

## CONVERSION OF LACTOSE TO CALCIUM LACTOBIONATE BY SOME BACTERIAL SPECIES

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### ABSTRACT

Bacterial conversion of lactose to calcium lactobionate was carried out by twelve strains of bacteria. The strain *Bacillus* spp. E showed high lactose oxidizing activities, when cultivated at 30°C for 7 days in a medium (pH 6) containing (g/100ml): lactose ,5; KH<sub>2</sub>PO<sub>4</sub>,0.06 ; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.025; corn steep liquor ,0.75 ml ; 1 drop of soybean oil ; 1.5ml of 20% urea solution and CaCO<sub>3</sub>,2.5 added during inoculation. The oxidizing activity was the highest where a relatively high lactose conversion (82%) was obtained at a lactose concentration of 50g/l . The activity remained almost stable between pH 5 and 6 and high conversion efficiency ( about 90%) was achieved at pH 0.6.

**Key words:** *Bacillus* spp. E, lactose, bacterial conversion., calcium lactobionate .

### 1. INTRODUCTION

Lactobionic acid (4-O- $\alpha$ -D-glucopyranosyl-D-gluconic acid) is an oxidation product of lactose, which can be prepared efficiently by bromine oxidation or electrolytic oxidation (Miyamoto *et al.*,2000).The biochemical formation of lactobionate by microorganisms, *e.g.*, the proper strains of the genus *Pseudomonas* , has been demonstrated (Stodola and Lockwood,1947) and it has been reported that the economical and industrially viable processes provide the production of lactobionate in quantity ,in good yield, and in high purity(Maruo,*et al.*,1994). The use of glucose dehydrogenase (fructose) of *Zymomonas mobilis* for the continuous production of lactobionic acid from lactose in an ultrafiltration membrane reactor was reported(Satory *et al.*,1997). Glucose dehydrogenase (EC1.1.1.47) from *Pseudomonas graveolence* (Nishizuka and Hayaishi,1962) catalyzes oxidation of lactose ,maltose and cellobiose, producing their aldobionic- $\delta$ -lactone in the presence of an appropriate hydrogen acceptor.Biological reactions have general advantages such as high selectivity for reactions as well as substrates,high efficiency ,simple reaction systems, mild reaction conditions and avoidance of poisonous chemicals (Murakami *et al.*,2008). Lactobionic acid (lacA) has several practical potential uses such as ingredient in solutions stabilizing organs before transplanation. This is

based on the excellent metal – chelating properties of the acid that reduces oxidative damage to tissue during storage and preservation of organs caused by certain metal ions (Southard and Belzer,1995). Furthermore, as a component of a water-soluble antibiotic, erythromycin lactobionate , and a functional saccharide that promotes intestinal absorption of minerals (Hirakata *et al.*,1992 ; Murakami *et al.*, 2006a). Among these examples of practical and potential usefulness, we are interested in high aqueous solubility of the mineral salts of lacA.Two glycosyl moieties and carboxyl group enable the high solubility: calcium lactobionate dissolves at approximately 62 g/100 ml ,which is almost 10-60 times higher than calcium gluconate , lactate and citrate 28,000 times higher than calcium carbonate , an ingredient commonly used for foods and beverages. Highly soluble calcium salt, *e.g.*, calcium chloride, has bitter taste,whereas calcium bionate has no peculiar and relatively high threshold concentration (Murakami *et al.*,2008). Here, we screened a microorganism for testing its ability to oxidize lactose to lactobionic acid and describe the culture conditions for efficient lactobionic acid production by the isolated strain *Bacillus* spp. E as a potent calcium lactobionate producer.

### 2.MATERIALS AND METHODS

#### 2.1.Microorganisms and growth conditions

Different microorganisms were collected from different local habitats and others were kindly obtained from the Center of Culture of Microbiological Food Irradiation Department, National Center for Radiation Research and Technology, Nasr City, Egypt .

