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Studying the influence of nitrogen source on lactic acid production from whey permeate by immobilized *Lactobacillus bulgaricus Lb-12*.

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ABSTRACT

Aiming to enhance the lactic acid production (g/l) and lactose utilization from whey permeate in a step toward eco-friendly management of this serious dairy waste and cost-effective production of very important chemical, several nitrogen sources including yeast extract, casein hydrolysate and other two cheaper sources the ground corn and the defatted soybean were used. Fermentations were performed as batch fermentations in 100 ml static flasks. Yeast extract and casein hydrolysate were added separately by the ratios 0.5 – 2 % to whey permeate and then flasks were inoculated with free and Ca alginate immobilized *Lactobacillus bulgaricus Lb-12*. Corn and soy were extracted separately in permeate in the ratio of 10 % and the clear supernatants were fermented by free and immobilized cells. Mineral salts (0.2 g/l $MgSO_4$ and 0.05 g/l $MnSO_4$) were added. Control experiment contain only whey permeate. Lactic acid yield increased with increase in yeast extract concentration in immobilized cells than free ones. Mineral salts appeared to enhance production. The increase in lactic acid yield and productivity was greater in case of casein hydrolysate by 42% and 0.062 g/l.h respectively. Soy and corn extracts showed promising results when partially replaced yeast extract (150 gram replaced 17.5 gram yeast extract) thus lactic acid yield increased by 42% and 82% for soy and corn respectively. Due to its high potential, corn was chosen for further combinations in different ratios with yeast extract. The highest of lactic acid 26g/l was obtained for 20% corn and 0.5% yeast extract. Under full controlled (pH, temperature, agitation) fermentor system, the yield reached 44 g/l with productivity of 0.88 g/l.h and 88% efficiency, achieving complete lactose utilization. For lactic acid production, corn successfully replaced yeast extract resulting incomplete lactose exhaustion and enhancing the acid production by 322%, and saving time and energy by 27.7%.

Keywords: *Lactobacillus*, Immobilization, Lactic acid production, Nitrogen source, whey permeate.

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INTRODUCTION

Lactic acid is an organic acid (2-hydroxypropionic acid) occurs widespread in nature. It is one of the most versatile ingredients applied to wide range of applications such as food formulation and preservation worldwide. In this field, more than 50 % of both synthetic and fermentation derived lactic acid is used for this purpose. It is used as an acidulant, preservative, for improving egg white whip ability, flavor improvement of beverages and vinegar pickled vegetables, prevention of discoloration of fruits and vegetables and in the form of calcium lactate, as an additive in milk powders [1]. The most common use of high quality 'heat stable' food or pharmaceutical grade lactic acid is for the production of calcium stearoyl-2-lactylate (CSL), which is used mostly in baking as a 'dough conditioner' and sodium stearoyl-2-lactylate (SSL) which acts as an emulsifier. In addition, lactic acid is used for producing Lacto pickles from orange fleshed sweet potato [2].For medical application, it is used in the production of biodegradable plastic made of polylactic acid (PLA). PLA is a biodegradable, biocompatible, non-toxic and eco-friendly polymer and its composites are currently used in medical implants, tissue engineering, orthopedic devices, drug delivery systems [3]. Materials based on PLA and its co-polymers have also been designed to replace metal and other non-absorbable polymers as therapeutic aids in surgery [4].

Chemical synthesis is based on such lactonitrile derived from acetaldehyde and cyanide, sorbitol under alkaline hydrothermal conditions, or transformation of biomass chemo-catalytically [5]. Due to the high price of lactic acid, reduction of lactic acid production cost through utilization of inexpensive substrates and improvement of lactic acid production and productivity has become an important goal, so the direct microbial synthesis is preferred. Microbial production of lactic acid through fermentation is advantageous, where it enables the synthesis of either one of the two lactic acid isomers with high optical purity, concentration and productivity. Also, utilization of inexpensive substrates by microbial strains results in significant commercial availability [6].

