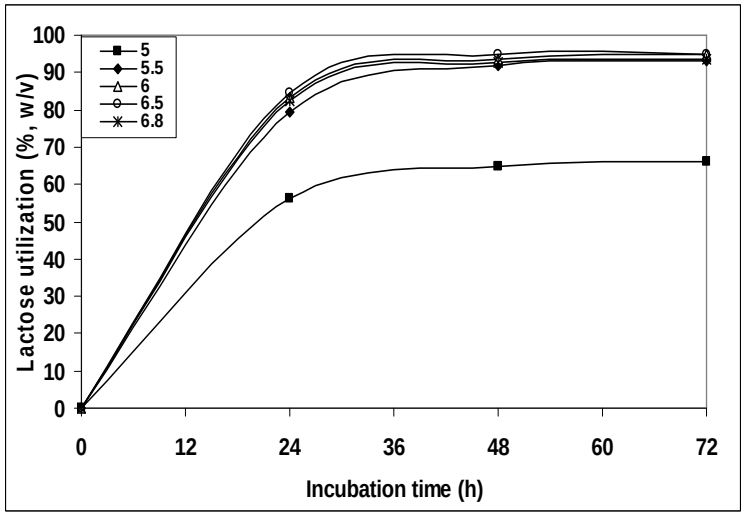

Figure 2 - Lactose utilization in whey by *L. casei* with pH as a function

However, at higher and lower pH levels, a decrease in the both the function was observed, with insignificant decrease at pH 6.0 and 6.8. A pH range of 6.0-6.5 has been reported optimal for lactic acid production using *L. casei* strain (Krischke et al., 1991). However, pH 5.5 has been used for lactic acid production using *L. helveticus* by Ghaly et al. (2004).

The hydrogen ion concentration of medium has the maximum influence on the microbial growth. The pH affects at least two aspects of microbial cells, i.e. functioning of its enzymes and the transport of nutrients into the cell. It limits the synthesis of metabolic enzymes responsible for the synthesis of new protoplasm. The pH values also affect the RNA and protein synthesis. When micro-organisms are grown on either side of their optimum pH range, there may be an increased lag phase.

From the above observations, a pH 6.5 was considered optimal for maximum lactic acid production. In the subsequent experiments, the pH of the fermentation medium was adjusted to 6.5.

Effect of inoculum age

To find the effect of inoculum age on lactic acid production, whey medium was inoculated with 16-28 h old cultures. An increase in the lactose utilization and lactic acid production was observed when bacterial culture of 16-20 h old was used (Fig. 3). The maximum lactose utilization and lactic acid production of 95.62% (w/v) and 33.71 g/L, respectively was observed with 20 h old bacterial culture. Insignificant decrease in these functions was observed with 24 h old culture. However, suppression in both the functions was observed, when 28 h old growth was used. The low lactose conversion with inoculum age of 16 h could be attributed to the fact that bacterial culture might have not yet entered in log phase of growth. The maximum lactose conversion observed with inoculum of 20 h, could be due to the exponential phase of the bacterial culture used as an inoculum. A 20 h old culture of *L. helveticus* for lactic acid production has been used by Roy et al. (1986). However, Gandhi et al. (2000) used 24 h old culture of *Lactobacillus* cultures for lactic acid production. The use of 24 h old culture of *L. casei* has also been reported for lactic acid production (Krischke et al., 1991).

Since, 20 h bacterial culture displayed maximum lactic acid production, it was selected for further studies.