Those were *Pseudomonas aeruginosa*, *Ps.oleoverance* and *Murxella phenylpyruvica 118* and *Mycobacterium* spp. NRRLB-3805. In addition *Ps.* spp. were kindly obtained from the Culture Collection of Natural and Microbial Products Chemistry Department of National Research Centre. Also, *Bacillus* spp. K, *Bacillus* spp. M , *Bacillus* spp. A , *Bacillus* spp. C , *Bacillus* spp. E and *Bacillus* spp. G were isolated from honey and identified by phylogenetic neighbour-joining tree obtained by 16S rDNA sequence analysis of the tested isolated and other *Bacillus* spp. present in the gene bank database, NRC and *Ps. florescens* was kindly obtained from Navy American Medical Research Unit III ,Cairo, Egypt.

## 2.2. Stock cultures

The experimental organisms were maintained on agar slopes of the following specific medium (g/l): glucose, 10, peptone, 6, yeast extract, 3, meat extract, 1.5 and agar, 20. The pH was adjusted to 6.5. All the slant cultures were stored in a refrigerator with regular transfer every month.

## 2.3. Preparation of cell suspension and standard inocula

Two ml of cell suspension of bacteria (prepared from 7-day old slant) were allowed to grow in 250 ml Erlenmeyer flasks containing 50 ml of fermentation medium for 72 h at 30°C using reciprocal shaker (200 r.p.m.). Then 5ml (the density of the cells was  $12 \times 10^7$  spores) from the resulted cultures were used as standard inocula (otherwise stated).

## 2.4. Fermentation procedures

The organisms used were cultivated in 250 ml Erlenmeyer flasks , each containing 100 ml of a fermentation medium composed of (g/l): lactose ,100 ,  $\text{KH}_2\text{PO}_4$ , 0.6  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.25, 5ml of corn steep liquor and 3 drops of soybean oil were added to each culture as an antifoaming agent, the flasks were sterilized at 121°C for 20 min. Then inoculated with ( $12 \times 10^7$ ) spores inoculum suspension of bacterial cells (3 day old cultures) , 1ml of sterile 20% urea was added to each culture flask unless otherwise stated (Stodola and Lockwood, 1947). Flasks were agitated on a reciprocal shaker at 200 r.p.m. and 30°C for 14 days, the contents of each flask were centrifuged at

4000 r.p.m. to determine the residual lactose and calcium lactobionate contents.

## 2.5. Assay of lactose consumed

Lactose was measured by the phenol –sulfuric acid method (Dubois *et al.*, 1956).

## 2.6. Determination of lactobionic acid

### 2.6.1. Qualitative determination

Solution of samples of calcium lactobionate after passing on cation exchange resin Amberlite IR-120 (H+) and chromatographed on paper chromatography (Whatman No. 1) by descending technique with the solvent system : n-butanol : acetic acid : water (2:1:1, v/v), and detection was performed with aniline-xylose or bromocresol blue reagent (Smith and Seakins, 1976). Authentic sample of lactobionic acid was used as reference.

### 2.6.2. Quantitative determination

The contents of each flask were centrifuged. Fifteen ml of the broth were neutralized to pH 7.0 by  $\text{Ca}(\text{OH})_2$  and filtered. To each filtrate, about 1 mg of Eriochrome Black T and 2 ml of  $\text{NH}_4\text{Cl}$  buffer were added. The mixture was then titrated with standardized 0.1 M disodium salt of EDTA (Das and Nandi, 1969). The end point was indicated by faint blue color. Calcium lactobionate present in the broth was calculated from the data according to the calculation:

$0.1\text{M EDTA} \equiv 4.008 \text{ mg of calcium lactobionate}$

$\text{Ca. lactobionate (\%)} =$

$$\frac{\text{Weight of calcium lactobionate produced}}{\text{Total sugar}} \times 100$$

## 2.7. Optimization of the fermentation parameters

### 2.7.1. Time course

From inoculated flasks by *B.* spp. E (5ml =  $12 \times 10^7$  spores) containing fermentation medium and lactose as substrate , samples were collected after 3, 7, 14, 17 and 21 days of incubation for lactobionic acid determination.

### 2.7.2. Culture age

Aliquots (50ml) of the nutritive medium (Naim *et al.*, 2002) were inoculated with  $12 \times 10^7$  spores of cell suspension. Flasks were shaken (200 r.p.m.) at 30°C for 24, 48, 72 and 96 h .The fermentation process was achieved by adding the inoculum (5ml =  $12 \times 10^7$  spores, unless otherwise stated) separately added to the production medium in each flask and incubated for 7 days.

