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Full Length Research Paper

Effect of amino acid requirements on the growth and lactic acid production of *PEDIOCOCCUS ACIDILACTICI* culture

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The aim of this research was to investigate essential groups of amino acids and effect of amino acid supplementation. *P. ACIDILACTICI* culture was grown in chemically defined medium (CDM) with initial pH 6.35 and at 30°C. To minimize the number of experiments, the experimental design was based on grouping the metabolic amino acid pool. The selected amino acid groups were the following: a) phenylalanine and tyrosine; b) lysine, methionine, isoleucine; c) leucine and valine and d) histidine. The concentration of each selected amino acid in original CDM was 50 mg/l while its concentration in the modified CDM was reduced to 25 mg/l. Concentrations of biomass, lactic acid, glucose, and selected amino acids were measured. It was found that amount of Ile, Met, and Lys added in CDM-grown *P. ACIDILACTICI* was directly affected lactic acid production rate. Limitation of Phe, Tyr, Leu, Val, and His concentration added had the largest effect on cell growth and lactic acid production. The maximum specific growth rate (μ_{max}) and the lactic acid production rate obtained from fermentation in modified CDM with 25 mg/l of Phe and Tyr (Run # A) and Leu and Val (Run # C), as compared with fermentation in original CDM, were improved by a factor of 3.4 and a factor of 2.7, respectively. The sub-optimal composition of amino acids in modified CDM was 25 mg/l of Phe, Tyr, Leu and Val at pH 5.5-5.8.

Key words: Lactic acid bacteria, *Pediococcus acidilactici,* lactic acid, amino acid.

INTRODUCTION

Lactic acid bacteria (LAB) are commonly defined as gram-positive, non-spore forming, catalase-negative, and vary in morphology from long, slender rods, which frequently form chains. LAB are facultative anaerobic bacteria with a carbohydrate fermentative metabolism and produces lactic acid as the major end-product. The main genera of LAB include *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* (Axelsson, 1998). LABs are widely used in the manufacturing of foods and beverages, such as dairy fermentations, meat and fish fermentations, vegetable fermentations, and sourdough fermentation (Leroy and Devuyst, 2004). The product of LAB fermentation, lactic acid, is applied as a preservative, acidulant and flavourant in food processing (Liu, 2003), as an intermediate in pharmaceutical and cosmetic manufacture and in the manufacture of biodegradable polylactic acid polymers (Martin, 1996).

The growth of LAB is optimum at pH 5.5-5.8, 25-40°C and the organisms have complex nutritional requirements for amino acids, peptides, nucleotide bases, vitamins, minerals, fatty acids and carbohydrates. Mills and

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Thomas (1981) suggested that amino acids, peptides and proteins may be important as nitrogen sources for cell growth. LAB can be stimulated to grow faster and reach higher cell densities in complex medium containing more easily convertible nitrogen such as amino acids or proteins with small molecular weight. Evidently, in the studies of Jensen and Hammer (1993) and Niven et al. (1998) *Lactococcus lactis* used as starter cultures for the manufacture of cheese are fastidious organisms that have complex amino acid requirements, that is, Glu, His, Ile, Leu, Met and Val being essential for most strains. Juillard et al. (1995) observed that biphasic logarithmic growth of *L. lactis* in milk which was consistent with milk oligopeptides as an essential nitrogen source during the early stages of growth, and proteolysis becoming more important during the later stages. Chemically-defined medium (CDM) have been studied to sustain the growth of most strains of *Streptococcus thermophilus* (Letort and Juillard, 2001). It was found that a minimal medium containing 20 components, including one carbohydrate source, six amino acids, two metallic ions, six vitamins and urea allowed for growth of 13 out of 15 *S. thermophilus* strains. Growth of the two last strains, ST 23 and ST 8 required the presence of additional amino acid, His (0.1 g/l) and His (0.1 g/l) and Pro (0.15 g/l), respectively. Growth rates of the strains ranged from 0.38 to 0.64 h^{-1}, and final populations were about 10^8 cfu ml^{-1}. The study of Letort et al. (2002) indicated that the growth rate of *S. thermophilus* ST18 was 3.3 h^{-1} when milk was supplemented with glutamine (2.6 g/l) and methionine (1.0 g/l). Leu, Met and Gly were essential to the growth of *Pediococcus pentosaceus* (Fernández et al., 2003). When these amino acids were independently removed from medium, biomass concentration were 90% lower than in the complete medium.