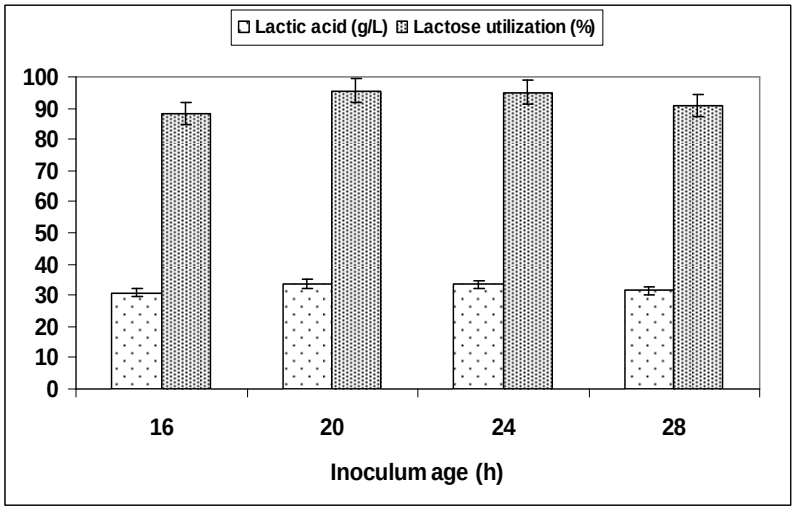

Figure 3 - Lactose conversion to lactic acid by *L. casei* in whey with inoculum age as a function. Bars indicate the standard deviation from triplicate determinations.

Effect of inoculum size

To study the influence of inoculum size on the lactic acid production, different inoculum levels (1-5%, v/v) were added to the fermentation medium (Fig. 4). The lactose utilization and lactic acid production increased with the increase in inoculum size up to 2% (v/v), thereafter no improvement in both the functions was observed. The maximum lactic acid production of 33.72 g/L was observed with 2-4% (v/v) inoculum of bacterial culture. The low lactic acid production at 1% (v/v) inoculum level could be attributed to the low density of starter culture.

The use of 2% (v/v) inoculum for the lactic acid production has been reported in earlier studies also (Roy et al., 1986; Gandhi et al., 2000). However, the higher inoculum (3%, v/v) has also been used for lactic acid production (Chiarini et al., 1992).

From the above observations, an inoculum of 2-4% (v/v) could be considered optimal for achieving maximum lactic acid production using 20 h old bacterial culture, however, keeping in view the economics of the process, 2% (v/v) inoculum size was used in the subsequent studies.

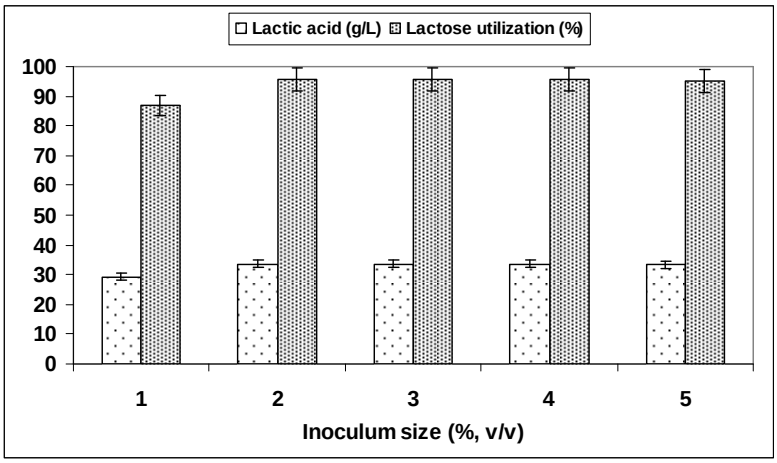

Figure 4 - Lactose conversion to lactic acid by *L. casei* in whey with inoculum size as a function. Bars indicate the standard deviation from triplicate determinations.

Effect of temperature

To find the optimum temperature for lactic acid production, whey medium after inoculation was incubated at a temperature range of 30-45°C. The lactose utilization and lactic acid production increased with increase in the temperature up to 37°C; however, an insignificant decrease in the both the functions was found at 40°C (Fig. 5). Other tested temperatures displayed low values of lactose utilization and lactic acid production. The maximum lactic acid production of 33.72 g/L was observed at 37°C.