### 2.7.3. Inoculum size

Aliquots (50ml) of the production medium inoculated with different inoculum size ( 1ml of cell suspension =  $10^7$  spores, 2ml =  $3 \times 10^7$ , 3ml =  $5 \times 10^7$ , 4ml =  $8.75 \times 10^7$  and 5ml =  $12 \times 10^7$ ) of the selected *B.* spp. E.

### 2.7.4. Fermentation medium

*B. spp. E* was cultivated on four different media composed of (g/100ml) :

**2.7.4.1.** lactose ,10; KH<sub>2</sub>PO<sub>4</sub>,0.06; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.025; corn steep liquor, 0.5 ml ; 1 drop of soybean oil ; 1ml of 20% urea and CaCO<sub>3</sub>,2.5 added during inoculation (Stodola and Lockwood, 1947).

**2.7.4.2.** lactose,7 ; polypeptone, 0.5; dried yeast extract 0.1; NH<sub>4</sub>NO<sub>3</sub>,0.2; NaCl, 0.05; KH<sub>2</sub>PO<sub>4</sub>, 0.13;K<sub>2</sub>HPO<sub>4</sub>, 0.04; MgSO<sub>4</sub> anhydrous, 0.05; FeSO<sub>4</sub>. 7H<sub>2</sub>O, 0.001; and CaCO<sub>3</sub>,1 (Kiryu *et al.*,2008).

**2.7.4.3.** ← lactose, 5;KH<sub>2</sub>PO<sub>4</sub>,0.15; MgSO<sub>4</sub> anhydrous, 0.03; Peptone, 1.5 and CaCO<sub>3</sub>,1 ( Miyamoto *et al.* ,2000 ).

**2.7.4.4.** lactose ,10; corn steep liquor, 3ml; yeast extract, 0.02 and CaCO<sub>3</sub>, 1 (Murakami *et al.*,2006b).

**2.7.5. Concentration of lactose**

The effect of various concentrations of lactose on the lactobionic acid production was investigated. The tested concentrations were 25,50,100 and 150 g/l. In all treatments, the fermentation medium was initially adjusted at pH 6.5.

**2.7.6. Concentration of urea**

The effect of different concentrations of urea (20%, w/v) on the fermentation of lactose to calcium lactobionate by *B. spp. E* was studied. The tested concentrations (ml/100ml) were 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 respectively.

**2.7.7. Concentrations of corn steep liquor**

The effect of different levels of corn steep liquor (concentration 0.4 gm/100ml) 2.5, 5.0, 7.5, 10.0 ml/l in the fermentation medium (I) on the lactobionate production was investigated .

**2.7.8. pH-value**

The effect of pH value was studied by adjusting the fermentation medium (I) before autoclaving at different pH values (4-8) using Ca(OH)<sub>2</sub> or dilute HCl (pH range 4-9).

**3.RESULTS AND DISCUSSION**

**3.1. Screening for the most active bacteria**

Twelve bacterial strains were screened for their ability to convert lactose to calcium lactobionate. Those belonging to the genera *Pseudomonas*, *Moraxella*, *Bacillus* and *Mycobacterium* could convert lactose to calcium lactobionate in the presence of calcium carbonate in the fermentation medium (I) after 14 days of fermentation (Table 1). The microorganisms belonging to the genera *Pseudomonas* (*Ps. spp.* , *Ps. fluorescens*, *Ps. aerogenosa* and *Ps. oleoverance*), all are able to convert lactose to calcium lactobionate with different yields .The highest yield of calcium lactobionate (43%) was recorded with *Ps. aerogenosa* after 14 days of fermentation processes. On the other hand, the highest level (50%) of calcium lactobionate was recorded with *B. spp. E*. *Morxella phenylpyruvica 118* and *Mycobacterium spp. NRRLB-3805* recorded the lowest yield ( 16% and 18%), respectively. Collectively, *B. spp. E* and *Ps. aerogenosa* proved to be the most promising organisms for lactose bioconversion. Nisizuka and Hayaishi (1962) reported that lactose dehydrogenase of *Ps. graveolence* especially catalyzes oxidation of lactose, maltose and cellobiose, producing their aldobionic-δ-lactone in the presence of an appropriate hydrogen acceptor.

