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Improvement of Fermentation Processing Conditions for the Production of Lactic Acid by Immobilized *Lactobacillus delbrueckii ssp. bulgaricus* Lb-12.

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ABSTRACT

Aiming to enhance the yield of lactic acid from the bio-waste; whey permeate with achieving complete utilization of lactose, the followed strategy in this work is the evaluation of environmental conditions; pH, temperature, inoculation level and shaking speed. Fermentation processes were carried at different pH values (5.5, 6.5 and 7.5), separately considering A whey permeate media (WPM) with corn extract; (10%) and yeast extract; 0.25% without pH adjustment as control. pH of the fermentation media can drive the process through controlling the membrane-bound proton translocating ATPase system or interfering active sites of functioning enzymes that drive the metabolic process. At pH 5.5, the fermentations were performed at 37ºC, 40ºC and 42ºC. Incubation temperature is suggested to have a remarkable influence on nutrients and metabolites distribution, and the maximum carbon flux toward lactic acid at the pyruvate node was obtained at the optimal temperature. In addition, inoculation ratio was evaluated at three different levels 1%, 2% and 3%. By applying the successful conditions; pH 5.5, temperature 37ºC and inoculation ratio 2%, on bench-scale fermentation, the maximum lactic acid production reached 26 gl^{-1}, and lactose utilization 88% after 72 h fermentation. Under the full controlled fermenter conditions, shaking speed was studied at (50, 80 and 100 rpm) for more achievements. The utmost lactic acid 44 gl^{-1} was obtained with complete lactose consumption after only 52 h fermentation period.

Key words: Lactic acid, fermentation conditions, *Lactobacillus delbrueckii ssp. bulgaricus* Lb-12.

Introduction

Lactic acid is now considered a promising in many fields. In the field of 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 (Narayanan *et al*., 2004). 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 (Ahmann and Dorgan, 2007).

In concern with lactic acid production through fermentation, it is believed that microbial metabolic activities are significantly affected with changing of environmental conditions. Temperature and pH are the key environmental parameters that affect the lactic acid fermentation process. The influence of pH was investigated on the production of lactic acid from starch using *Lb. manihotivorans* LMG18010T and *Lb. plantarum* A6, when pH was adjustedat 6.0, lactic acid had markedly increased saving half of fermentation time (Guyot *et al*., 2000). Also, Mussatto *et al*., (2008) achieved lactic acid yield (0.99 gram lactic acid produced per gram glucose consumed) with decrease in residual sugar at the same pH value. The main aim of this work is bioconversion of all lactose content of WPM to lactic acid achieving the highest yield and conversion efficiency through optimization of fermentation conditions.

Materials and Methods

Preparation of biomass for the producing strain:

The current utilized strain; *Lactobacillus dulbrueckii subsp. bulgaricus* Lb-12 (Agricultural Research Service Culture Collection, Illinois, USA) was grown and maintained on MRS broth medium (De Man *et*

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al., 1960). The pure culture was inoculated into sterilized skimmed milk tubes and incubated for 24 hours at 37°C and then stored in freezer as a stock culture to be used for fermentation. Upon inoculum preparation, the stock culture was left to melt and 1ml was transferred to MRS broth tube. Reculturing, every 48 h, for three times, was performed to obtain high active cells for immobilization and inoculation to fermentation medium (Panesar *et al*., 2010). A biomass of 10^8 cell per ml was obtained by anaerobic growing of the culture in a 250 ml Erlenmeyer flask containing 50 ml of MRS broth medium (Panesar *et al*., 2007). Then the flask was incubated at 37°C for 20 h without shaking (Benthin and Villadsen, 1995).

The strain biomass was obtained by centrifugation at 4000 xg for 15 min at 4 °C, and then washed with 0.1% wv^{-1} sterile peptone (Bacto, Difco Laboratory, USA). The pellets were suspended in 5ml of 0.1% wv^{-1} peptone and mixed with equal volume of Sodium alginate solutions, (Fluka BioChemika, USA), to yield a final alginate concentration of 2% wv^{-1}. The mixtures were added drop-wisely to sterile gently stirred 1% wv^{-1} $CaCl_2$, through a needle forming beads which entrapped bacterial cells. After 30 min of gelification, the 2 mm-diameter beads were washed twice with sterile saline, to remove free cells and excess calcium ions, and then the beads were rinsed with 0.1% sterile peptone and stored in peptone at 4 °C. About $1.6x10^9$ CFU were immobilized in one gram beads.

Using chitosan-type I of lower molecular weight and a lower viscosity, the obtained beads were coated according to Zhou *et al*., (1998).

Preparation of fermentation media:

Whey permeate medium (WPM) with 50 gl^{-1} lactose was used as a basal medium and supplemented with corn and yeast extracts at 10% and 0.25% wv^{-1} respectively as nitrogen source (Mawgoud *et al*., 2016).

