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Full Length Research Paper

Preliminary study on metabolic regulation and control of L-valine fermentation in a newly screened L-valine producing *Brevibacterium flavum* strain

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A L-valine hyper-producer *Brevibacterium flavum* XQ-6 (Leu^{l}, Ile^{l}, AHV^{r}, $\alpha\text{-}AB^{hr}$, $2\text{-}TA^{hr}$) was obtained, which could be resistant to high concentration of α-amino-butyric acid (α-AB) and 2-thiazolealanaine (2-TA). The metabolic network of XQ-6 was regulated with the addition of amino acids, organic acids, vitamins, bases and other organic things. The carbon flux was directed to L-valine by manipulating the specific activity of α-ketoglutarate dehydrogenase complex (KGDH). With a combinational regulation strategy, the highest L-valine concentration of 67.7 g/L was achieved.

Key words: L-valine, *Brevibacterium flavum*, HPLC, metabolic analysis.

INTRODUCTION

L-valine is an essential amino acid mainly used for pharmaceutical purposes and as precursor in chemical synthesis (e.g., anti-viral drugs). It has a market volume of approximately 500 tons per year with relatively high market prices (Magnus et al., 2006; Eggeling et al., 2001). In the metabolism of *Brevibacterium flavum*, L-valine is formed in four steps beginning with the condensation of two pyruvate molecules to acetolactate by acetolactate synthase (Figure 1) (Leyval et al., 2003). The same enzymes catalyzing the formation of L-valine are also involved in the formation of the other branched chain amino acids, L-leucine and L-isoleucine. The direct precursor of L-valine is α- ketoisovalerate, which is also used for L-leucine synthesis as well as for the formation of pantothenate (Hűser et al., 2005).

L-valine can be produced by chemical synthesis (D, L-valine), fermentation and by extraction from animal raw materials. Nowadays, the way of fermentative production is getting more important due to a higher flexibility of produced amounts and the rising sensibility against risks from products based on animal origins.

The advances in the amino acid fermentation industry are closely connected with screening or selection of suitable putative production organisms and subsequent improvement of the production strains. Our attempts at strain improvement have relied on classical mutagenesis and screening procedures, which focused not only on deleting competing pathways and eliminating feedback regulations in the biosynthetic pathways but also many other things which are important to the practice on the large scale in the plant.

Brevibacterium lactofermentum NO.487 which could be resistant to high concentration of 2-thiazolealanaine (2-TA) was obtained by Tsuchida from *B. lactofermentum NO.*2256 by N-methyl-N'-nitro-soguanidine (NTG) treatment in 1975. And the mutant produced 31 mg/mL L-valine in the medium containing 10% of glucose (Kinoshita, 1975).

Using *Corynebacterium glutamicum* as a starting strain for mutagenesis, a L-valine hyper-producer was obtained by Katsumada in 1993, which could produce 26 mg/ml of L-valine. And a strain which produced 39 mg/mL of L-valine was obtained by Katsumada in 1996 (Katsumada, 1993). More recently, L-valine excretion by *C. glutamicum*

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Abbreviations: EMP, Embden-Meyerhof-Parnas pathway; **HPLC,** high performance liquid chromatogratography; **NTG,** N-methyl-N'-nitro-soguanidine; **HMP,** hexose monophophate pathway; **KGDH,** α-ketoglutarate dehydrogenase complex; **α-AB,** α-amino-butyric acid; **DES,** diethylstilboestrol; **2-TA,** 2-thiazolealanaine.

