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LACTIC ACID PRODUCTION BY MIXED CULTURES OF *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* GROWN ON DEPROTEINIZED WHEY

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ABSTRACT

Lactic acid is considered to be a very important organic acid, which used in different food, dairy and other pharmaceutical industries. Therefore, the aim of this work focused on the production of lactic acid with free and co-immobilized mixed cultures which revealed that: starch hydrolyzate medium gave the highest lactic acid concentration. However, deproteinized whey was found in the third order of lactic acid production. Deproteinized whey was used for further studies as a by-product from cheese manufacture. 96 hours and 10% yeast extract induced the production of lactic acid. The increase of lactic acid production in the fermentation media, as end product, repressed the production of lactic acid. 37°C was found as the optimum temperature for lactic acid production. Lactic acid production was increased gradually by increasing of inoculum size up to 3.0% (v/v, inoculum size to the production media). Higher production of lactic acid was obtained by co-immobilized mixed culture of *L. delbrueckii* subsp. *bulgaricus* and *S. saliverius* subsp. *thermophilus*. Increasing the incubation time of co-immobilized mixed cultures, increased the development of lactic acid concentration up to the end of fermentation time.

Keywords: Lactic acid production, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus salivarius* subsp. *thermophilus*, Co-immobilized-mixed culture, Deproteinized whey.

INTRODUCTION

Lactic acid (2-hydroxypropionic acid) is one of the most widely used organic acids. It is considered to be a very important chemical compound with significant application in the pharmaceutical, cosmetics and, especially, in the food industries (including meat, fish and cereal application, dairy products and confectionery). New application of lactic acid such as biocompatible plastics made from polyacetate, have the greatest potential for expanding the market of lactic acid. Polyacetate is becoming important as an intermediate material to produce biodegradable polyester, which form a plastic with good tensile strength, thermoplasticity, fabricability and biodegradability material. Also, lactic acid can be used as a foodstock for the chemical and biochemical production of other organic acids such as acetic, propionic acid. On the other hand, ammonium lactate is an excellent source of non-proteic nitrogen for feed rations (Abdel-Naby & Lee, 1992; Demirci *et al.*, 1993; Norton *et al.*, 1994; Parajo *et al.*, 1997; Payot *et al.*, 1999 and Omar, 2003).

Production of lactic acid is currently carried out by either chemical or biotechnological methods. During, the past few years, the fermentative route to lactic acid has increased its participation in the overall market from 50% up to 65%. For food applications, the products obtained by biotechnological producers are the preferred ones by both industry and consumers, because its "natural" origin avoides the introduction of "synthetic" products in the food chain (Chahal, 1989; Parajo *et al.*, 1997 and Omar, 2003).

Lactic acid bacteria are an important group of industrial starters cultures, applied in the production of fermented foods like yoghurt, cheese, dry sausage, sauerkraut and sauerdough. Also, these bacteria (*Streptococcus*, *Pediococcus*, *Lactococcus* and *Lactobacillus*) are widely used to produce great amount of lactic acid using sugars as carbon source (Parajo *et al.*, 1997). Abdel-Naby & Lee (1992) and Demirci *et al.* (1993) have reported the utilization of hydrolyzates from starchy substrates for the same purpose, but these substrates are directly utilizable as foods. The production of lactic acid from whey permeate has been reported by using *Lactobacillus casei*, *Lactococcus lactis*, *Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* showing high rate of released 22 to 27 g/L (Roukas & Kotzekidou, 1996; Reinheimer *et al.*, 1997 and Champagne & Gardner, 2001). Also, the fermentative production of lactic acid can be carried out by using raw materials containing starch, cellulose and lignocellulosic substances, since these polysaccharides can be hydrolyzed to give glucose solutions. Lactic acid bacteria were used for converting glucose and other intermediate materials into lactic acid (Parajo *et al.*, 1997; Chakraborty & Dutta, 1999 and Sunhoon *et al.*, 2000).

Therefore, this work deals with the production of lactic acid from deproteinized whey by using free and encapsulated cells of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*. Optimal conditions for this organic acid production were also evaluated.