**Table (1): Potentiality of some bacterial isolates for calcium lactobionate production.**

Bacterial isolates	Calcium lactobionate	
	(g/100ml)	Yield (%)
<i>Bacillus spp. A</i>	2.5	25
<i>B. spp. C</i>	2.5	25
<i>B. spp. E</i>	5	50
<i>B. spp. G</i>	2.1	21
<i>B. spp. K</i>	2.6	26
<i>B. spp. M</i>	2.6	26
<i>Morxella phenylpyruvica 118.</i>	1.6	16
<i>Mycobacterium sp NRRLB-3805.</i>	1.8	18
<i>Ps. aeruginosa.</i>	4.3	43
<i>Ps. fluorescens.</i>	2.1	21
<i>Ps. oleoverance</i>	2.8	28
<i>Pseudomonas spp.</i>	2.7	27

Conditions : inoculum size, 5ml; culture age,72 h; medium number (I); incubation , 14 days; shaking condition 200 r.p.m.; lactose concentration 100 g/l.

### 3.2. Optimization of bioconversion fermentation parameters

#### 3.2.1. Fermentation time course

The optimal bioconversion time of lactose markedly depends on the type of microorganism. The maximal calcium lactobionate yield (55%) was detected after 7 days using *B. spp. E*, while relatively lower yields (44 and 33%) were recorded with *Mycobacterium spp. NRRLB-3805* and *Ps. spp.* after 3 days (Table 2). On the other hand, yields of calcium lactobionate increased gradually to reach their maxima (39% and 30%) with *Ps. fluorescens* and *Ps. oleoverance* after 17 days. The present comparison assessed the superiority of *B. spp. E* as a potent microorganism, the calcium lactobionate increased and finally reached 5.5 gm/100ml (55% yield) after 7 days.

#### 3.2.2. Time course of cultivation

It was clearly evident that, the lactose oxidizing activity proved to be the enzymic reaction dominating at all the growth phases (24-96 h) of *B. spp. E*. However, comparatively higher yield of calcium lactobionate (72%) was formed in the stationary phase of the growing cells 48 h (Fig.1). Similar results were reported by Murakami *et al.* (2002) and Murakami *et al.* (2006b).

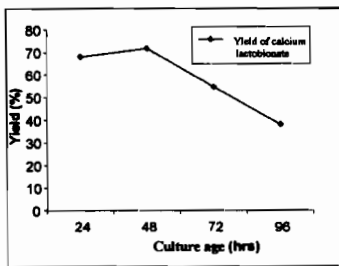


Fig. (1): Effect of culture age on the production of calcium lactobionate.

### 3.3. Effect of inoculum size of *B. spp. E* on calcium lactobionate production

A maximum yield of calcium lactobionate (79%) was obtained using 3ml of cell suspension ( $5 \times 10^7$  spores) of 48 hrs (Fig.2). The increase in

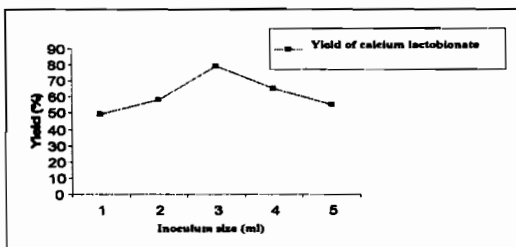


Fig. (2): Effect of inoculum size on the production of calcium lactobionate.

the level of cell suspension ( $5 \text{ ml} = 12 \times 10^7$  spores) brought about the cell autolysis (Murakami *et al.*, 2006a).

#### 3.3.1. Fermentation medium

Results in (Fig.3) clearly indicate that medium I (Stodola and Lockwood, 1947) proved to be the most favourable formulation convert lactose to calcium lactobionate (79% yield). The superiority of medium (I) may be due to the presence of corn steep liquor and urea as nitrogen source which promoted cell growth and production of calcium lactobionate and soybean oil as antifoaming agent (El-Minofi *et al.*, 1994).