Large amounts of agro industrial residues are generated from diverse economic activities that represent one of the energy rich resources available and when not properly discharged or used, add to environmental pollution. The most common substrate is the dairy waste; the whey, which is the remaining liquid after separation of milk fat and casein from whole milk, that represents a serious environmental pollutant and the major problem of the dairy industry [7].Production of cheese whey in the world is estimated to be over 10^8 tons per year. And Egyptian milk production in 2011 was 5.8 million tons [8]. Cheese whey is an important source of environmental pollution since 10 Liters of cheese whey is produced from 1 kg cheese with high carbohydrate, protein and lipid contents. Whey is composed of protein (12%), lactose (sweet whey 73%, and acid whey 68%) and the mineral content [9]. For economic and environmental management, whey is applied to Ultrafiltration using such a spiral wound membrane composed of hydrophilic polyamide, with a molecular mass cut off of 30,000 Da to obtain the whey concentrate and permeate [10]. From economic industrial view, whey permeate whey is more suitable for lactic acid production where it contains a high significant amount of lactose [11].

Providing of fermentation media with essential growth factors, amino acids, vitamins and several organic acids reported to improve lactic acid production. Hujanen and Linko, [12] studied the effect of eleven nitrogen sources, including yeast extract and some grains extracts individually and in combinations, on the production of lactic acid by two homofermentative strains of *Lactobacillus casei.* Their results showed yeast extract which was considered the best enhancer was efficiently replaced with barely malt sprouts and grass extract from an economic view. Other cheap sources such as urea, corn-steep liquor, malt sprout also showed significant enhancing lactic acid production [13]. Another source is casein, which was hydrolyzed using five proteases with different degrees of hydrolysis. Zhang *et al*., [14] used their ultrafiltrated fractions showing significant enhances in lactic acid production by *S. thermophilus* and *L. bulgaricus.* Corn is an important source of starch (as a major component), proteins and fat [15]. Among cheap agricultural sources are corn and soybean. Corn by-products, containing fermentable sugars, proteins and fat [15] were used efficiently for lactic acid production [16]. Soybean has high importance among oleaginous seeds in the world due to its availability and its highly contents of high quality proteins, essential amino acids, calcium, phosphorus, iron, vitamins (especially A and B) and vegetable oil [17]. However, few lactic acid bacteria have a considerable potential to hydrolyze soybean at the level of free amino acid [18]. Neither Corn nor soy protein were previously extracted in permeate for this purpose. Lactic acid production was common by both free and immobilized cells, but immobilized cells system keeps High cell densities (HCD) exposed to nutrients all time of fermentation and

allows easy recovery of the cells from fermentation media after the end of the process to be reused more times [19]. Different methods and different matrices were used for immobilization of lactobacillus spp. immobilization by cheap and readily available alginate gels is simple and gentle keeping cell viability [20].

Lactobacillus delbrueckii subsp. bulgaricus LB-12 was reported in literature as not suitable for lactic acid production from supplemented casein whey permeate [10].

The aim of this study is to optimize fermentation media components through investigation of potential of some cheap natural nutrients to replace the expensive ones for *Lb. bulgaricus* to completely utilize permeate lactose achieving maximum and cost-effective lactic acid production.

MATERIALS AND METHODS

Bacterial strain growth and inoculum preparation

*Lactobacillus dulbrueckii*subsp. *bulgaricus* Lb-12 DRI-VAC*,* (provided by the Northern Regional Research Laboratory. Illinois, USA) was grown on the MRS broth medium, which is recommended for the detection and enumeration of lactobacilli [21]. Pure strain was inoculated from MRS broth, after successive subculturing, in to sterilized skimmed milk tubes with screw caps, incubated at 37^{o}C for 24 hours and then stored in freezer as a stock culture to be used for fermentation. The stock culture was thawed at room temperature and 1ml transferred to MRS broth tubes. Reculturing every 48 h up to three generations [22] to remove the remains of fermented milk and obtain cell with high activity to be immobilized and inoculated to fermentation medium. An inoculum of 10^8 cells per ml was obtained by growing the anaerobic culture in a 250 ml Erlenmeyer flask containing 50 ml of MRS broth medium [23].The flask was incubated at 37°C for 20 h without shaking [24].