Investigation for the effect of fermentation pH:

Fermentation processes were carried at different pH values (5.5, 6.5 and 7.5), separately, while pH was adjusted with 5N Sodium hydroxide as described (Afifi, 2011). A WPM with corn extract; (10%) and yeast extract; 0.25% without pH adjustment was taken as control.

Estimation of incubating temperature:

Fermentation flasks, inoculated with immobilized *Lb. bulgaricus* cells, were examined for growth and lactic acid production at 37°C, 40°C and 42°C (Goranov *et al*., 2013).

Assessment of inoculum size:

Fermentation flasks were inoculated with *Lb. bulgaricus* cells entrapped in Chitosan-coated Calcium alginate beads. Immobilized cells were loaded in three ratios (1%, 2% and 3% wv^{-1}) to evaluate the effect of different initial cell densities (Panesar *et al*., 2010).

Shaking effect in the fermenter:

A WPM was supplemented with corn and yeast extracts (20% and 0.5% respectively) in a full controlled fermenter. The process was operated at pH; 5.5, temperature; 37°C and 2% inoculum size. No aeration was applied due to the anaerobic nature of Lactobacillus strains. Shaking was investigated at 50, 80 and 100 rpm while static flask fermentation was considered as control.

Analytical procedures:

Immobilized cell count, $CFUg^{-1}$ beads, was investigated together with concentration of the produced lactic acid, gl^{-1}, and concentration of the residual unconsumed lactose, gl^{-1}, as indicators for the studied variables of the fermentation processes.

Immobilized cells counting:

For immobilized cells enumeration, Beads (0.1 g) were liquefied in 100 ml of 1% sterilized sodium citrate solution (pH 6) and serially diluted with 0.1% peptone (Zhou *et al*., 1998). Serial dilutions of free and immobilized cells were made by transferring 1 ml of cell suspension to 9 ml saline, then 1 ml from each dilution was cultured on MRS agar plates and incubated at 37°C for 48 h under anaerobic condition (Chávarri *et al*., 2010).

Estimation of residual lactose:

Estimation of lactose was performed by the modified colorimetric method of Dubois *et al*., (1956).

Estimation of the produced lactic acid:

The produced lactic acid after fermentation was quantitatively assayed by Taylor method, (Taylor, 1996). Samples of the produced lactic acid after full controlled fermentation by *Lb. bulgaricus* Lb-12 were also analyzed by HPLC (Agilent Technologies, Palo Alto, USA) (Kishore *et al*., 2013).

Statistical analysis:

The experiments were accomplished following a complete randomized design where the obtained data were subjected to analysis of variance (ANOVA) according to Snedcor and Cochran modified method using Mastate program (Snedcor and Cochran, 1980). The least significant differences were used to compare means of treatments at probability 5% following Walter and Duncan procedure (Walter and Duncan, 1969).

Results and Discussion

The present study was carried out to maximize the produced lactic acid achieving maximum lactose utilization through controlling fermentation parameters.

Results presented in Table (1. a) shows the role of pH control on lactic acid production and lactose utilization. The maximum acid production ($22.5 gl^{-1}$) with maximum productivity $0.72\ gl^{-1}h^{-1}$ was obtained at pH 5.5 without a significant difference from pH 6.5 where lactic acid was produced as $22 gl^{-1}$ with maximum productivity $0.74 gl^{-1}h^{-1}$. These results were agreed by those of Wee *et al*., (2005) in that there was no difference in lactate production when pH was changed from 5.5 to 6. At pH 6.5, the maximum lactose utilization exceeded that at pH 5.5 by 4%. Cell growth was observed to increase at the first 48 h supporting the growth-associated lactic acid production, while after 48h and after the growth had been ceased, the non-growth associated lactic acid was produced. In contrast, pH 7.5 showed a lower acid concentration ($16.9 gl^{-1}$)even than the control ($17.8 gl^{-1}$) with $0.575 gl^{-1}h^{-1}$ as a maximum productivity, confirming the observation of Rhee and pack, (1980) who increasingly obtained lactate using *Lb. bulgaricus* only at pH range 5 - 6, while lactose utilization exceeded that at 6.5 by 3%. This enhanced productivity supported that of Yoo *et al*., (1996). In addition, Tuli *et al*., (1985) obtained the maximum lactic acid production by immobilized *Lb. casei* from whey permeate at pH 5.5. Furthermore, Krischke *et al*., (1991) obtained the maximum acid production and harvested the largest biomass at pH between 6 and 6.5 by the immobilized *Lb. casei* sub sp *casei* grown on whey permeate as well. At pH 7.5, no more 16.9 g l^{-1} lactic acid was produced and maximum productivity did not exceed 0.575 g $l^{-1}h^{-1}$. Amrane (2001) found that the optimal pH for lactic acid production was 5.9 while the present study reported that the optimal was pH 5.5 while acid yield was the greatest (0.7258 g g^{-1}) at pH 5.5, while the lowest (0.42 g g^{-1}) was obtained at pH 7.5.