The temperature is also one of the important factors, which influences the activity of metabolic/cell enzymes. Enzymes are most active at optimum temperature and enzymatic reaction proceeds at maximum rate. However, below and above optimal temperature reaction rate is decreased which causes the problems in cell metabolism.

The optimal temperature for growth of lactic acid bacteria varies between the genera from 20 to 45°C (Wood et al., 1995; Dicks et al., 1995). In fermentations using *L. delbrueckii*, and *L. bulgaricus* a temperature of 45°C, or higher may be maintained (Buchta, 1983). *L. helveticus*, and *L. acidophilus* can be used in a temperature range of 37-45°C. Krischke et al. (1991) used 37°C temperature for lactic acid production using *L. casei*. However, a temperature of 28°C has also been reported optimal for *L. casei* in a separate study (Nabi et al., 2004).

From the above observations, a temperature range of 37-40°C was considered optimal for lactose conversion to lactic acid using bacterial cells; however, a temperature of 37°C was selected for further experimentation.

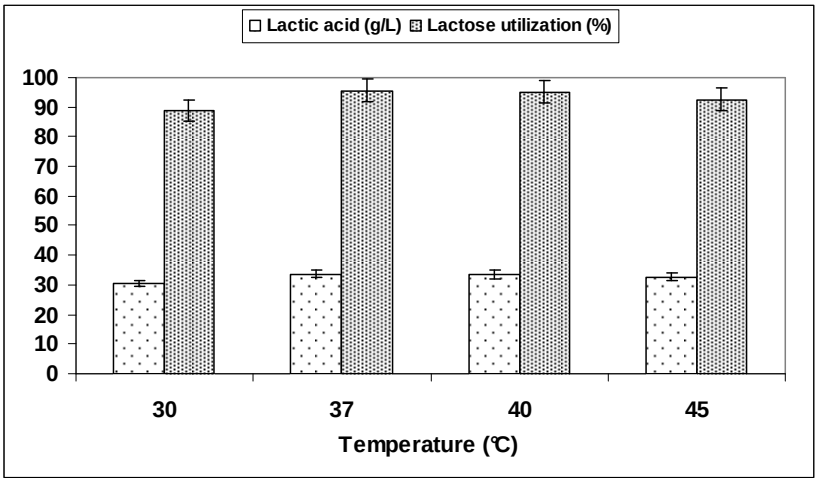

Figure 5 - Lactose conversion to lactic acid by *L. casei* in whey with temperature as a function. Bars indicate the standard deviation from triplicate determinations

Effect of agitation

To study the effect of agitation on lactic acid production by the bacterial culture, the cultivation was carried under stationary condition (control) in a biological oxygen demand incubator and shaking condition (100 rpm) on an orbital shaker (Fig. 6). The agitation mode of cultivation did not support any increase in lactose conversion as compared to the culture maintained under stationary condition, which could be attributed to the microaerophilic nature of the bacteria.

The earlier studies have also supported the stationery mode for lactic acid production. Gandhi et al. (2000) used stationary conditions for the lactic acid production using different lactobacilli cultures (*L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. casei* etc.). However, stationary conditions for growth and agitation mode for fermentation have also been reported in some earlier studies (Roy et al., 1986).

Since, no difference was observed for lactic acid production with agitation, stationary mode was selected in further investigations.