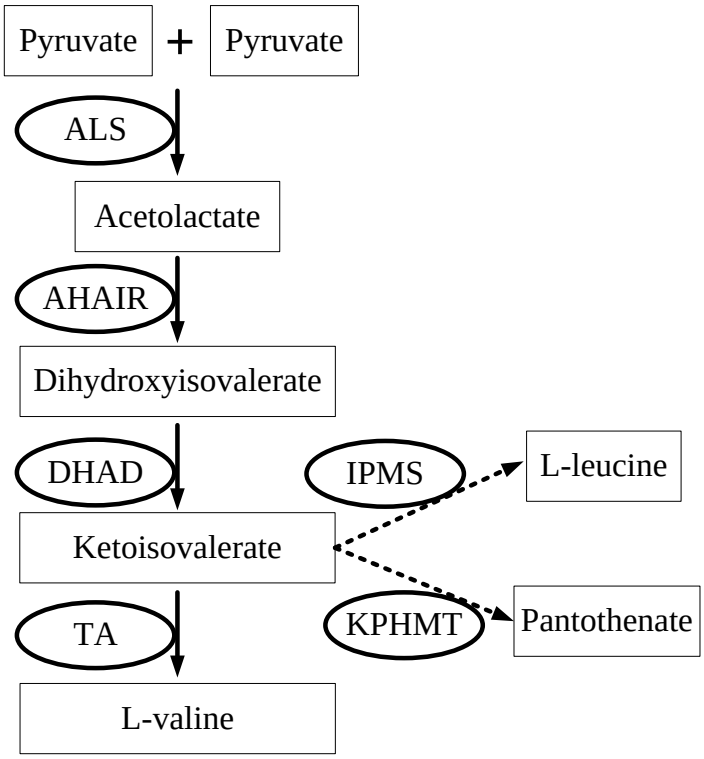

Figure 1. Biosynthesis of L-valine in *Brevibacterium flavum*. Abbreviations: ALS, acetolactate synthase; AHAIR, acetohydroxy acid isomeroreductase; DHAD, dihydroxy acid dehydratase; TA, transaminase; IPMS, isopropylmalate synthase; KPHMT, ketopantoate hydroxymethyltransferase.

strains modified by recombinant DNA techniques has been reported (Radmacher et al., 2002; Leyval et al., 2003).

However, the amounts of the L-valine produced by the strains mentioned above seem to be too small for the industrial production of L-valine. And genetically modified strains can not keep steady on a large scale production in the plant now. Accordingly, our newly screened L-valine producing strain *B. flavum* XQ-6 is quite appreciable, which is not only a L-valine hyper-producer, but also can keep very steady on a large scale in the plant.

For the successful development of biotechnological processes, an effective redirection of metabolic fluxes towards the desired product is essential (Sahm et al., 2000; Lee et al., 2005). The substrate, in many cases glucose, should be converted to the product of interest very effectively.

In order to strengthen the metabolic path of L-valine production by XQ-6, the metabolic network was analyzed. And the metabolic network was regulated with the addition of amino acids, organic acids and vitamins. To direct more carbon fluxes to L-valine biosynthesis pathway, carbon fluxes into tricarboxylic acid cycle (TCA) should be cut down. KGDH (α-ketoglutarate dehydrogenase complex) is a key enzyme in TCA cycle and to decrease the carbon fluxes into TCA cycle, one of the effective ways is to set back carbon fluxes in TCA cycle, that is, inhibit the activity of the KGDH. For the inhibition of the KGDH, addition of hydrogen peroxide, a specific inhibitor of KGDH (Tretter and Adam-Vizi, 2000; Applegate et al., 2008), could be considered to decrease the KGDH activity.

In this study, a combinational regulation strategy for enhanced L-valine production based on simultaneous addition of H_2O_2, the amino acids and biotin, was proposed. The effectiveness of the strategy was testified experimentally.

MATERIALS AND METHODS

Microorganisms and medium

B. flavum XQ5122, an original producer of L-valine, was kept in our laboratory. The identification of this strain was performed according to the descriptions of Buchanan (1984). It was maintained on agar slants and subcultured every month. The culture medium used for the storage of *B. flavum* XQ5122 contained (g/L): peptone 10, beef extract 10, yeast extract 5, NaCl 2.5, agar strip 20, adjusted to pH = 7.0 with 0.1 M of HCl or NaOH.

Mutant ZQ-2, a producer of L-valine, is isolated from N-methyl-N'-nitro-soguanidine (NTG) mutagenesis on strain V4-153 which is a mutant of *B. flavum* XQ5122.

XQ-6, a producer of L-valine, is isolated from NTG and diethylsulfate

Table 1. Effect of various amino acids on L-valine production by XQ-6.

Addition of amino acids	0.1 g/L	0.4 g/L
	L-valine produced (g/L)	
L-alanine	58.5	53.5
L-histidine	56.8	56.5
L-glutamic acid	55.7	55.9
L-aspartic acid	55.8	54.9
L-isoleucine	57.2	52.3
L-leucine	56.3	50.4
L-phenylalanine	55.1	54.2
L-lysine·HCl	56.9	55.8
L-proline	55.2	55.4
Glycine	57.9	54.1
L-glutamine	56.1	55.8
L-methionie	55.9	52.8
L-threonine	57.8	55.7
L-cysteine	56.2	55.9
L-valine	57.6	48.9
L-arginine	56.9	56.0
Not added	58	

Fermentation medium: glucose 12.5, $(NH_4)_2SO_4$ 4, KH_2PO_4 0.1, $MgSO_4·7H_2O$ 0.05, corn-steep (65% dry wt.) 0.5, and pH = 7.0.

(DES) mutagenesis on strain Mutant ZQ-2.

Fermentation tests of producers of L-valine

Fermentation tests for determining the maximum concentration of L-valine obtained by mutants were carried out in batch culture. The culture medium used for the preparation of the inoculum of *B. flavum* mutants contained (g/L): glucose 2.5, $(NH_4)_2SO_4$ 0.5, KH_2PO_4 0.1, $MgSO_4·7H_2O$ 0.05, corn-steep (65% dry wt.) 0.5, $CaCO_3$ 1.0, and adjusted to pH = 7.0 with 0.1 M of HCl or NaOH.