Table 1 a: Influence of pH value on the fermentation process.

pH values	Lactic acid (gl-1)			Residual lactose (gl-1)		
	24 h	48 h	72 h	24 h	48 h	72 h
Control	$11.8^{C}\pm0.2$	$13.3^{C}\pm0.12$	$17.8^{C}\pm0.1$	$39.5^{A}\pm1.41$	$36^{A}\pm1.1$	$15.8^{A}\pm0.9$
5.5	$17.3^{A}\pm0.3$	$18.9^{A}\pm0.5$	$22.5^{A}\pm0.31$	$25^{C}\pm0.67$	$22^{B}\pm0.8$	$14^{B}\pm0.31$
6.5	$17.8^{A}\pm0.31$	$18.5^{A}\pm0.34$	$22^{A}\pm0.12$	$22^{C}\pm0.55$	$19^{C}\pm0.7$	$12^{C}\pm0.23$
7.5	$13.8^{E}\pm0.31$	$16.5^{B}\pm0.3$	$16.9^{C}\pm0.41$	$20^{E}\pm0.51$	$14^{E}\pm0.8$	$10.5^{D}\pm0.11$

This positive effect of pH control came in agreement with Burgos-Rubio *et al*., (2000) who found that the optimal pH was around 5.6. He reported that lowering in pH was combined with growth reduction till pH 4.4, where the growth was clearly reduced as a result of lactic acid accumulation in un-dissociated form. The positive influence of pH may be explained by several mechanisms; in the glycolytic non-respiring bacteria, the membrane-bound proton translocating ATPase which represents the most important homeostatic system , that drives the uptake of solutes through proton gradient, requires a narrow pH difference (internal and external) to facilitate the H^+ movement against the concentration gradient (Hutkins and Nannen, 1993).

Table 1 b: Effect of pH variation on growth of the immobilized *Lb. bulgaricus*.

pH values	Incubation time (hours)		
	24 h	48 h	72 h
Control	$2.1x10^{8\ D}$±0.01	$4.9x10^{7\ D}$±0.02	$4\ x10^{8\ H}$±0.009
5.5	$2.3x10^{9\ B}$±0.04	$3x10^{10\ B}$±0.01	$1.8x10^{10\ F}$±0.01
6.5	$4x10^{10\ A}$±0.01	$2.0x10^{10\ C}$±0.03	$3x10^{10\ H}$±0.009
7.5	$8x10^{8\ C}$±0.01	$4x10^{10\ A}$±0.008	$3x10^{10\ C}$±0.001

In addition, some enzymes which drive metabolic reactions were believed to have, in their active sites, ionic groups that must be in the suitable form (H^+ or OH^-) for function. Non-optimal pH condition contributes to alteration of the active site form resulting in enzyme inactivation (Yuwono and Kokugan, 2008).Therefore, in determination of the effective incubating temperature for maximal production of lactic acid from whey permeate; the experimental pH was adjusted to be 5.5.

At pH 5.5, results presented in Table (2. a) shows the role of incubation temperature on lactic acid production and lactose utilization. Maximum acid production by the immobilized *Lb. bulgaricus* at 37ºC, where the produced lactic acid was 23.6 g l^{-1}, with maximum productivity 0.645 g $l^{-1}h^{-1}$ exceeding amount of acid obtained at 40 ºC and 42 ºC by about 59.57%.Residual lactose at the end of fermentation was 13.5 g $l^{-1}$and immobilized cell biomass $2.3x10^{10}$ CFU g^{-1} beads (Table 2.b).

Table 2 a: Influence of incubating temperature on the fermentation process.

Incubating temperature (^OC)	Lactic acid (gl^{-1})			Residual lactose (gl^{-1})		
	24 h	48 h	72 h	24 h	48 h	72 h
37	15.5^{A}±0.2	21^{A}±0.12	23.6^{A}±0.4	32.1^{C}±0.77	28^{A}±1.1	13.5^{B}±0.4
40	12^{B}±0.3	13^{B}±0.5	15.6^{B}±0.39	32^{C}±1.1	27^{A}±0.8	22.5^{A}±0.31
42	10^{C}±0.31	12.5^{B}±0.3	14^{B}±0.41	28.5^{E}±1.01	10.3^{E}±0.7	25^{A}±0.23

Under the same conditions, Idris and Suzana, (2006) obtained the maximum lactic acid using the immobilized *Lb. delbrueckii*. Also, Adamberg *et al*., (2003) reported that increasing in temperature from 25 to 38°C, had increased the specific growth rate which decreased above 38°C. This may be explained by the depletion of nutrients from the fermentation media and lactic acid accumulation and other metabolites as well (Aasen *et al*., 2000). However, another observation was recorded by Aghababaie *et al*., (2014) who found thatcell growth and acid production by *Lb. bulgaricus* were optimal at 44°C.