Currently, the primary genera of LAB used as meat starter culture is *Pediococcus acidilactici*. It is homo-fermentative, producing large amounts of lactic acid from carbohydrate. Properly selected, physiologically active starter culture will ensure the required pH decrease, safety of sausage as well as improve uniformity of product in the sense of flavour, appearance and texture and shorten the production cycles. *P. acidilactici* has numerous nutritional requirements for growth, especially amino acids as nitrogen sources. However, a complex laboratory medium cannot be used for the examination of nutritional requirements of the strain. Up-to-date available information relating to amino acid catabolism in LAB is relatively little (Christensen et al., 1999). The aim of this work was to investigate essential groups of amino acids and effect of amino acid additions.

MATERIALS AND METHODS

Bacterial strain

The *P. acidilactici* used in this study was granted from National center for Genetic Engineering and Biotechnology culture collection, Thailand. *P. acidilactici* was routinely grown in CDM at 30°C for 24 h and then stored at -80°C in fresh medium containing 16% glycerol.

Medium preparation

The chemically-defined medium (CDM) consisted of free amino acids, vitamins, metabolic ions and nucleic acid bases as shown in Table 1. Stock solutions of tryptophan, tyrosine, cysteine and adenine and stock solutions of other individual nutrients were sterilized by filtration through a 0.22 µm pore-size membrane and autoclave, respectively. The CDM was prepared by adding appropriate volumes of each concentrated stock solution to obtain the final concentration of individual compounds (Table 1). The medium was then adjusted to pH 6.35 with 2 N NaOH or 2 N HCl.

Culture condition

P. acidilactici culture-grown original CDM was the baseline experiment of its amino acid requirements. To minimize the number of experiments, the experimental design was based on grouping the metabolic degradation of amino acids to common metabolic intermediates. The interested amino acid groups were the following: a) phenylalanine and tyrosine; b) lysine, methionine, isoleucine; c) leucine and valine and d) histidine (Ayad et al., 1999). The experiments were divided into five batches as shown in Table 2. Each experiment was performed in duplicate with two different flasks.

P. acidilactici was precultured on CDM and incubated at 30°C for 24 h. Cultures were then transferred to the CDM in Table 2. Inoculation of growth medium was at 10% (v/v). *P. acidilactici* was cultured in a 3 L Erlenmeyer flask with a working volume of 1.2 L. After inoculums *P. acidilactici*, culture was not centrifuged to preparation of cells from the medium.

Therefore, the remaining medium in inoculums was mixed with the growth medium prepared as listed in Table 2. The initial pH of culture was 6.35. The shaker was controlled at 30°C and 120 rpm. Samples were aseptically taken every 6 h following the start of incubation until stationary phase.

Biomass concentration

Cell density was measured by spectrophotometric measurements (U1201, Shimadzu, Tokyo, Japan) at 600 nm (OD_{600}) and then converted to biomass concentration (g/l).

The correlation of optical density was Y (g/l) = (0.3684 × OD) +0.1228.

Chemical analysis

The culture sample was centrifuged at 4000·*g* for 15 min (CN180 nüve, Nkata, Turkey) to separate cells. The supernatant was then analyzed.

Glucose concentration

The phenol-sulfuric acid method (Dubois et al., 1956) was applied to determine glucose concentration in samples. The absorbance at 490 nm was then measured.

Table 1. Composition of the chemically-defined medium.