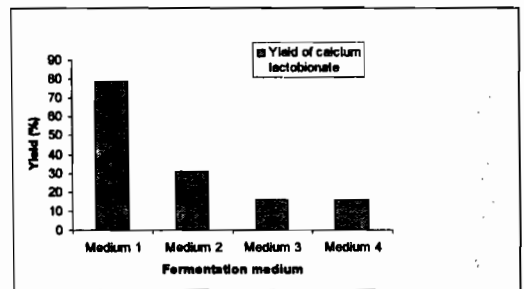


Fig.(3): Effect of fermentation medium on the production of calcium lactobionate.

#### 3.3.2. Effect of lactose level

Mixtures containing different concentrations of lactose (25, 50, 100, 125 and 150 gm/l) and 3 ml of cells were incubated at 30°C for 7 days. As shown in Fig.4 a relatively high lactose conversion (82%) was obtained at lactose concentration 50 g/l. On the other hand, the decrease in lactose concentration to 25 g/l leads to a slight reduction in calcium lactobionate yield (64%), while the yield sharply decreased to (23%) at 150 g/l lactose. This might be due to the substrate inhibition of the enzyme or a decrease of the penetration rate of lactose through cell membrane (Miyamoto *et al.*, 2000; Murakami *et al.*, 2006a).

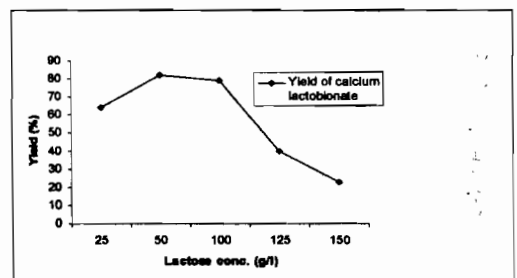


Fig. (4): Effect of lactose concentration on the production of calcium lactobionate.

**Table (2): Effect of fermentation time course on the yield (%) of calcium lactobionate.**

Bacterial isolates	<i>Bacillus</i> spp. <i>E</i>		<i>Mycobacterium</i> sp NRRLB-3805		<i>Ps. fluorescens</i>		<i>Pseudomonas oleoverance</i>		<i>Ps. spp.</i>	
	Calcium lactobionate (g/100ml)	Yield (%)	Calcium lactobionate (g/100ml)	Yield (%)	Calcium lactobionate (g/100ml)	Yield (%)	Calcium lactobionate (g/100ml)	Yield (%)	Calcium lactobionate (g/100ml)	Yield (%)
3	4.4	44	4.4	44	1	10	1.4	14	3.3	33
7	5.5	55	4	40	1.6	16	1.8	18	1.6	16
14	5	50	1.8	18	2.1	21	2.8	28	2.7	27
17	2.7	27	1.5	15	3.9	39	3	30	1.6	16
21	2	20	1.5	15	2.8	28	1.3	13	1.2	12

### 3.3.3. Effect of different levels of urea

Urea concentrations greatly affected the production of calcium lactobionate. The maximal yield (84%) was obtained at 1.5ml/100ml of 20% urea solution (Fig.5). The higher and lower concentrations decreased the calcium lactobionate production (El-Minofi *et al.*, 1994).

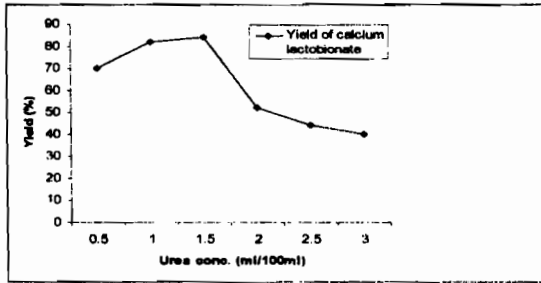


Fig. (5): Effect of urea concentration on the production of calcium lactobionate.

### 3.3.4. Effect of corn steep liquor concentration

The best level of corn steep liquor which gives the highest yield (90%) of calcium lactobionate was 7.5ml/l (Fig.6). Relatively lowest yields (50, 70, 84 and 33%) of calcium lactobionate were recorded at 1, 2.5, 5 and 10ml/l of corn steep liquor. It is likely to assume that corn steep liquor at 7.5 ml/l provides cells with specific constituents that activate their sugar-oxidizing enzyme (El Minofi *et al.*, 1994).