MATERIALS AND METHODS

Microorganisms and culture conditions:

Lactobacillus delbrueckii subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* were kindly obtained from Dairy Dept., Fac. of Agric., Mansoura Univ., Mansoura, Egypt. These strains were propagated at 37°C in MRS broth and the inocula were obtained from cultures cultivated in 200 ml of MRS broth for 48 hours. The inocula contained 1.4×10^{10} cfu/ml of *Lactobacillus delbrueckii* subsp. *bulgaricus* and 1.3×10^{10} cfu/ml of *Streptococcus salivarius* subsp. *thermophilus*. The pH of broth was adjusted to 6.0 with 1.0 N NaOH.

***Media used for* lactic acid production:**

1- Enzymatic starch hydrolyzate medium Abdel-Naby and Lee (1992) basal medium:

The medium was used for lactic acid production with batch culture technique. The medium consisted of (g/L): 5.0 yeast extract; 2.0 succinic

acid; 0.2 K_2HPO_4, 0.2 KH_2PO_4; 0.6 $MgSO_4.7H_2O$; 0.03 $MnSO_4.H_2O$; 25 enzymatic hydrolyzate of starch (as reducing sugars). The pH was adjusted to 6.5 before autoclaving.

Preparation for enzymatic hydrolyzate, glucoamylase was supplied from Sigma Chemical Co. St. Louis, USA. One unit of enzyme activity was defined as the amount of enzyme, which liberates one µmole of glucose per min.

Enzymatic hydrolysis:

100 ml of 10% soluble starch in 0.1 M phosphate buffer pH 6.5 was incubated with 1000 units of glucoamylase in an incubator shaker (150 rpm) at 50°C for 24 h. The hydrolyzed was filtered off and immediately heated at 100°C in a boiling water both for 5 min and stored at 4°C until using (Abdel-Naby and Lee, 1992).

2-MRS broth:

The de Man, Rogasa and Sharpe (MRS) broth was used for lactic acid production, which consists of (g/L), 20 glucose, 10 peptone, 10 beef extract, 5 yeast extract, 5 sodium acetate, 2 sodium citrate, 0.2 K_2HPO_4, 0.058 $MgSO_4.7H_2O$, 0.12 $MnSO_4.H_2O$, 1 ml tween 80 and 1000 ml distilled water, pH 6.5 (20 g agar for MRS agar slants used for maintenance the bacterial strains.

3- Deproteinzed whey medium:

Whey obtained by coagulating unsalted milk with rennet was obtained from unit of Dairy Products, Dairy Dept., Fac. Agric., Mansoura Univ., Mansoura, Egypt. It contained 5% (w/v) lactose and had a pH of 6.0. Protein precipitation was induced by heating the whey at 90°C for 20 min. Precipitated proteins were removed by centrifugation at 4000 g for 15 min. The pH of the supernatant was adjusted to 6.5 with 0.5 N NaOH, and the medium was sterilized at 121°C for 20 min. After cooling, the whey was supplemented with 0.2% yeast extract, 0.02% peptone, 0.04% $MgSO_4.7H_2O$, 0.007% $MnSO_4.4H_2O$ and 2.0% $CaCO_3$. The solutions of nutrients were sterilized separately (Roukas and Katzekidou, 1996).

Fermentation conditions:

The fermentation was carried out in 250 ml Erlenmeyer flasks, each containing 50 ml from each medium. The inoculation was performed by the addition of 2.0% free cell (1:1) of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* and incubated at 37°C for 24 hours under stationary conditions.

Co-immobilized mixed cultures were performed by the addition of 10 g of gel beads to the culture medium.

Gel beads were prepared as follows: 59% (w/v) sterile aliginic acid sodium salt solution (Sigma, A-2033), then extruded drop by drop with a peristatic pump into a sterile 2.0% $CaCl_2$ solution at room temperature with continuous stirring (Roukas and Kotzekidou, 1996).

Analytical techniques:

The concentration of viable cells entrapped in Ca-alignate beads was determined by dissolving six beads in 10 ml of 0.3 M sodium citrate for 20

min with continuous stirring. The number of cells liberated from the gels was determined by plate counting, using MRS agar medium (Roukas and Kotzekidou, 1996).

Lactic acid concentration was calculated according to the method described by Lawrence (1975).