Immobilization

Classic entrapment method

The cells were harvested by centrifugation at 4000 g for 15 min at 4 °C, washed with 0·1% (w/v) sterile peptone (Bacto, Difco Laboratory) and recentrifuged. The pellets were suspended in 5ml of 0·1% (w/v) peptone and mixed with equal sodium alginate solutions to yield a final alginate concentration of 2%(w/v).The mixtures were added dropwise to sterile gently stirred (1%w/v $CaCl_2$or $BaCl_2$[25])through a needle. Alginate drops solidified upon contact with CaCl2, formed beads and thus entrapped bacterial cells After 30 min gelification, the 2mm diameter beads were washed twice with sterile saline to remove excess calcium or barium ions and unimmobilzed cells and then rinsed with 0.1% sterile peptone and stored in peptone at 4 °C, and was then subjected to fermentation [26].About 1.6x10^9CFU were immobilized in one gram beads.

Chitosan membrane coating on Ca-alginate beads:

Ca-alginate beads were coated with type I chitosan. Type I has a lower molecular weight, thus a lower viscosity (14 mPas in1% w/v solution). Chitosan is generally insoluble at pH levels above 6·5, so an aqueous solution was prepared by dissolving 0·4 g in 90ml distilled water acidified with 0·4ml of glacial acetic acid to achieve a final chitosan concentration of 0·4% (w/v). After dissolution, 1mol l-1 NaOH was added to adjust the pH to between 5·7 and 6. This solution was filtered (Whatman no. 4), and the volume adjusted to 100ml. Alginate beads with cells were washed with distilled water and immersed (about 12 g) in 100 ml of chitosan solution with gentle shaking for 40 min on an orbital shaker. Coated beads were then filtered and washed with sterile peptone and stored in peptone at 4 °C [26].

Free and immobilized cell count

For immobilized cells enumeration, Beads (0.1 g) were liquefied in 100 ml of 1% sterilized sodium citrate solution (pH6.0) and serially diluted with 0.1% peptone [26]. Dilutions of free and immobilized cells were transferred in to plates and count determined using MRS agar according to [21].The plates were incubated at 37°C for 48h under anaerobic condition [27].

Preparation of fermentation media

Permeate collection and preparation

Permeate, a cheese making waste containing approximately 5% (w/v) lactose was obtained, in ice form, from Animal production research Institute, Agricultural research Centre, Giza, Egypt. Firstly, the permeate was analyzed for lactose content, then protein precipitation was induced by heating the whey permeate at 90°C for 20 min. Precipitated proteins were removed by centrifugation at 4,000 rpm for 15 min. and then kept at a cold storage facility at -25°C to minimize microbial and enzymatic degradation. Prior to fermentation, clarified medium was supplemented with salt and then sterilized at 121°C for 20 min [23].

Preparation of yeast extract and casein hydrolysate

The two complex nutrients (yeast extract and casein hydrolysate) were separately added to whey permeate, as sources of vitamins, nitrogen, amino acids and carbon and then sterilized at 121°C for 20 min. The composition of yeast extract and casein hydrolysate was listed as provided by their manufacturing companies. Yeast extract (a water-soluble portion of yeast cells autolysate) contains ≥ 10-11.8% of total nitrogen and amino nitrogen of 4.5-5.8% and as a source of naturally occurring vitamin B complex, while Lactamine AA (a pancreatic hydrolysate of casein) contains ≥ 11% total nitrogen and ≥ 6% amino nitrogen and high in tryptophane.

Preparation of corn extract and soy whey

Corn (Zea maize), a nutrient supplement, was obtained kindly from a corn farm; Menofia governorate, Egypt. Before fine grinding, the grains were washed with sterile water and still to dry.

Defatted soy bean powder with 48% protein, a nutrient supplement was purchased from Agricultural research centre; Giza, Egypt.

Ground corn and soy protein were separately extracted in whey permeate as described by [28]. The nutrient was added to permeate in a 20% w/v. the pH was adjusted to 8.5 and the mixture was shaked for 30 min. at room temperature. After centrifugation at 10000xg for 20min. at 4°C. The soluble proteins in the supernatant were precipitated by lowering pH to 4.5. The resulting slurry was kept for 4 hours at 4°C and centrifugation repeated to obtain the extract which after sterilization was ready for fermentation. Control experiment was not supplemented.