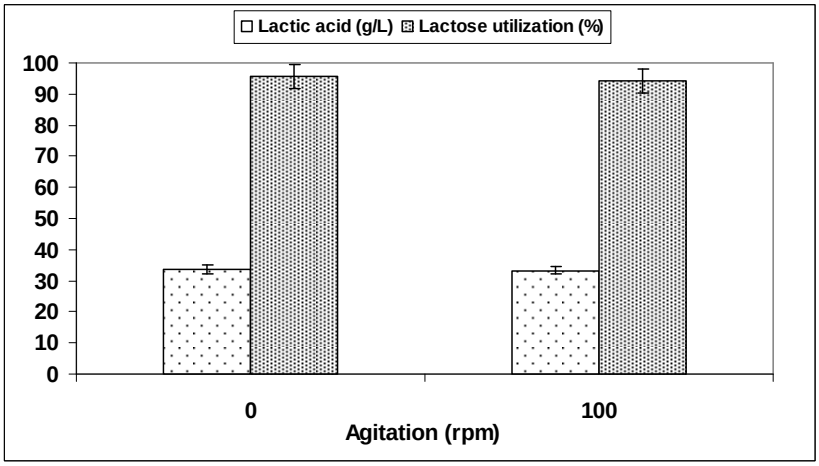

Figure 6 - Lactose conversion to lactic acid by *L. casei* in whey with agitation as a function. Bars indicate the standard deviation from triplicate determinations.

Effect of incubation period

To find out the optimal incubation time for the maximal lactose utilization and lactic acid production, the whey medium inoculated with bacterial culture was incubated for 48 h under the above optimized conditions. The samples were drawn at specified time intervals and the results obtained are presented in Fig. 7. As evident from the results, an increase in lactose utilization and subsequent lactic acid production was found up to 36 h and thereafter no improvement in both the functions was observed. This could be attributed to the growth of the culture reached to the stationary phase and as a consequence of metabolism, micro-organisms continuously change the characteristics of the medium and the environment. A maximum lactic acid production of 33.73 g/L with lactose utilization of 95.62% (w/v) was observed after 36 h of incubation. The reduction in fermentation period is additionally advantageous to improve the economics of the process. Therefore, an incubation time of 36 h was considered optimal for maximum lactose conversion to lactic acid.

The incubation period of 48 h has been generally used for lactic acid production using different lactobacilli cultures (Chiarni et al., 1992; Gandhi et al., 2000; Kumar et al., 2001). Thus, reduction in the fermentation period along with high lactose conversion to L(+) lactic acid are the advantages of the developed process.

From the observations made during the process optimization studies, it could be concluded that maximum lactose conversion to lactic acid was obtained with the process conditions of pH 6.5, temperature 37°C and inoculum size 2% (v/v) of 20 h old culture under stationary conditions with an incubation of 36 h. The different optimal conditions reported by various workers for maximum lactic acid production could be explained by the differences in the nature of the strains and medium composition used in their studies. The above optimized process parameters can be used in scale up studies in further investigations.

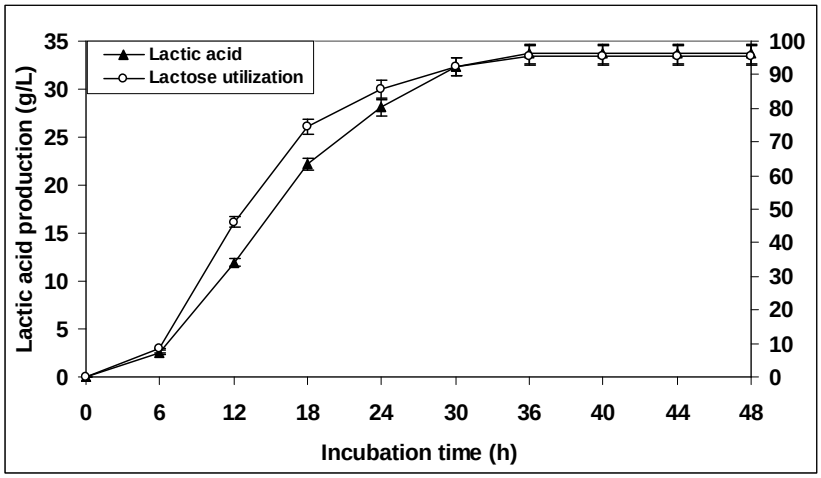

Figure 7 - Lactose conversion to lactic acid by *L. casei* in whey with incubation period as a function. Bars indicate the standard deviation from triplicate determinations.

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