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Supplementation of whey with glucose and different nitrogen sources for lactic acid production by *Lactobacillus delbrueckii*

Vasanthy Arasaratnam, Appadurai Senthuran, and Kandiah Balasubramaniam

Department of Biochemistry, Faculty of Medicine, University of Jaffna, Kokuvil, Sri Lanka

Lactobacillus delbrueckii *was grown at room temperature in static culture. When whey (total sugar 30 g l^{-1}) was supplemented with different concentrations of yeast extract, lactic acid production increased. When glucose (20 g l^{-1}) was added to whey, 20 g l^{-1} yeast extract supplementation was found to be most suitable. Among the different nitrogen sources supplemented to whey [yeast extract, peptone, soya flour, and $(NH_4)_2SO_4$, having the same elemental nitrogen level of 3.1 g l^{-1}], yeast extract was the best. The effect of yeast extract could be due to its vitamin B content. Therefore, whey was supplemented with different nitrogen sources and vitamin B complex, and no significant improvement in lactic acid production was observed. As yeast extract supplementation was not economically attractive, we decided to use a proportion of $(NH_4)_2SO_4$ and yeast extract. When the elemental nitrogen ratio of $(NH_4)_2SO_4$ to yeast extract was 3:1, the substrate use and efficiency of lactic acid production were same as in whey supplemented with 20 g l^{-1} yeast extract.*

Keywords: Lactic acid; whey; fermentation; nitrogen sources

Introduction

The main by-product of the cheese industry is whey. Whey contains between 40 and 50 g l^{-1} of lactose and 1.3 g l^{-1} of elemental nitrogen.[1] This nutrient-rich whey could be fermented to lactic acid by bacteria. Lactic acid has been produced from whey in developed countries,[2] and it has been proved to be an economically feasible process while reducing the wastage of biologic oxygen demand (BOD) substrate.[1] The aim of this study was to produce lactic acid from whey in static culture at room temperature. In this article we describe the supplementation of whey with glucose and different nitrogen sources to improve lactic acid production.

Address reprint requests to Dr. V. Arasaratnam, Department of Biochemistry, Faculty of Medicine, University of Jaffna, Kokuvil, Sri Lanka
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Materials and methods

Microorganism

Lactobacillus delbrueckii NRRL-B445 (recently identified as *Lactobacillus casei* subsp. *rhamnosus*), a homofermentative lactic acid producer, was used. The strain was stored in glycerol (12% w/w

Preparation of inoculum

A loopful of *L. delbrueckii* from 2-day-old slant was inoculated to 25ml MRS broth (52 g l^{-1}) and allowed to grow at room temperature in static culture for 24 h. From this, 1 ml was transferred to 10 ml MRS broth taken in a 100 ml conical flask and incubated at room temperature for 24 h. A 10% (v/v) inoculum containing $1.12–1.3 \cdot 10^8$ cells ml^{-1} was used as inoculum.

Analytic methods

Samples removed at different time intervals were centrifuged. Cells obtained by the centrifugation of the medium were washed twice and the dry weight[4] was taken. The supernatants were ana-

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lyzed for lactic acid[5] and total sugar.[6] Viable cell count[7] was taken by appropriately diluting culture medium and spread-plating on MRS-agar plates.

Whey supplemented with yeast extract

Yeast extracts of different amounts was added to whey to bring the final concentration to 0, 10, 20, and 30 g l^{-1}. Sterilized solid $CaCO_3$ (30 g l^{-1}) was mixed to this medium. The media were inoculated with 10% (v/v) *L. delbrueckii* and incubated at room temperature in static culture. At 12 h intervals, the total sugar, lactic acid, and dry weight of the cell were monitored.

Whey supplemented with yeast extract and glucose

Whey containing different concentrations of yeast extract were prepared and mixed with $CaCO_3$ and inoculated with *L. delbrueckii* as above. When the sugar concentration decreased to 10 g l^{-1}, solid glucose was added to bring the total sugar concentration to 30 g l^{-1} while adding a calculated amount of $CaCO_3$.