Preparation of mineral salts

A stock solution of mineral salts was prepared by dissolving 20 grams of MgSO4 and 5 grams of MnSO4 in one liter of distilled water. Fermentation flasks were supplemented with 0.5 ml of stock to reach the final concentration of 0.2 g.l-1 and 0.05 g.l-1 respectively [21].

Preparation of the fermentor

The laboratory bench-top bioreactor (Bioflo 3000) System manufactured by the New Brunswick Scientific Co., Inc., 44 Tallmadge Road, P.O. Box 4005, Edison, New Jersey 08818-4005, U.S.A. was prepared for the batch fermentation process. The 5 liters reaction vessel of working volume 3 liters was sterilized, by autoclaving at 121°C at 15 PSIG for 20 minutes, after removing of all probes and probe cables. For batch fermentation, medium was sterilized with the vessel.

Process parameters adjustment was computerized. PH (ranged from 2 to 12), which is controlled by PID controller which operates two peristaltic pumps connected acid and base addition ports, was adjusted to 5.5. Temperature of 37°C was selected (in the range of 5 to 80 °C). It is controlled by a microprocessor based PI (Proportional and Integral). And agitation, by a top locate removable servo motor connected to agitation shaft with a multi-jaw coupling, was adjusted to 50, 80 and 100 rpm [29] and [30].

Sampling, through a sampler which is attached to a sampling tube extending to the lower portion of the vessel. The sampler has a rubber suction bulb to facilitate collection of representative samples without contamination. A 25mL screw cap container serves as a reservoir. Six samples were analyzed through 48 hours for lactose and lactic acid. The seventh sample showed complete lactose consuming terminating the experiment.

Fermentation process

All experiments were performed in static flasks [31] for 72 h without pH control [32]. All flasks were inoculated with bacterial strains at 2% w/v [22].

Lactic acid estimation

The lactic acid produced was quantitatively assayed by [33], which utilizes hot concentrated sulphuric acid effects, which include oxidation of lactic acid to acetaldehyde, which subsequently forms a chromogenic complex with p-phenyl phenol in presence of copper. The extracts were centrifuged at 8000g and the supernatants were used for lactic acid estimation. 0.5 ml of supernatant was added with 3mL of 96% sulphuric acid, followed by heating for ten minutes in boiling water bath for ten minutes, then cooling it to room temperature for about 30 minutes. The cool solution was added with 50 µl 4% copper sulphate and 100 µl p-phenyl phenol (prepared by dissolving 1.5% of the reagent in 95% ethyl alcohol) which provided a chromogenic complex. The absorbance for lactic acid is measured in a UV-VIS double beam spectrophotometer at 570nm [33] and[34].Standard curve was drawn by using a standard solution of lactic acid (Riedel-de Haen) and preparation for measurement by the same procedures of Kimberly Taylor method.

Furthermore, samples of the application experiment were analyzed by HPLC system was a HP 1100 chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with an auto-sampler; quaternary pump and a diode array detector were used. The quantitation was integrated by Chemstation chromatographic software interfaced to a personal computer. The analytical column was ZORBAX Eclipse XDB C18 column (15 cm x 4.6 mm I.D., 5 µm, USA). The isocratic elution was performed with phosphate buffer (10 mM, pH 3.0) and methanol at 95:5 %v/v ratios as mobile phase. The flow rate was maintained at 0.7 ml/min. The effluent was monitored at a wavelength of 220nm. Identification of lactic acid was performed by comparison with the retention times of standard material [35].