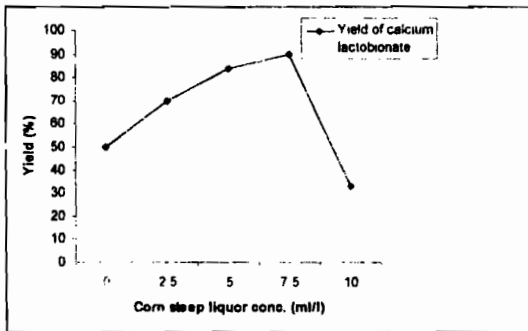


Fig. (6): Effect of corn steep liquor concentration on the production of calcium lactobionate.

### 3.3.5. pH value

The effect of pH value on the production of calcium lactobionate was studied by suspending cells cultured for 7 days in 50 gm lactose/l with pH values ranging from 4.0 to 8.0 (Fig.7). The maximal yields (87 and 90%) after 7 days fermentation were obtained at pH 5.0 to 6.0. The pH of the flask culture could be maintained within the range by the addition of calcium carbonate as a potential-alkali (Miyamoto *et al.*, 2000 and Murakami *et al.*, 2006b).

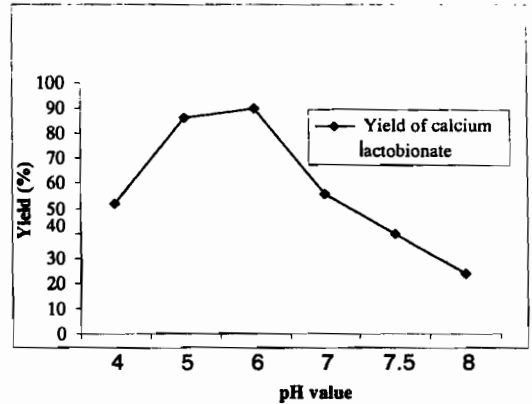


Fig. (7): Effect of pH on the production of calcium lactobionate.

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### التحول الميكروبي للاكتوز بواسطة بعض أنواع من البكتيريا إلى لاكتوبيونات الكالسيوم

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#### ملخص

تهدف الدراسة الحاليه إلى إختبار كفاءة بعض أنواع من البكتيريا لتحويل اللاكتوز إلى لاكتوبيونات الكالسيوم وذلك لما له من أهميه فى بعض الصناعات الدوائيه كما أنه يدخل فى صناعة بعض الاغذيه والمشروبات. اشتملت الدراسه على إختبار مقدرة ١٢ عزله بكتيريه من مختلف الأنواع والمصادر. كما أجريت بعض الدراسات الفسيولوجيه لمعرفة أفضل الظروف التى تودى إلى أعلى إنتاجيه من لاكتوبيونات الكالسيوم.

تفوقت السلالة البكتيريه *Bacillus* spp. E فى القيام بهذا التحول وتكوين لاكتوبيونات الكالسيوم بنسبة ٥٠% بعد أربعة أيام. كما وجد أن البيئه الغذائيه المكونه من (جم/ ١٠٠ مللتر): لاكتوز ٥؛ فوسفات البوتاسيوم الأحادي ٠.٠٦؛ كبريتات ماغنسيوم ٠.٢٥ إلى ٠.٧٥ مللتر من مستخلص نقيع الذرة وقطرة واحدة من زيت فول الصويا ويضاف ١.٥ مللتر من محلول اليوريا (٢٠%) و ٢.٥ جرام من كربونات الكالسيوم عند البدء فى الحقن والأس الهيدروجينى ٦ ودرجة الحرارة ٣٠ درجة مئوية أدت إلى تحويل اللاكتوز إلى لاكتوبيونات الكالسيوم بأعلى كفاءه (٩٠%).

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المجلة تخميه لكلية الزراعة - جامعة القاهرة - المجلد (٦١) العدد الثالث (يوليو ٢٠١٠): ٣٢٥-٣٣١.