Whey supplemented with different sources of nitrogen

Whey containing $CaCO_3$ (30 g l^{-1}) was mixed with peptone (14.5 g l^{-1}), yeast extract (20 g l^{-1}), soya flour (34.5 g l^{-1}), or $(NH_4)_2SO_4$ (10 g l^{-1}). A control medium was prepared by adding an equal volume of sterilized distilled water (pH 6.5). The experiment proceeded as described above. Viable cell count was also monitored.

Whey supplemented with vitamin B complex and different nitrogen sources

To whey containing either peptone (14.5 g l^{-1}) or $(NH_4)_2SO_4$ (10 g l^{-1}), vitamin B complex (5 g l^{-1}) was added. Respective control media were prepared by avoiding the vitamin B complex. The pH of tests and controls was adjusted to 6.5. To compare the additive effect of the B complex with these nitrogen sources, yeast extract was taken alone and the experiment proceeded as before.

Supplementation of whey with different proportion of $(NH_4)_2SO_4$ and yeast extract as nitrogen sources

In whey, different proportions of $(NH_4)_2SO_4$ and yeast extract were dissolved (*Table 1*) and sterile $CaCO_3$ (30 g l^{-1}) was added. The experiment proceeded as described above.

Table 1 Supplementation of whey with different proportions of inorganic [$(NH_4)_2SO_4$] and organic (yeast extract) nitrogen sources

		Nitrogen source supplemented			
		$(NH_4)_2SO_4$		Yeast extract	
Flask	Ratio of elemental nitrogen $(NH_4)_2SO_4$/yeast extract	Weight (g)	Elemental nitrogen (g)	Weight (g)	Elemental nitrogen (g)
A	00/100			2.0	0.21
B	25/75	0.247	0.053	1.5	0.157
C	50/50	0.495	0.105	1.0	0.105
D	75/25	0.742	0.157	0.5	0.053
E	100/00	0.989	0.21		

Results and discussion

Whey supplemented with yeast extract

Whey has 1.3 g l^{-1} elemental nitrogen and 30 g l^{-1} total sugar. When whey was fermented by *L. delbrueckii* while the pH was maintained at 6.5 by the addition of 30 g l^{-1} $CaCO_3$, 20 g l^{-1} lactic acid was obtained at 72 h. To improve lactic acid production and decrease the downstream processing cost, whey was supplemented with varying concentrations (0, 10, 20, and 30 g l^{-1}) of yeast extract. In all cases, the initial total sugar level was kept at 30 g l^{-1}. Growth of *L. delbrueckii* increased with an increase in yeast extract supplementation up to 10 g l^{-1}, above this there was no significant increase in growth (*Figure 1*). *Lactobacilli* have complex nutrient requirements.[2] Therefore, it is essential to supplement whey with some commercially available growth supplements such as yeast extract, casamino acids, etc.[8] When whey was supplemented with yeast extract (10 g l^{-1}), lactic acid production was increased from 12 to 22.5 g l^{-1} with a concurrent increase in substrate use from 73% to 86.6% [Substrate use (%) = (Total sugar consumed [g]/Initial total sugar [g]) · 100] (*Table 2*). Further, when the medium was supplemented with yeast extract, the time taken for maximum lactic acid production also decreased from 72 to 48 h. The yeast extract supplementation not only increased the bacterial yield, but reduced the time required for the completion of fermentation. This could be due to the substances such as amino acids, peptides, vitamins, and several organic acids including pyruvic and glyceric acid in yeast extract.[2,9] Supplementation of whey with >10 g l^{-1} of yeast extract gave no further increase in lactic acid production and substrate use (*Table 2*). This could be due to carbon source limitation. Hence, an experiment was designed to increase the concentration of carbon source by introducing glucose at an appropriate time when the total sugar concentration declined to about 10 g l^{-1}. Here, 30 g l^{-1} was selected as the highest sugar concentration in the medium as higher sugar concentrations can exert an inhibitory effect.[10]