Lactose estimation

This is the most widely used colorimetric method to date for determination of carbohydrate concentration in aqueous solutions [36]. The basic principle of this method is that carbohydrates, when dehydrated by reaction with concentrated sulfuric acid, produce furfural derivatives. Further reaction between furfural derivatives and phenol develops detectible color. The standard procedure of this method is as follows. A 2 ml aliquot of a carbohydrate solution is mixed with 1 ml of 5% aqueous solution of phenol in a test tube. Subsequently, 5 ml of concentrated sulfuric acid is added rapidly to the mixture. After allowing the test tubes to stand for 10 min, they are vortexed for 30 s and placed for20 min in a water bath at room temperature for color development. Then, light absorption at 490 nm is recorded on a spectrophotometer. Reference solutions are prepared in identical manner as above, except that the 2 ml aliquot of carbohydrate is replaced by DDI water. The phenol used in this procedure was redistilled and 5%phenol in water (w/w) was prepared immediately before the measurements. [37].

RESULTS AND DISCUSSION

This study was conducted to investigate the production of high-valued lactic acid from the environmental pollutant whey permeate by both free and immobilized *Lactobacillus delbrueckii subsp. bulgaricus LB-12*cells using two commercial nutrients and other two cheaper agricultural ones. The addition of nutrient supplements considerably enhanced lactic acid yield and lactose utilization when compared with control. Results in tables (1.a and 1.b) showed that increasing of yeast extract addition from 0.5 to 2% resulted in increasing of the total lactic acid yield from 126% to 188% and lactose utilization from 4% to 27%. The fermentation enhancing effect of yeast extract was reported by [38] and this could have been due its high b-vitamin content [39]. Mineral salts MnSO4 and MgSO4) addition also contributed to enhancing acid yield by

23.27% and sugar utilization by 2.6%.These results were disagreed by [40] who reported that the lactate dehydrogenase (LDH) system of *Lactobacillus bulgaricus* did not require Mn^{+2} for catalytic activity. Here, it can be explain by that the two strains might be different. However, [41] found that MnSO4 when added with yeast extract to a fermentation medium inoculated with *Lactobacillus casei* the fermentation time, required for maximum lactic acid yield and sugar conversion, reduced to 1/5 and reduced yeast extract requirement. Alsocell growth (free and immobilized) was shown to be positively affected by increasing addition of both yeast extract and casein hydrolysate (tables 2.a and 2.b), but not significantly by mineral salt addition.

	with salts					without salts			
fermentation	Time(hours / YE concentration	0	24	48	72	0	24	48	72
Control	-----		2.97	3.14	3.4	0.5	2.97	3.14	3.4
lactic acid g/l	0.50%	0.5	5.1	5.9	7.7		4.89	4.97	5.83
	1%		6	6.5	8.4		5.83	6.08	6.5
	2%		7.6	7.98	9.8		7.1	7.78	7.95
Control	-----		32.6	32	31		32.6	32	31
residual lactose g/l	0.50%	50	32	30	28.8	50	37	34	31.1
	1%		22	21.6	18.7		32.6	32	29
	2%		21.1	20	17.3		23.5	21	18.8
Control	-----		6.27	5.54	5.49		6.27	5.54	5.49
(log CFU/ml)	0.50%	6.3	6.77	6	6.813	6.6	6.778	6	6.813
	1%		6.83	5.95	6.485		6.84	5.95	6.485
	2%		6.6	6.07	6.699		6.477	6.146	6.5185

Table 1a: Effect of yeast extract (YE) on free cells

	with salts					without salts			
Fermentation	Time(hours) / YE concentration	0	24	48	72	0	24	48	72
Control		0.5	3.91	4.67	4.84	0.5	3.91	4.67	4.84
lactic g/l	0.50%		6.5	7.3	8.4		6.53	7.53	7.61
	1%		6.7	7.9	9		7.53	7.61	8.61
	2%		7.15	8.75	9.99		9.23	9.65	10.42
Control		50	34	29.3	27.5	50	34	29.3	27.5
residual lactose g/l	0.50%		30	27	20		32	31.7	31
	1%		20	18	15.3		25	24.1	24
	2%		13	12.7	11		23.5	21.1	18.5
Control		8	8.477	8.04	8.2	8	8.47	8.04	8.2
(log CFU/g)	0.50%		8.845	8.845	9.114		8.3	8.845	9.114
	1%		8.845	9.08	9.146		8.845	9.08	9.146
	2%		8.477	9.114	9.2		8.477	9.114	9.2

Table 1b: Effect of yeast extract (YE) on Ca-alginate immobilized cells.