RESULTS AND DISCUSSION

The suitability of enzymatic hydrolysate of starch, MRS broth and deproteinized whey for production of lactic acid was investigated. The mixed cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* were cultivated in the basal medium for 48 h under static conditions. The data in Table (1) show that the mixed cultures were able to produce lactic acid in the fermentation media contained starch hydrolyzate or MRS broth and/or deproteinized whey. Since this polysaccharide (starch hydrolyzate) can be hydrolyzed to give glucose solutions, which can be used to form lactic acid. Therefore, starch hydrolyzate induced the productivity of lactic acid, which produced the highest amount of this organic acid. At the same time, deproteinized whey enhance the productivity of lactic acid and come in the third order. MRS medium was found in the second one for lactic acid production. The results also revealed that all three media induced the productivity of lactic acid, but the difference of lactic acid production between these media are negligible. Starch hydrolyzates were used as substrate for lactic acid production are directly utilizable as food. Therefore, deproteinized whey as a by-product in cheese manufacture was used for further studies to produce lactic acid as an important preservative material in fermented milks and other food industries. Highest amount of lactic acid was produced by lactic acid bacteria grown on deproteinized whey was reported by Nortcn *et al.* (1994); Roukas & Kotzekidou (1996 & 1998).

Table (1): Effect of different substrates on lactic acid production by mixed cultures of *L. delbrueckii* subsp. *bulgaricus* and *S. salivarius* subsp. *thermophilus*.

Substrate	Lactic acid concentration (g/L)
Starch hydrolyzate	14.8
MRS broth	13.9
Deproteinized Whey	12.6

Effect of time-course on lactic acid production:

The obtained results on the effect of time-course on lactic acid production by the tested mixed cultures are illustrated in Fig. (1). The results showed that lactic acid formation was detected in the beginning of the fermentation time and increased gradually up to 96 hours, thereafter, lactic acid production decreased. Lactic acid productivity reached its maximum (20.6 g/L) after 96 hours. These results also revealed that there is a relationship between lactic acid production and length of the fermentation time up to 96 hours. Similar results were reported by Hujanen *et al.* (2001) and Omar (2003).

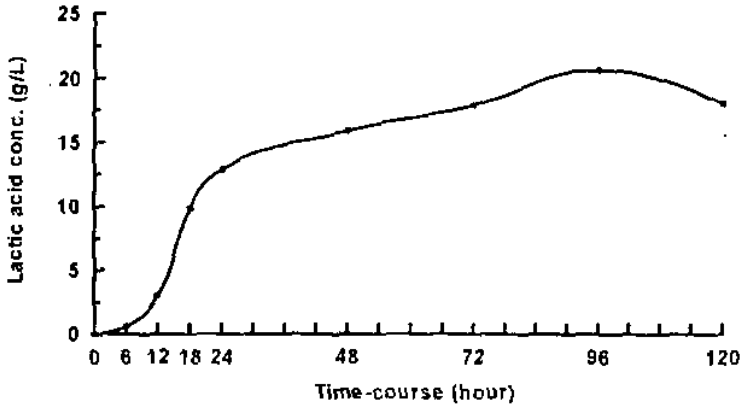

Fig. (1): Effect of time-course on lactic acid production by mixed culture of lactic acid bacteria.

Effect of supplementation of media with different nitrogenous sources on lactic acid production:

To study the effect of different nitrogenous sources on lactic acid production, the production media was supplemented with different nitrogen sources as well as different concentration of yeast extract. The results in Table (2) show that casein, meat extract and peptone decreased the production of lactic acid. Yeast extract was found as the best favorable nitrogen source. All concentrations of yeast extract used induced the productivity of lactic acid, but with different extent. The variation of lactic acid production between 10, 12.5 and 15% yeast extract was negligible, therefore, 10% yeast extract was chosen for further studies as the best nitrogen source. Higher levels showed negligible improvement of lactic acid concentration. Abdel-Naby & Lee (1992); Shahbaz *et al.* (1996) and Krishnan *et al.* (1998) reported similar results.

Effect of the end product:

The results illustrated in Fig. (2) show that the addition of lactic acid to the production media as the end product inhibited the productivity of lactic acid through the fermentation. Also, the results showed that the increasing of lactic acid added to the production media decreased the production of lactic acid and the inhibitory effect reached about 92% with level of 30 g/L lactic

acid added. It could be conclude that the level of lactic acid corresponding to a pH 3.9-3.8 critically limited lactic acid production. These results are in accordance with the findings reported by Gatje & Gotteschalk (1991) and Abdel-Naby & Lee (1992).