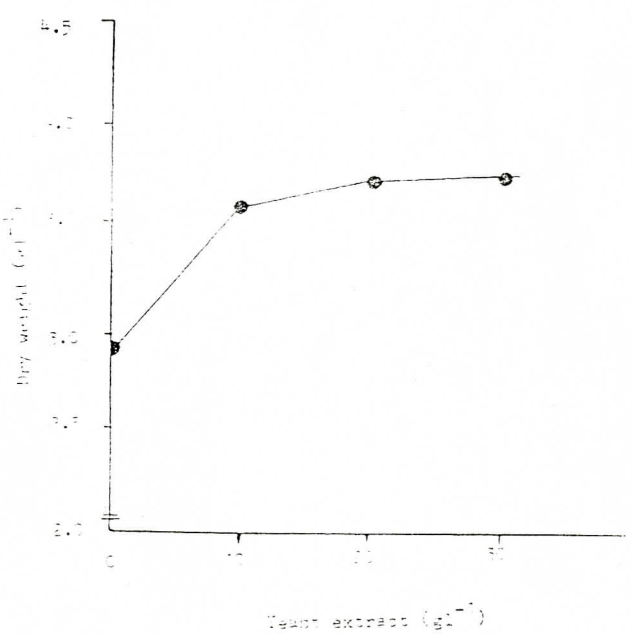

Figure 1 Effect of supplementing whey with yeast extract on growth of *Lactobacillus delbrueckii*

Table 2 Effect of supplementing whey with different concentrations of yeast extract on lactic acid production and residual total sugar left by *delbrueckii* as a function of time[a]

	Yeast extract ($g\ l^{-1}$)							
	0		10		20		30	
Time (h)	RTS ($g\ l^{-1}$)	LA ($g\ l^{-1}$)	RTS ($g\ l^{-1}$)	LA ($g\ l^{-1}$)	RTS ($g\ l^{-1}$)	LA ($g\ l^{-1}$)	RTS ($g\ l^{-1}$)	LA ($g\ l^{-1}$)
0	30.0		30.0		30.0		30.0	
12	24.5	5.0	22.0	7.0	22.5	7.0	21.0	8.0
24	20.0	7.5	16.0	10.5	15.0	12.0	15.5	13.0
36	16.5	10.0	10.0	17.5	9.0	18.0	10.0	17.5
48	13.5	12.0	4.0	22.5	3.0	23.0	3.0	22.5
60	11.0	15.5	3.0	23.0	3.0	23.5	3.0	24.0
72	8.0	20.0	3.0	23.0	3.0	24.5	3.0	24.0

[a]RTS, residual total sugar; LA, lactic acid

Whey supplemented with yeast extract and glucose

To increase lactic acid production, solid glucose (20 $g\ l^{-1}$) was added to whey supplemented with different concentrations of yeast extract when the total sugar level in the media had fallen to around 10 $g\ l^{-1}$. In all of the conditions considered, the total sugar level had fallen to about 10 $g\ l^{-1}$ at 36 h (*Table 3*). Sterile $CaCO_3$ (20 $g\ l^{-1}$) was also added, as the pH at this time had dropped to 5.0. The pH was maintained in the range of 6.5–5.0. Addition of yeast extract from 0 to 20 $g\ l^{-1}$ increased lactic acid production from 27.5 to 40 $g\ l^{-1}$ at 84 h. However, a further increase in yeast extract concentration had very little effect on increasing the lactic acid production (*Table 3*). From the results it can be concluded that supplementing whey with yeast extract and maintaining the total sugar concentration at 30 $g\ l^{-1}$ led to increased lactic acid production. Among the three conditions considered, whey supplemented with yeast extract (20 $g\ l^{-1}$) and glucose (20 $g\ l^{-1}$) at 36 h seems to be the best (*Table 3*). It was reported that supplementation of 30 $g\ l^{-1}$ yeast extract to enzyme-thinned corn starch was the best in respect to the rate of lactic acid production, but not for total lactic acid production by *L. amylovorus*.[11] Yeast extract in the range from 3 to 30 $g\ l^{-1}$ stimulated the rate of lactic acid production, and concentrations above 30 $g\ l^{-1}$ did not improve the production further. In our experiment, an increase in yeast extract concentration beyond 20 $g\ l^{-1}$ did not improve lactic acid production. The reduced requirement for yeast extract under our experimental conditions may be due to the higher assimilable protein content of whey than that in the enzyme-thinned cornstarch. Hence, supplementation of whey with 20 $g\ l^{-1}$ yeast extract was selected to avoid the limitation in essential growth factors for the bacteria.