Compound	Concentration (g/l)	Compound	Concentration (g/l)
Glucose	10	Glycine	0.05
CH_3COONa	2	L-Asparagine	0.05
Tween 80	1	L-Tryptophan	0.05
$Na_2HPO_4.H_2O$	1.75	L-Serine	0.05
KCl	0.75	L-Alanine	0.05
$MnSO_4.H_2O$	7.675×10^{-3}	L-Phenylalanine	0.05
$MgSO_4.7H_2O$	0.2	L-Histidine	0.05
Sodium gluconate	10	L-Isoleucine	0.05
p-Aminobenzoic acid	4×10^{-4}	L-Leucine	0.05
Pyridoxal	5.02×10^{-4}	L-Methionine	0.05
Nicotinic acid	0.001	L-Lysine	0.05
Folic acid	2×10^{-4}	L-Proline	0.05
Ca Panthotenate	0.001	L-Threonine	0.05
Riboflavin	0.001	L-Valine	0.05
Thiamine	0.001	L-Arginine	0.05
Adenine	5×10^{-6}	L-Tyrosine	0.05
Guanine	1×10^{-5}	L-Cysteine	0.05
Uracil	1×10^{-5}	L-Glutamine	0.05

Table 2. Concentrations of interested amino acid in each experiment.

Run #	Amino acids	Concentration (mg/l)
Control	original CDM	50
A	Phe and Tyr	25
B	Lys Met and Ile	25
C	Leu and Val	25
D	His	25

Concentrations of other amino acids were listed original CDM in Table 1.

Lactic acid concentration

Lactic acid was determined by a spectrophotometric method with an enzymatic reagent (R-Biopharm, Darmstadt, Germany). 1 ml of 0.6 M glycylglycine and 0.1 M L-glutamate buffer, pH 10, 0.2 ml of 0.04 M NAD^+, 0.8 ml of distilled water, 20 µl of 20 mg/ml glutamate pyruvate transaminase suspension and 0.2 ml of sample were placed in a cuvette, mixed and allowed to incubate for 5 min before measuring the absorbance at 340 nm, the value of which is A_1. 20 µl of 5 mg/ml L-lactic dehydrogenase suspension was then added and after mixing was allowed to stand for 20 min before the absorbance was measured. This is A_2. The difference between A_2 and A_1 is ΔA_S. The same procedure but substituting 0.2 ml of sample by distilled water was used to determine the blank value, ΔA_B. The concentration in g/l was calculated from $C = 0.160\,(\Delta A_S - \Delta A_B)$.

Free amino acid analysis

The concentration of amino acid was determined by high-performance liquid chromatography (HPLC). Derivatization was performed using ortho-phthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC). Derivatized samples were analyzed by reversed phase high-performance liquid chromatography (RP-HPLC) (Agilent 1000 Series, Palo Alto, CA, USA) equipped with a DAD detector (Agilent 1200 Series, Palo Alto, CA, USA). The separation was performed using an amino acid column 200×2.1 mm (Agilent Technologies) and guard column (Hypersil ODS, 20×2.1 mm, Agilent Technologies). The gradient was used between two solvents: A, 20 mM sodium acetate pH 7.2 containing 0.018% triethylamine and B, 20% of 100 mM sodium acetate pH 7.2 containing 40% acetonitrile and 40% methanol. The detection wavelength was 338 nm.

RESULTS AND DISCUSSION

The influence of essential groups of amino acids on the growth of *P. acidilactici* was investigated. Fermentations were performed in chemically-defined medium (CDM) which have different amino acid concentration. The experiments were divided into five batches as shown in Table 2.