Table (2.a and 2.b) showed that the maximum lactic acid produced without nutrient supplementation was 4.84 g/l. With the addition of casein hydrolysate by 0.5, 1 and 2% reached 11.48, 11.91 and 14.88 g/l respectively. Lactic acid productivity was maximally enhanced due casein hydrolysate supplementation by 0.062 g/l.h over yeast extract, but lactose utilization rate was lower. This observation confirmed what was reported by [42]who found that increasing nutrient supplementation up to 1% increased lactose utilization rate from 1.35 g/l.h to 3.25 g/l.h for casein hydrolysate and 4.15 g/l.h for yeast extract. That was supposed

that the higher tryptophan content of Casein hydrolysate than yeast extract may give the former a higher potential. NAD –dependent D-lactate dehydrogenase structure of *Lb. bulgaricus* was literature to contain tryptophan [43]. As shown in tables (set 1, set 2) at the first 48 hours, the majority of lactic yield was obtained through the growth associated mechanism. After growth had been ceased due to the depletion of nutrients [45], the non-growth associated lactic acid was produced at lower rate. This behavior of the two nutrients toward improving lactic acid production was reported by many researchers and that is because they provided the fermentation media with growth factors, short peptides and carbonaceous compounds[42].

with salts						without salts			
fermentation	Time(hours) / CH concentration	0	24	48	72	0	24	48	72
Control		0.5	2.97	3.14	3.4	0.5	2.97	3.14	3.4
lactic acid g/l	0.50%		7.18	9.3	11.48		6.12	8	9.35
	1%		8	10.3	11.91		6.9	8.18	10.2
	2%		10.1	12.9	14.88		8.65	10.89	12.33
Control		50	32.6	32	31	50	32.6	32	31
residual lactose g/l	0.50%		42.1	37.4	27.8		46.1	41	34.2
	1%		41.5	39.1	33.5		42.3	37.6	34.4
	2%		36.4	24.3	17		41.1	34.6	25.4
Control		6.3	8.3	9.34	9	6.6	8.3	9.34	9
(log CFU/ml)	0.50%		8.78	9.25	8.6		8.23	9	8.95
	1%		8.95	9.36	9.23		8.477	9.25	9.04
	2%		9.52	9.756	9.43		8.9	9.36	9.04

Table 2a: Effect of casein hydrolysate (CH)on free cells.

with salts						without salts			
fermentation	Time(hours) / CH concentration	0	24	48	72	0	24	48	72
Control		0.5	3.91	4.67	4.84	0.5	3.91	4.67	4.84
lactic acid g/l	0.50%		6.38	9.78	11.9		5.95	8.5	10.63
	1%		7.23	11.9	13.6		5.95	9.78	11
	2%		9.36	13.2	14.88		8.72	11	13.2
Control		50	34	29.3	27.5	50	34	29.3	27.5
residual lactose g/l	0.50%		46.2	35.5	25		48	40	31.8
	1%		42.2	31.6	13.7		46	34	32
	2%		39.5	23	15.8		40.4	31.9	23.4
Control		8	8.32	8.6	8.69	8	8.32	8.6	8.69
(log CFU/g)	0.50%		8.83	9.08	9.2		7.9	9.3	9.55
	1%		8.95	9.447	9.43		8	9.415	9.78
	2%		8	9.114	8.9		8.94	9.43	9.38

Table 2b: Effect of casein hydrolysate (CH) on Ca-alginate immobilized cell

Results of tables (3.a and 3.b) revealed that corn which was extracted by 10% in permeate, showed enhanced lactic acid yield and lactose utilization somewhat better than 0.5% yeast extract. This enhancement was affected with mineral salts addition and was better in case of immobilized cell fermentation.