Whey supplemented with different nitrogen sources

When whey was supplemented with 20 $g\ l^{-1}$ yeast extract, the elemental nitrogen level was 3.1 $g\ l^{-1}$. The effects of supplementing whey with different nitrogen sources such as

Table 3 Effect of supplementing whey with yeast extract at time 0 and with glucose at an appropriate time in lactic acid production and residual total sugar left by *Lactobacillus delbrueckii* as a function of time[a]

	Yeast extract ($g\ l^{-1}$)							
	0		10		20		30	
Time (h)	RTS ($g\ l^{-1}$)	LA ($g\ l^{-1}$)	RTS ($g\ l^{-1}$)	LA ($g\ l^{-1}$)	RTS ($g\ l^{-1}$)	LA ($g\ l^{-1}$)	RTS ($g\ l^{-1}$)	LA ($g\ l^{-1}$)
0	30.0		30.0		30.0		30.0	
12	23.0	4.0	20.0	7.0	19.0	9.0	18.0	9.0
24	20.0	7.0	15.0	11.0	13.0	13.0	12.0	13.5
36	15.0	10.5	10.0	12.5	9.5	17.5	9.0	18.0
36	30.0		30.0		30.0		30.0	
48	23.0	17.0	21.0	21.5	15.5	27.5	13.5	28.0
60	17.0	22.0	16.0	26.5	10.5	35.0	10.0	36.0
72	15.0	25.0	12.0	30.0	6.0	38.0	6.0	39.0
84	14.5	27.5	10.0	35.0	4.0	40.0	3.0	41.0

[a]RTS, residual total sugar; LA, lactic acid; 36–20 $g\ l^{-1}$ glucose was added to whey at 36 h

Table 4 Effect of supplementing whey with different nitrogen sources on growth of and substrate use, production, yield, efficiency, and production rate of lactic acid by *Lactobacillus delbrueckii*[a]

	Nitrogen source supplemented					
	0	Yeast extract	Peptone	Soya flour	$(NH_4)_2SO_4$	
	48 h	48 h	48 h	48 h	48 h	60 h
Lactic acid ($g\ l^{-1}$)	12.0	24.5	22.5	23.0	20.0	23.5
Viable cells ($ml^{-1} \cdot 10^9$)	2.4	3.8	3.6	3.8	2.8	3.1
Substrate use (%)	73.3	92.0	85.7	90.0	83.0	100.0
Efficiency (%)	90.0	94.0	93.0	89.0	87.4	89.3
Yield (%)	66.7	81.6	75.0	76.7		78.5
Production rate ($g\ l^{-1}\ h^{-1}$)	0.27	0.57	0.44	0.47	0.36	

$$^a\text{Yield} = \frac{\text{Lactic acid produced}}{\text{Theoretical yield on the basis of total sugar supplied}} \cdot 100$$

peptone, yeast extract, soya flour, and $(NH_4)_2SO_4$ having the same elemental nitrogen level (3.1 $g\ l^{-1}$) on the performance of *L. delbrueckii* were compared. Supplementation with different nitrogen sources has increased lactic acid production compared with unsupplemented whey (control). However, when the effects of different nitrogen sources on lactic acid production were considered, the difference was not appreciable (*Table 4*). *Lactivacillus delbrueckii* reached maximum growth at 48 h in medium containing organic nitrogen sources, and the viable cell number was almost same. In $(NH_4)_2SO_4$-supplemented medium, maximum growth was reached later in organic nitrogen source-supplemented medium. Ammonium ions influence the metabolism of certain amino acids in *Lactobacilli*[12] by their incorporation with either α-ketoglutarate or glutamate.[13] Thus, when $(NH_4)_2SO_4$ is used, the inorganic nitrogen should first be converted to amino acids and then used for the synthesis of proteins needed for growth and lactic acid production. This reason may explain the observed delay in growth and lactic acid production in whey supplemented with $(NH_4)_2SO_4$. However, in the case of organic nitrogen sources such as peptone, soya flour, and yeast extract, the amino acids could be directly obtained by protein hydrolysis. The rate of lactic acid production at 36 h was highest (0.5 $g\ l^{-1}\ h^{-1}$) in whey supplemented with yeast extract (*Table 4*). A suggestion was made that yeast extract must contain either a specific peptide or other growth factors which increase the rate of growth and lactic acid formation.[11] Among the different organic nitrogen sources, yeast extract seems to be the best and could be attributed to its B-complex vitamins.[11] Therefore, an experiment was formulated to study the effect of vitamin B complex on the performance of *L. delbrueckii* in whey supplemented with different nitrogen sources.