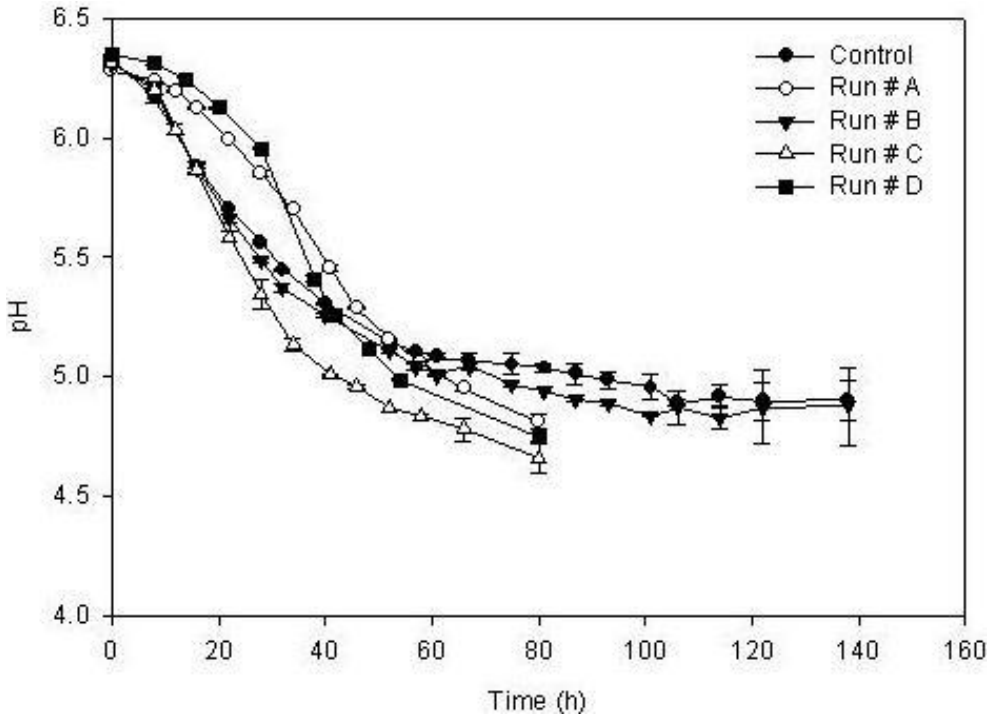

Figure 1. Profiles of pH in CDM-grown *P. acidilactici* culture.

Table 3. Effect of amino acid concentration on the growth of *P. acidilactici* and lactic acid production.

Batch	Amino acids	Concentration (mg/l)	Glucose consumption rate $\frac{dS_{glu}}{dt}$ (g/l-h)	Specific growth rate μ_{max} (h^{-1})	Biomass Yield $Y_{x/s}$ (g cell / g glu)	Lactic acid production rate $\frac{dC_{Lac}}{dt}$ (g/l-h)
Control	original CDM	50	0.061	0.018	0.269	0.23
A	Phe and Tyr	25	0.006	0.059	0.981	0.66
B	Lys, Met and Ile	25	0.046	0.036	0.034	0.14
C	Leu and Val	25	0.024	0.061	0.923	0.57
D	His	25	0.056	0.122	0.315	0.43

Change in pH

The change in pH of the fermentation of *P. acidilactici* in each CDM is shown in Figure 1. The initial pH of all batches was about 6.35. The pH was drastically dropped during the growth phase between 12 and 48 h of fermentation and then slowly decreased after 48 h. At the end of fermentation, pH of *P. acidilactici* fermented in Run # A (25 mg/l Phe and Tyr), Run # B (25 mg/l Lys Met and Ile), Run # C (25 mg/l Leu and Val), and Run # D (25 mg/l His) were 4.81, 4.88, 4.66, and 4.75, respectively, while that of *P. acidilactici* fermented in original CDM (control) was 4.90. The decrease in pH values corresponded to the production of organic acids mainly lactic acid by LAB (Komprda et al., 2004).

Glucose consumption rate

Glucose consumption rate is shown in Table 3. The result indicated that *P. acidilactici* grown in original CDM (control) had the highest glucose consumption rate at 0.061 g/g·h whereas *P. acidilactici* grown in modified CDM with 25 mg/l of Phe and Tyr (Run # A) and modified CDM with 25 mg/l Leu and Val in CDM (Run # C) showed the lowest at 0.006 g/g·h and 0.024 g/g·h, respectively. Nevertheless, $Y_{X/S}$ obtained from Run # A and Run # C were higher than the result of control experiment. The amino acid concentration in Table 4 showed that Phe and Tyr concentration in Run # A and Val concentration in Run # C were intensely decrease. These indicated that Phe, Tyr, and Val were essential for facilitating growth of

Table 4. Changes in amino acid concentration in CDM during fermentation of *P. acidilactici.*