Table (2): Effect of supplementation production media with different nitrogen source on the production of lactic acid by mixed cultures of *L. delbrueckii subsp. bulgaricus* and *S. salivarius* subsp. *thermophilus*.

Nitrogen source	Lactic acid concentration (g/L)
Casein 5.0%	
Meat extract 5.0%	
Peptone 5.0%	
Yeast extract	
5.0%	
7.5%	
10.0%	
12.5%	
15.0%	
Control (basal medium)	

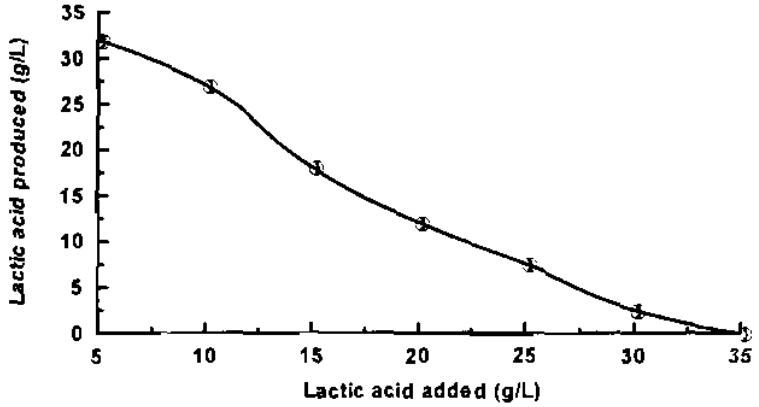

Fig. (2): Effect of lactic acid concentration in the culture medium on lactic acid productivity.

Effect of incubation temperature:

It is shown from the results illustrated in Fig. (3) that the incubation temperature greatly affected the production of lactic acid. The development of lactic acid increased greatly up to 37°C, then declined with much more amount. 37°C was found as the optimum temperature for lactic acid production. Above or below, acid production was repressed. Cultivation above 40°C greatly inhibited the formation of acid, however, complete

inhibition was observed at 50°C. These results are in agreement with those reported by Hujanen & Linko (1996) and Hujanen *et al.* (2001).

Effect of inoculum size:

The results in Fig. (4) show that lactic acid concentration increased greatly with the increase of inoculum size up to 3.0%, then lactic acid increased slightly. This means that the increasing of inoculum size up to 3.0% enhanced the development of lactic acid. The increase of inoculum size above 3.0% resulted in competitive nutrition between the enumeration of inoculum size, thus, the increase of the lactic acid formation was slight. Therefore, 3.0% inoculum size was found as the optimum inoculum size used for lactic acid production. Omar (2003) reported similar results.

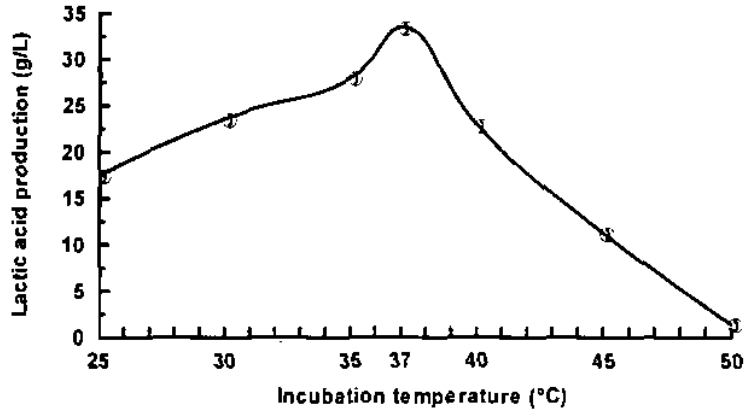

Fig. (3): Effect of incubation temperature on lactic acid production.

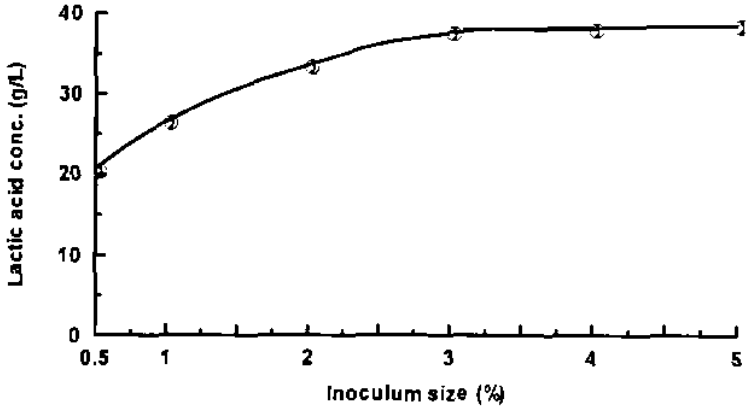

Fig. (4): Effect of different inoculum size on lactic acid production.