Table 5 Comparative studies on the additive effect of vitamin B complex in whey supplemented with either $(NH_4)_2SO_4$ or peptone on the dry weight of and substrate use efficiency, and lactic acid production by *Lactobacillus delbrueckii*

	Nitrogen source				
	Yeast extract	Peptone	Peptone (vitamin B complex)	$(NH_4)_2SO_4$	$(NH_4)_2SO_4$ (vitamin B complex)
Lactic acid ($g\ l^{-1}$)	25.0	22.5	23.5	22.0	24.5
Dry weight ($g\ l^{-1}$)	3.8	3.4	3.6	3.1	3.5
Substrate use (%)	96.6	86.6	93.4	93.4	100.0
Efficiency (%)	91.3	86.5	90.5	78.5	81.7

Whey supplemented with vitamin B complex and different nitrogen sources

Vitamin B complex was added to whey supplemented with different nitrogen sources. The growth, efficiency, and yield of lactic acid production by *L. delbrueckii* were almost similar in vitamin B complex-supplemented and -unsupplemented media (*Table 5*). These results show that addition of vitamin B complex had no significant influence on *L. delbrueckii.* Therefore, it could be concluded that the pronounced difference in lactic acid production in whey supplemented with yeast extract could possibly be due to some other growth-promoting factors present in yeast extract. Although sugar use was complete in whey supplemented with $(NH_4)_2SO_4$ medium (*Table 4*), the yield was not optimal; therefore, we decided to use a combination of organic (yeast extract) and inorganic $[(NH_4)_2SO_4]$ nitrogen sources.

Supplementation of whey with different proportions of $(NH_4)_2SO_4$ and yeast extract

Whey was supplemented with different proportions of $(NH_4)_2SO_4$ and yeast extract while keeping the elemental nitrogen content constant (3.1 g l^{-1}) and the total sugar level at 30 g l^{-1}. Growth of *L. delbrueckii* (presented as dry weight) was almost the same in all cases. Lactic acid produced at 48 h was almost similar in all cases (*Table 6*), but the lactic acid produced at 36 h in the media containing $(NH_4)_2SO_4$ and yeast extract was higher compared with $(NH_4)_2SO_4$ alone. Substrate use was highest when the ratio of $(NH_4)_2SO_4$ to yeast extract was 3:1, where 90% of the substrate was used with 95% of efficiency [Efficiency (%) = (Lactic acid produced (g)/Lactose consumed [g]) × 100] (*Table 6*). Performance of *L. delbrueckii* in this medium was comparable to whey supplemented with 20 g l^{-1} yeast extract. Therefore, with whey proteins, 7.5 g l^{-1} of $(NH_4)_2SO_4$ and 5 g l^{-1} yeast extract (reduced level) supplementation seem to give the same results as those of 20 g l^{-1} yeast extract. Thus, using $(NH_4)_2SO_4$ with yeast extract in a 3:1 ratio would reduce the cost of nitrogen source by 75%.

Table 6 The effect of supplementing whey with different proportions of inorganic $[(NH_4)_2SO_4]$ and organic (yeast extract) nitrogen sources on the performance of *Lactobacillus delbrueckii* at 48 h

$(NH_4)_2SO_4$/ yeast extract	Lactic acid (g l^{-1})	Dry weight (g l^{-1})	Substrate use (%)	Efficiency (%)
100/00	22.5	3.2	86.6	84.3
75/25	23.0	3.5	90.4	95.6
50/50	23.5	3.6	83.5	94.5
25/75	24.5	3.6	90.2	94.5
00/100	25.0	3.7	89.6	95.6

Conclusions

These results show that lactic acid production could be increased by supplementing whey with yeast extract and $(NH_4)_2SO_4$ as nitrogen sources and glucose as carbon source.

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