Batch	Amino acid concentration (mg/l)							
	Phe	**Tyr**	**Lys**	**Met**	**Ile**	**Leu**	**Val**	**His**
Control								
0 h	46.07 ± 2.15	47.75 ± 2.17	32.94 ± 3.79	41.26 ± 3.37	47.34 ± 2.01	49.23 ± 3.62	47.12 ± 2.21	61.07 ± 9.82
24 h	28.42 ± 2.62	36.12 ± 1.26	26.39 ± 2.19	38.11 ± 2.13	34.89 ± 1.58	46.41 ± 3.21	45.02 ± 3.27	55.82 ± 4.09
72 h	18.82 ± 1.46	22.99 ± 2.41	16.40 ± 1.35	33.17 ± 1.22	32.97 ± 1.30	43.18 ± 2.35	41.54 ± 3.71	44.75 ± 2.12
Run # A								
0 h	20.83 ± 2.74	21.15 ± 0.90	29.23 ± 3.76	48.22 ± 5.50	40.54 ± 0.95	58.97 ± 8.81	44.92 ± 4.92	51.20 ± 5.59
24 h	4.29 ± 0.60	6.28 ± 1.66	19.80 ± 1.58	35.59 ± 3.77	37.84 ± 0.60	53.81 ± 4.31	42.21 ± 1.94	47.46 ± 4.94
72 h	0.00 ± 0.00	0.00 ± 0.00	12.08 ± 1.94	32.20 ± 3.75	31.58 ± 0.77	37.36 ± 3.72	39.09 ± 2.27	42.16 ± 2.04
Run # B								
0 h	50.30 ± 0.28	53.22 ± 3.97	14.24 ± 2.97	27.76 ± 3.84	27.11 ± 2.31	51.16 ± 4.92	43.28 ± 3.19	57.23 ± 7.41
24 h	35.67 ± 4.14	41.26 ± 4.41	10.89 ± 2.41	24.07 ± 2.34	25.86 ± 2.45	35.84 ± 2.68	34.98 ± 4.65	43.74 ± 3.86
72 h	18.55 ± 2.25	30.51 ± 1.79	6.42 ± 1.09	16.84 ± 1.07	23.07 ± 1.01	24.11 ± 1.36	27.51 ± 0.21	41.78 ± 2.05
Run # C								
0 h	45.67 ± 4.47	33.36 ± 2.56	29.31 ± 4.23	48.45 ± 3.46	44.15 ± 4.56	23.87 ± 3.11	20.73 ± 2.79	50.59 ± 3.17
24 h	29.69 ± 2.21	5.44 ± 0.34	21.53 ± 2.58	39.27 ± 2.27	34.89 ± 4.03	21.33 ± 1.46	16.22 ± 2.73	34.81 ± 1.37
72 h	17.76 ± 2.30	0.00 ± 0.00	15.23 ± 1.69	33.34 ± 2.62	30.66 ± 3.22	20.13 ± 1.33	13.93 ± 2.45	22.60 ± 2.36
Run # D								
0 h	42.11 ± 4.06	39.53 ± 3.95	15.11 ± 1.15	33.39 ± 2.61	38.20 ± 2.64	52.06 ± 2.59	34.28 ± 3.41	25.42 ± 1.95
24 h	27.29 ± 3.11	25.51 ± 2.11	3.00 ± 0.43	23.53 ± 0.83	15.35 ± 1.51	22.49 ± 0.57	16.31 ± 2.76	20.43 ± 1.88
72 h	12.00 ± 0.87	12.86 ± 1.86	1.47 ± 0.16	17.74 ± 0.71	8.20 ± 0.53	14.00 ± 0.63	8.91 ± 0.38	18.53 ± 0.53

Initial concentration of studied amino acid was reduced to 25 mg/l.

P. acidilactici. Glucose consumption rates obtained from Run # B (25 mg/l Lys Met and Ile) and Run # D (25 mg/l His) were 0.046 and 0.056 g/g·h, respectively.

One of the important criteria for lactic acid bacteria is the initial conversion of sugars to lactic acid, the primary acid responsible for decreasing the pH in fermented medium (Lee et al. 2006; Hammes and Knauf 1994). Furthermore, carbohydrates or sugars are a primary source of energy for microorganism. According to Adamberg et al. (2006), LAB could produce their major energy requirements by fermenting amino acids and thus utilizing carbohydrates for biomass synthesis.