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with salts					without salts			
Time(hours) / fermentation	0	24	48	72	0	24	48	72
Control	0.5	1.44	2.81	3.5	0.5	1.44	2.81	3.5
lactic acid g/l		5.52	7.23	8.16		5.52	5.53	6.8
Control	50	49	48.1	39.4	50	49	48.1	39.4
residual lactose g/l		44.8	39	13.2		45.4	41.1	23.2

Table 3a: Effect of corn extract on free cells

with salts					without salts			
Time(hours) / fermentation	0	24	48	72	0	24	48	72
Control	0.5	2.55	3.4	4.6	0.5	2.55	3.4	4.6
lactic acid g/l		5.9	7.28	9.19		5.9	6.6	8.8
Control	50	48.4	47.1	38.1	50	48.4	47.1	38.1
residual lactose g/l		42	36.6	11.2		43	38.4	16.9

Table 3b: Effect of corn extract on Ca-alginate immobilized cells.

In contrast to corn extract, soy extract (10%) showed lower pattern in both lactic acid production and lactose utilization than both corn and yeast extract (table 3.c and 3.d).

with salts					without salts			
Time(hours) / fermentation	0	24	48	72	0	24	48	72
Control	0.5	1.34	2.11	3.57	0.5	1.34	2.11	3.57
lactic acid g/l		5.7	6.01	7.2		5.53	5.95	6
Control	50	49	48.1	42.9	50	49	48.1	42.9
residual lactose g/l		41.1	40.1	35.4		46	43	39.8

Table 3c: Effect of soy protein on free cells

Time(hours) / fermentation	0	24	48	72	0	24	48	72
Control	0.5	2.18	3.35	4	0.5	2.18	3.35	4
lactic acid g/l		5.8	7.23	9.29		5.31	6.8	8.5
Control	50	48.5	47	45	50	48.5	47	45
residual lactose g/l		40.5	37.4	29.5		45	41.5	34.8

Table 3d: Effect of soy protein on Ca-alginate immobilized cells

Lactic acid production rate was high for immobilized cells than free cells. This observation agreed what was reported by [30] who explained this by the adverse effect of low pH caused by accumulated lactic acid (product inhibition) which can better tolerated by immobilized cells than the free ones. Results obtained by[19]showed that lactic acid productivity achieved by immobilized cells in the first cycle was high as 145% of that of free cells. In this study, the performance of immobilized cell system may be described as that the beads act as active cell spots which protect the entrapped cells that reproducing and continuously transmitted to fermentation media. One more probable reason is the availability of nutrients residues from the reactivation broth that retained by the gel material. As in table (3.e and 3.f) two gel matrices, Calcium alginate and Barium alginate were investigated for their abilities to keep HCD, which accompanied by higher conversion efficiency of lactose to lactic acid. Table (3.e) showed little differences between the effects of the two matrices on lactic acid production by the producing strain, but the difference was clear in both viable cell count and lactose utilization. Higher lactose utilization and cell count in the case of Calcium alginate than Barium alginate referred to the higher efficiency of Ca^{++} than Ba^{++}. These results was disagreed with results of[44] in that Ba alginate is physically and chemically more stable than Ca alginate, but [46] found that under mechanical stress, Ca alginate beads only were existed. Furthermore, [47] reported that Ba alginate beads couldn't withstand high temperature. For lactic acid production, [48] obtained the maximum lactic acid concentration (60 g/l) by Ba alginate immobilized cells after 96 h fermentation. Other study showed the maximum lactic acid (> 70 g/l) obtained was by Ca alginate immobilized cells after 120 h [49]. Chitosan coated Ca alginate beads showed enhanced lactic acid and all other criteria than the Ca and Ba. This coating keep HCD in more efficient manner that represented by improving lactose utilization by 2% and 18% for Ca and Ba alginate, respectively. Similar results, reported by [50] revealed that the rate of cell release during long-term fermentation was significantly affected by alginate/chitosan immobilization. Economically, Ca alginate was applied for further investigations.