Lactic acid production by co-immobilized cultures:

Results in Table (3) showed the production of lactic acid from deproteinized whey by co-immobilized cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* in Ca-

alginate beads. The results showed that co-immobilized cultures differed considerably in their capacity to produce lactic acid, compared with the free cells (Table 1). Highest concentration of lactic acid was produced with co-immobilized cells. Development of lactic acid also increased gradually with the prolongation of fermentation time. Maximum formation of lactic acid was observed at the end of fermentation time. The high ability of co-immobilized cells to produce lactic acid might be due to the protection of cells by immobilized matrix. Abdel-Naby & Lee (1992) and Roukas & Kotzekidou (1996 & 1998) reported that the immobilization of cultures and co-immobilization produced maximum concentration of lactic acid compared with other free ones.

Table (3): Effect of time-course on lactic acid production by co-immobilized mixed cultures.

Time (hours)	Lactic acid concentration (g/L)
0.00	0.0
6	2.4
12	5.3
18	9.7
24	14.4
48	33.9
72	40.5
96	51.6
120	57.2

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إنتاج حمض اللاكتيك بالمزارع المختلطة من *Lactobacillus delubrueckii* subsp. *bulgaricus* و *Streptococcus salivarius* subsp. *thermophilus* المنماة على الشرش منزوع البروتين

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نظرا لأهمية حمض اللاكتيك كأحد أهم الأحماض العضوية إستخداما فى مجال الصناعات الغذائية والألبان وكذلك الصناعات الغذائية والصيدلانية وصناعة مستحضرات التجميل وغيرها فقد هدفت الدراسة فى هذا البحث لإستخدام مزارع مختلطة والتى تستخدم فى إعداد الألبان المتخمرة وذلك لإنتاج حمض اللاكتيك بيولوجيا وذلك بتنمية هذه المزارع على بيئة الشرش منزوع البروتين كمنتج ثانوى من صناعة الجبن الدمياطى وذلك لتحويله إلى منتج إقتصادى هام مثال حمض اللاكتيك ، وقد أوضحت الدراسة النتائج التالية :

١- بيئة النشا المتحلل أعطت أعلى كمية من الحامض وقد حث الشرش المنزوع البروتين على إنتاج حمض اللاكتيك ولكنه جاء فى المرتبة الثالثة من حيث الإنتاج ولن إستخدم فى التجارب التالية نظرا لأنه مخلف عديم الفائدة سوف يحول إلى منتج إقتصادى هام هو حامض اللاكتيك .
٢- ٩٦ ساعة تحضين كانت هى المدة المثالية لإنتاج حمض اللاكتيك بدرجة عالية .
٣- ١٠% من مستخلص الخميرة حث على إنتاج أعلى كمية من الحامض .
٤- زيادة حمض اللاكتيك فى بيئة التخمير كناتج نهائى كان مثبطا لإنتاج الحامض .
٥- ٣٧°م هى درجة الحرارة المثالية اللازمة لإنتاج أعلى كمية من الحامض .
٦- بزيادة حجم اللقاح المستخدم حتى ٣% (حجم/حجم – لقاح إلى حجم بيئة الإنتاج) أدى إلى زيادة كمية الحامض المنتج .
٧- لوحظ إنتاج عالى من الحامض بكبسلة الخلايا المستخدمة فى عملية الإنتاج مقارنة بإنتاج الخلايا الحرة الغير مكبسلة وقد تزايد إنتاج الحامض حتى نهاية مدة التحضين .

وفى النهاية توصى الدراسة بإمكانية إنتاج حامض اللاكتيك من الشرش المنزوع البروتين والذى يعتبر ملوث للبيئة ومن ثم يمكن تحويله إلى منتج هام غذائيا وإقتصاديا وبخاصة عند إستخدام خلايا مكبسلة من البكتيريا المستخدمة .