Biomass concentration

The growth of *P. acidilactici* during fermentation process is illustrated in Figure 2. *P. acidilactici*

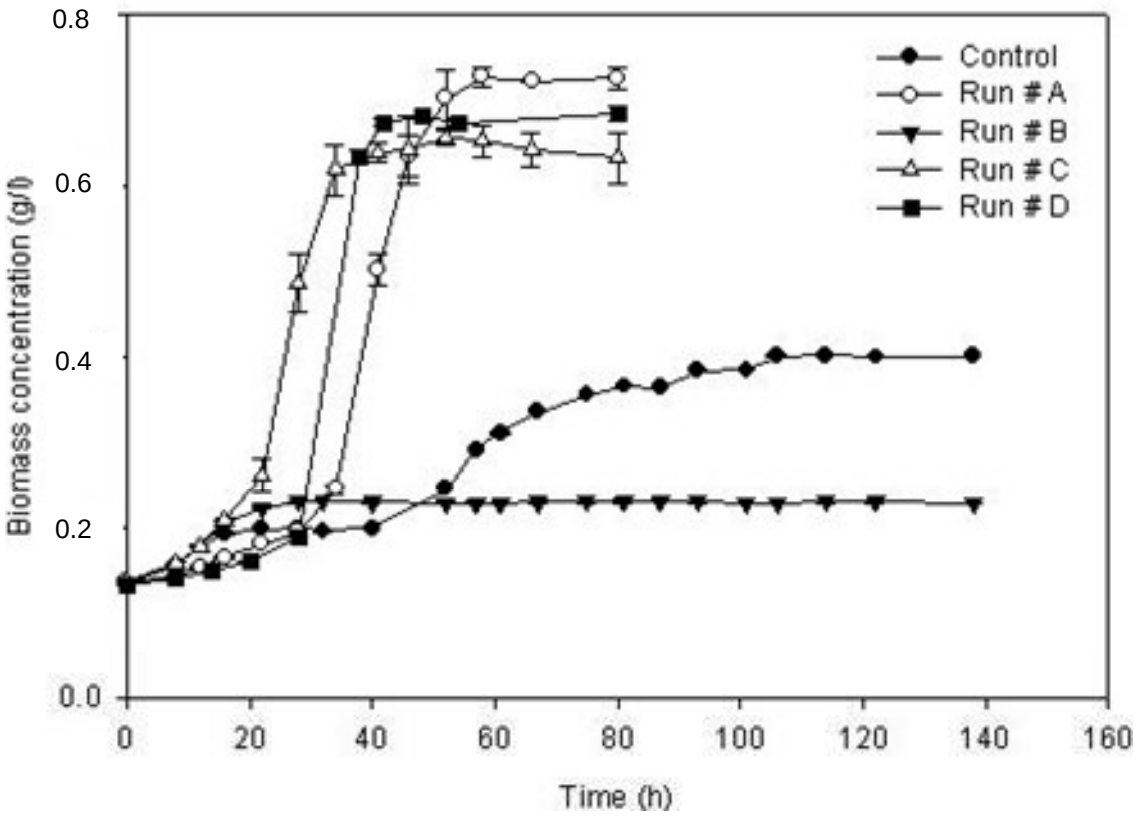

Figure 2. Profiles of biomass concentration in CDM-grown *P. acidilactici* culture *Lactic acid concentration.*

fermented in Run # A (25 mg/l Phe and Tyr), Run # C (25 mg/l Leu and Val), and Run # D (25 mg/l His) yielded the highest biomass concentration at 0.73, 0.66, and 0.68 g/l, respectively. The lowest biomass concentration (0.23 g/l) was observed in Run # B (25 mg/l Lys Met and Ile) batch, while biomass concentration of control experiment was 0.38 g/l. The maximum specific growth rate (μ_{max}) and biomass yield ($Y_{X/S}$) are shown in Table 3. The highest μ_{max} was observed in *P. acidilactici* grown in CDM with 25 mg/l His (Run # D) was 0.122 h^{-1}, whereas the lowest μ_{max} 0.018 h^{-1} was obtained from *P. acidilactici* grown in original CDM with 50 mg/l amino acids (control). The highest values of $Y_{X/S}$ obtained from *P. acidilactici* grown in CDM with 25 mg/l of Phe and Tyr (Run # A) and 25 mg/l of Leu and Val (Run # C) were 0.981 and 0.923 g cell/ g glucose, respectively. These $Y_{X/S}$ values were higher than the theoretical value of $Y_{X/S}$ based on glucose conversion to biomass, because Phe and Tyr (Run # A) and Val (Run # C) were depleted rather than glucose (Table 4). Compared to *P. acidilactici* grown in original CDM (control), μ_{max} and $Y_{X/S}$ obtained from *P. acidilactici* grown in 25 mg/l of Phe and Tyr in CDM were about 3.3-and 3.6- fold, respectively.