The culture medium used for the fermentation of *B. flavum* mutants contained (g/L): glucose 12.5, $(NH_4)_2SO_4$ 4, KH_2PO_4 0.1, $MgSO_4·7H_2O$ 0.05, corn-steep (65% dry wt.) 0.5, and adjusted to pH = 7.0 with 0.1 M of HCl or NaOH. For the fermentation, the first preculture was grown for 10 h in 250 ml shaking flasks containing 15 ml seed medium. Afterwards, 2 ml was transferred to a 500 ml shaking flask with 40 ml of fermentation medium. Temperature was maintained at 30ºC and the cultivations were finished after 72 h when glucose was totally consumed. Final extracellular L-valine concentrations were determined at that point.

Determination of biomass, amino acids and organic acids

Biomass concentration during fermentation was measured by optical density at k = 546 nm (Spectrometer UV-160, Shimadzu). Cell growth was determined by harvesting the mycelia pellets and freeze-drying them to a constant weight; the dry weight was expressed as gram per liter of the culture. Samples for determination of amino acids and organic acids were taken during cultivation and analyzed by HPLC as described in Brik-Ternbach et al. (2005) and Zelic et al. (2004), as well as glucose concentration using the AccuCheck sensor (Brik-Ternbach et al., 2005). As a quick and simple method to determine the concentration of L-valine in the broth, the paper chromatographic method of assay was applied, using a solvent system *n*-butanol-acetic acid-water (2:2:1 by volume) (Fink et al., 1963) and Klett-Summerson photo-electric colorimeter with a green filter (540 nm) (Mandal and Majumdar, 1970).

Metabolic flux analysis

The carbon flux through the primary metabolic pathways of XQ-6 thus needs to be estimated from the metabolic mass-balance equations based on a detailed description of the stoichiometry of all relevant bioreactions involved in cell growth and L-valine formation that include the EMP pathway and the HMP pathway.

RESULTS AND DISCUSSION

Obtainment of a newly screened L-valine producing strain

A valine producer stain V3-36 (Leu^-, Ile^-, $\alpha\text{-}AB^r$, AHV^r) was obtained from *B. flavum* XQ5122, which produces 2.3% L-valine in 10% glucose medium through DES treatment. Subsequently, Mutant ZQ-2 was isolated from NTG mutagenesis on strain V4-153, accumulating 4.2% - 4.5% of L-valine on average and 5.57% of L-valine as highest. Using ZQ-2 (Leu^l, Ile^l, $\alpha\text{-}AB^r$,AHV^r,$2\text{-}TA^r$) as a starting strain for mutagenesis by DES, NTG treatment, a L-valine hyper-producer XQ-6 (Leu^l, Ile^l, AHV^r,$\alpha\text{-}AB^{hr}$,_$2\text{-}TA^{hr}$) was obtained, which could be resistant to high concentration of α-amino-butyric acid (α-AB) and 2-thiazolealanaine_ (2-TA). "Leu^-" means that the strain can't grow without addition of Leu. The word "Ile^-" has the similar meaning. "Leu^l" means that the strain can supply some Leu by itself and it still needs the addition of Leu for its normal growth. And "Ile^l" has the similar meaning. The strain was cultured with shaking at (31 ± 1)ºC for 72 h, it could accumulate about 58 g/L of L-valine in a medium containing 14% glucose and 5% $(NH_4)_2SO_4$.

Effect of the addition of amino acids on L-valine production by *Brevibacterium flavum* XQ-6

Effect of amino acids on directing the carbon flux from glucose to L-valine was carefully investigated by single factor experiments, through adding L-alanine, L-histidine, L-glutamic acid, L-aspartic acid, L-isoleucine, L-leucine, L-phenylalanine, L-lysine·HCl, L-proline, glycine, L-glutamine, L-methionie, L-threonine, L-cysteine, L-valine and L-arginine into the broth. The results were indicated in Table 1. Based on the data in Table 1, the addition of many kinds of amino acids at 0.1 or 0.4 g/L had little effect on the L-valine production by *B. flavum* XQ-6, except some kinds of amino acids, that is, L-valine, L-leucine, L-isoleucine and L-methionie. To get detailed description of the effect on the regulation of the L-valine formation by the addition of different amounts of amino acids to the broth, L-leucine, L-isoleucine and L-methionie