Table 3e: Effect of different immobilization matrices on lactic acid production and lactose utilization

Time(hours) / fermentation	0	24	48	72
Lactic acid g/l (Ca alginate)	0.5	10.35	15.7	20
Lactic acid g/l (Ba alginate)	0.5	10.35	14.8	19.8
Lactic acid g/l (Ca/Chitosan)	0.5	10.8	17	20.5
residual lactose g/l (Ca alginate)	50	32	21	13.5
residual lactose g/l (Ba alginate)	50	30.3	23.2	21
residual lactose g/l (Ca/Chitosan)	50	31.5	21.5	12

Table 3f: Effect of different immobilization matrices on cell growth and cell release

Time(hours) / Beads count and cell release	0 h	24 h	48 h	72 h
immobilized CFU/g (Ca alginate)	8.3	10.6	10.04	10.27
immobilized CFU/g (Ba alginate)	8.3	10.06	9.699	10.146
immobilized logCFU/g (Ca/Chitosan)	8.3	10.08	10	10.3
Cell release log CFU/g (Ca alginate)	2	8.477	9.114	8
Cell release log CFU/g (Ba alginate)	2	8.613	9.2	8.6
Cell release log CFU/g (Ca/Chitosan)	2	8.14	9.08	8

Further studies (table 3.g) were performed using Ca alginate immobilized cells to enhance the production of lactic acid using corn due its successful substitution to 0.5% yeast extract. These studies aimed to investigate corn potential to replace higher concentrations of yeast extract. Three different concentrations, by which corn was extracted, were 10, 15 and 20% with 0.25% yeast extract. Table (3.h) showed an increase in the rate of lactose conversion to lactic acid and cell growth with increasing of corn concentration. The

accumulated lactic acid at the first 24 h revealed the faster adaptation of entrapped cells to 15% than 20% corn concentration, but the net lactic acid obtained at corn 20% exceeded what obtained at 15% by 6%. This enhanced performance of corn yeast combinations was agreed by last studies [51] and that may be explained by that the different components of the two nutrients filled the strain requirements for growth and metabolism. The residual non-utilized lactose stimulated further experiments on fermentation media composition. For 20% corn extract, different concentrations of yeast extract (0.25, 0.5 and 1%) were tested against permeate/10% corn as control. Experimental results showed greater increasing in lactic acid production rate (0.48 g/l.h – 0.53 g/l.h) at the first 48 h and lactose utilization reached 94%.

fermentation	Time(hours) / YE concentration	0	24	48	72
Control		0.5	5.9	7.28	9.19
lactic acid g/l	0.25%		13.7	23.17	24.7
lactic acid g/l	0.50%		16.08	24.5	26
lactic acid g/l	1%		17.64	25.8	26.2
Control		50	48.4	47.1	38.1
residual lactose g/l	0.25%		39.2	25	8
residual lactose g/l	0.50%		39	21	6
residual lactose g/l	1%		32	18	3
Control		8	8.5	8.1	8.3
Count CFU/g	0.25%		10.3	10	10.447
Count CFU/g	0.50%		9.6	10.2	10.08
Count CFU/g	1%		10.7	10.3	10.71

Table 3g: Effect of corn/yeast extract (YE) on lactic acid production, lactose utilization and cell growth ofCa alginate immobilized cells.

fermentation	Time(hours) / Corn concentration	0	24	48	72
control		0.5	3.91	4.67	4.84
Lactic acid g/l	10%		11.9	15.7	22
	15%		14.8	18.1	22.5
	20%		12.4	18.3	24
control		50	34	29.3	27.5
residual lactose g/l	10%		37	30	15
	15%		30	23	10
	20%		30	21	9
Control		8	8.47	8.04	8.2
Log CFU/g	10%		9.6	10.43	10.32
	15%		10.39	10.46	10.43
	20%		10.43	10.58	10.477

Table 3h: Effect of different concentrations of corn with minimum yeast extract (YE) concentration.

By application of the optimal culture conditions (20% corn and 0.5% yeast extract) in a full controlled fermentor system (pH 5.5, Temperature 37°C, agitation 80 rpm) the maximal lactic acid was obtained (44 g/l) with complete exhaustion of lactose. By this high rate of lactic acid production (0.846 g/l.h), the batch fermentation time was reduced by 27%.

CONCLUSION

For lactic acid production, the economics of the process became the most interested by many researchers. In a process toward optimization, two cheaper nutrients the corn and the soy were used to provide the media by some essential requirements. From an economic view, corn successfully replaced yeast extract in a full controlled fermentor system exhausting all lactose and enhancing the production by 322%, and saving time and energy by 27.7%.

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