This may be attained that limitation of concentration of Phe and Ty could stimulate fumarate and acetoacetate production in TCA cycle (Voet and Voet, 1995). Due to the increase of fumarate and acetoacetate, biomass production rate and biomass yield ($Y_{X/S}$) were higher, which in turn the lactic acid production rate were increased.

The experiment Run # B (25 mg/l Lys Met and Ile) gave the minimum $Y_{X/S}$ value of 0.034 g cell/ g glucose. It may be attained that limitation of Lys, Met, and Ile had an inverse effect on biomass and lactic acid synthesis (Table 3). Evidently, concentration of Lys, Met, and Ile were slightly consumed (Table 4).

Compared to *P. acidilactici* grown in control, μ_{max} and obtained from *P. acidilactici* grown in 25 mg/l of His $Y_{X/S}$ in CDM were 6.9 and 1.2 fold, respectively. It was pronounced that limitation of His concentration had a direct effect on biomass production.

These implied that the high concentration of Phe and Tyr (50 mg/l) resulted in substrate inhibition. It may be attributed that the synthesis of fumarate and acetoacetate in TCA cycle was inhibited by incomplete synthesis of Asp, Phe and Tyr in TCA cycle (Voet and Voet, 1995). As $Y_{X/S}$ decreased.

The evolution of lactic acid concentration was observed in all experiments as shown in Figure 3. The decrease of pH was attributed to organic acids, mainly lactic acid production by LAB. In comparison of *P. acidilactici* grown