Table 2 b: Effect of incubating temperature on the growth of immobilized *Lb. bulgaricus*

Incubating temperature (^OC)	Incubation time (hours)		
	24 h	48 h	72 h
$37\ ^OC$	$1.1x10^{9\ D}$±0.01	$2.0x10^{10\ C}$±0.02	$2.3x10^{10\ G}$±0.009
$40\ ^OC$	$4x10^{10\ B}$±0.04	$3x10^{10\ B}$±0.01	$8.2x10^{9\ H}$±0.009
$42\ ^OC$	$1.32x10^{10\ C}$±0.01	$4.1x10^{9\ E}$±0.008	$3.3x10^{9\ J}$±0.01

Qin *et al*., (2012) obtained the maximum production at 41 ºC as the optimal temperature for lactic acid production, suggesting that temperature has a remarkable influence on metabolite distribution, and the maximum carbon flux toward lactic acid at the pyruvate node was obtained at the optimal temperature. Stern and Frazier (1941) noted that *Lb. bulgaricus* left the log phase sooner at 49 ºC than at 37ºC, reporting a close relation between growth and acid production at 37ºC.

At optimal environmental conditions (pH 5.5 and incubating temperature 37ºC), the influence of microbial density was studied for obtaining the maximum lactic acid production (Table 3.a), where the maximum lactic acid production (26 gl^{-1}) was obtained at 2% inoculation, with maximum productivity 0.7 $gl^{-1}h^{-1}$. Residual lactose was 6 gl^{-1}; meaning that 88% of total lactose had been utilized. Immobilized biomass was $4x10^{10}$ CFU g^{-1} beads (Table 3.b).

Table 3 a: Influence of Inoculum volume on the fermentation process.

Inoculum volume % (w v^{-1})	Lactic acid (gl^{-1})			Residual lactose (gl^{-1})		
	24 h	48 h	72 h	24 h	48 h	72 h
1%	$12.5^{B}\pm0.2$	$15^{D}\pm0.2$	$17.7^{D}\pm0.2$	$31.6^{D}\pm0.9$	$29^{D}\pm0.2$	$20^{D}\pm0.2$
2%	$16.8^{A}\pm0.2$	$24.5^{A}\pm0.5$	$26^{A}\pm0.3$	$39^{A}\pm0.8$	$21^{D}\pm0.2$	$6^{D}\pm0.2$
3%	$13^{B}\pm0.2$	$22^{A}\pm0.6$	$25^{A}\pm0.6$	$28^{D}\pm1.8$	$19^{D}\pm0.2$	$5^{D}\pm0.12$

Results observed in fermenter experiment were confirmed by HPLC analysis which showed that lactic acid production was enhanced by 69%, lactose was completely utilized after 52 h. This great enhancement can be explained by that stirring of the fermentation media (80rpm) led to efficient distribution of both nutrients and metabolites, consequently exposing bacterial cells to every microgram of nutrient at the same time diluting metabolites around active cells. Panda and Ray, (2008) reported that lactic acid yield by still flask fermentation exceeded that of shake flask by about 25%. However, the results obtained by Coelho *et al*., (2011) revealed that bioreactor fermentation exceeded that of shake flask by about 5% and 0.5 for production and productivity respectively. Results of the current study confirmed results of Coelho team ascribing that enhancement for efficient distribution of both nutrients and wastes as mentioned above.

Table 3 b**:** Effect of inoculum volume on the growth of immobilized *Lb. bulgaricus*

Inoculum volume % (w v^{-1})	Incubation time (hours)		
	24 h	48 h	72 h
1%	$2.6x10^{10\ A}\pm0.2$	$2.1x10^{10\ D}\pm0.2$	$3.1x10^{10\ B}\pm0.2$
2%	$2.0x10^{10\ D}\pm0.2$	$2.5x10^{10\ B}\pm0.2$	$4x10^{10\ A}\pm0.2$
3%	$1.8x10^{10\ D}\pm0.2$	$2.1x10^{10\ D}\pm0.2$	$2.2x10^{10\ D}\pm0.2$

Conclusion

For enhancing lactic acid production through the way of improving fermentation conditions; pH, temperature, inoculum size and shaking speed, the best conditions were found to be 5.5, 37ºC, 2% and 80 rpm respectively.

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