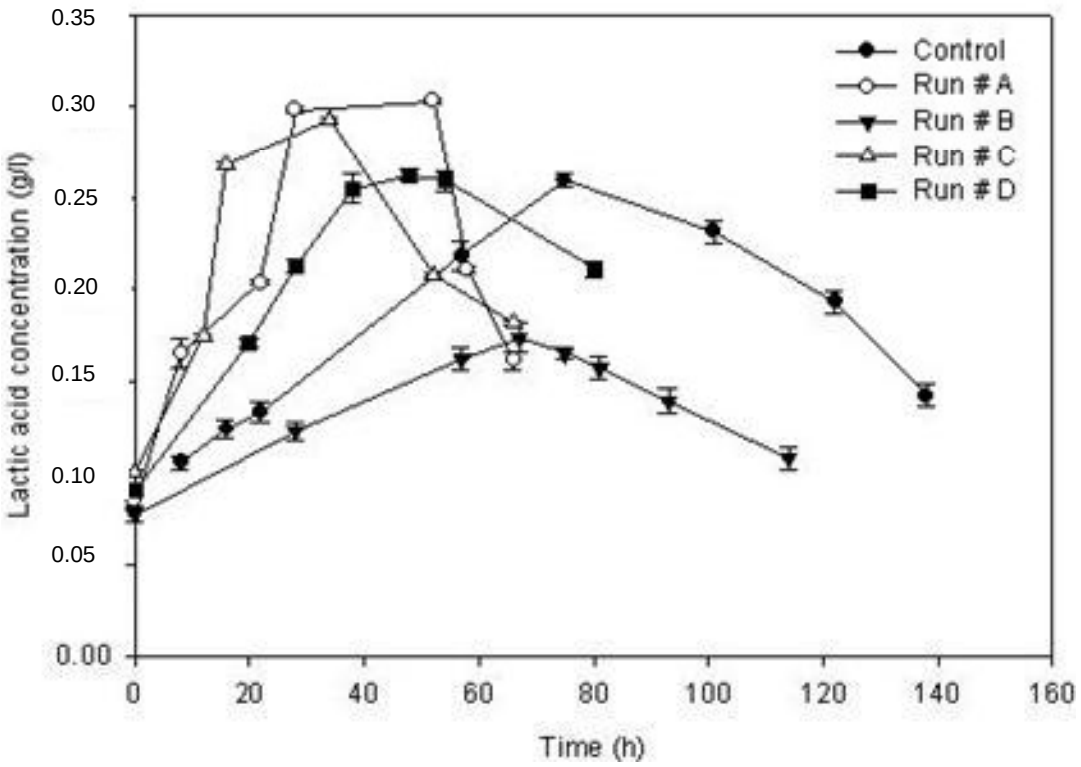

Figure 3. Profiles of lactate concentration in CDM-grown *P. acidilactici* culture.

in original CDM (control), 25 mg/l of Phe and Tyr (Run # A), 25 mg/l of Lys Met and Ile (Run # B), 25 mg/l of Leu and Val (Run # C), and 25 mg/l of His (Run # D) showed the increase of lactic acid concentration for the first 78, 52, 67, 34, and 48 h of fermentation, respectively.

Afterwards, the lactic acid concentration decreased towards the end of fermentation. Maximum values of lactic acid concentration for the control, Run # A, Run # B, Run # C, and Run # D batches were 0.25, 0.30, 0.17, 0.29, and 0.26 g/l, respectively. The initial lactic acid production rates in all batches are presented in Table 3. The maximum lactic acid production rate value at 0.66 g/l-h was obtained from *P. acidilactici* inoculated in CDM with 25 mg/l of Phe and Tyr (Run # A) whereas the experiment Run # B (25 mg/l Lys Met and Ile) yielded the lowest lactic acid production rate of 0.14 g/l-h. It indicated that the limited concentration of 25 mg/l of Phe, Tyr, Leu, and Val induced the production rate of lactic acid (Table 3). It was observed that lactic acid synthesis was *P. acidilactici* growth associated.

The results of glucose consumption rate, specific growth rate (μ_{max}), biomass yield ($Y_{X/S}$), and lactic acid production rate (dC_{Lac}/dt) are shown in Table 3.

Amino acid concentration

The result of essential amino acid concentration in CDM during fermentation of *P. acidilactici* is shown in Table 4.

It was observed that Phe and Tyr were drastically dropped during the growth phase of fermentation in all batches, especially in Run # A supplied with the limited concentration of 25 mg/l Phe and Tyr. In accompany with biomass yield and lactic acid production rate, *P. acidilactici* cultured in CDM with 25 mg/l Phe and Tyr (Run # A) caused the highest biomass yield and lactic acid production rate (Table 3). In contrast, the amount of Met, Ile, Leu, Val, and His was slightly decreased. It indicated that Phe and Tyr could enhance the growth and the lactic acid production of *P. acidilactici*, whereas the Ile, Met, and Lys concentration slightly affected the growth of *P. acidilactici* (Table 3). In the study of Fernández et al. (2003), Leu, Met, and Gly were essentials to growth of *Pediococcus pentosaceus*. Most strains of *Lactocuccus lactis* required essential amino acids such as Glu, His, Ile, Leu, Met, and Val (Jensen and Hammer, 1993).

For initial concentration of Lys, amongst ketogenic amino acids, carbon skeletons breakdown to acetyl-CoA or acetoacetate, Lys is non-aromatic amino acid. Thus, Lys is more easily degraded to acetoacetate than aromatic amino acids such as Phe and Tyr.

Conclusion

The most required amino acids for the growth and lactic acid production of *P. acidilactici* were Phe and Tyr. The

limitation of Phe, Tyr, Leu, Val, and His concentration (25 mg/l) significantly affected the production of biomass and lactic acid. Evidently, high substrate concentration of these five amino acids, that is, 50 mg/l, caused inhibition of cell growth. The results were found that the amount of Ile, Met, and Lys added in CDM-grown *P. acidilactici* had a direct effect on lactic acid production rate. It was suggested that limited concentration (25 mg/l) of Phe, Tyr, Leu, and Val were the sub-optimal composition of CDM for growth and lactic acid production of *P. acidilactici*.

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