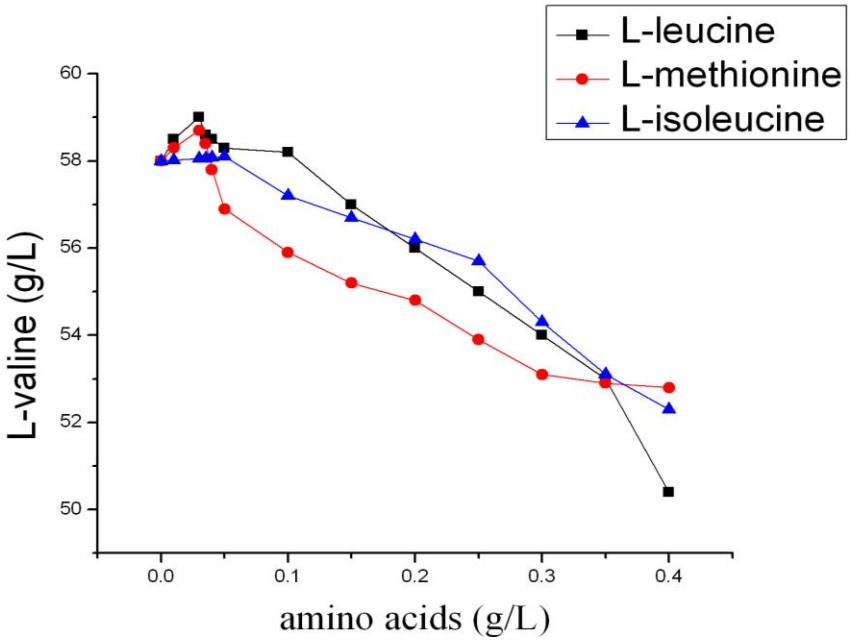

Figure 2. Effect of amino acids (L-leucine, L-methionine, and L-isoleucine) on L-valine concentration by XQ-6. Fermentation medium: Glucose 12.5, $(NH_4)_2SO_4$ 4, KH_2PO_4 0.1, $MgSO_4 \cdot 7H_2O$ 0.05, corn-steep (65% dry wt.) 0.5, and pH = 7.0.

were chosen for tests at sequential concentration. The results were illustrated in Figure 2. With more L-methionine and L-leucine fed into the broth, L-valine concentration increased firstly and then decreased. When L-methionie concentration was fed into the broth at 0.03 g/L, L-valine concentration reached the maximum value, that is, 58.7 g/L. The same phenomenon happened when L-leucine was added into the broth. When L-leucine concentration was fed into the broth at 0.03 g/L, the stain had a maximum production, that is, 59.0 g/L. Afterwards, when more L-leucine and L-methionine (more than 0.03 g/L) was presented in the broth, L-valine concentration decreased to 50.4 and 52.8 g/L, respectively. With more L-isoleucine fed into the broth, L-valine concentration increased when L-isoleucine concentration level was less than 0.05 g/L and decreased when L-isoleucine concentration level was more than 0.05 g/L. When L-isoleucine concentration was fed into the broth at 0.05 g/L, the strain had a maximum production, that is, 58.1 g/L. Afterwards, when more L-isoleucine (more than 0.03 g/L) was presented in the broth, L-valine concentration decreased to 52.3 g/L gradually.

During the process of the breeding of L-valine producing mutant, the biosynthesis pathway of L-isoleucine and L-leucine had been weakened and the biosynthesis pathway of L-valine had been strengthened. Under these circumstances, an increased carbon flux towards L-valine was achieved because there was less competitive pathways for carbon resources. Biosynthesis pathways of L-isoleucine and L-leucine were just weakened instead of being cut or blocked. And in this way, the strain could still have a very high production (58 g/L) without any addition of L-isoleucine and L-leucine in the broth. However, it could not offer enough L-isoleucine and L-leucine to maintain normal anabolism by itself. When exterior L-isoleucine and L-leucine under some concentration levels were added into the broth, the restriction of anabolism was broken and L-valine concentration increased. As the exterior L-isoleucine and L-leucine concentration increased to some levels, they inhibited and repressed the key enzymes involved in the biosynthesis of L-valine (Zohar et al., 2003).

Experiment to display the effect of the addition of L-leucine, L-isoleucine and L-methionine on the cell growth (dry cell weight, DCW) was carried out when the fermentation ceased and the results were illustrated in Figure 3. Generally, the outline of the curves in Figure 3 climbs slightly with the addition of amino acids. With more L-methionine, L-leucine and L-isoleucine fed into the broth, the cell growth increased, but the cell growth was always under 16 g/L. Maybe the addition of amino acids regulated the biosynthesis pathway of L-valine firstly. But when more and more amino acids were added into the broth, the *B. flavum* XQ-6 took the surplus amino acids as nitrogen source mainly.

Effect of the addition of other nutrients on L-valine production by *B. flavum* XQ-6

Ten kinds of organic acids at different levels were added into the broth, respectively. The results were indicated in

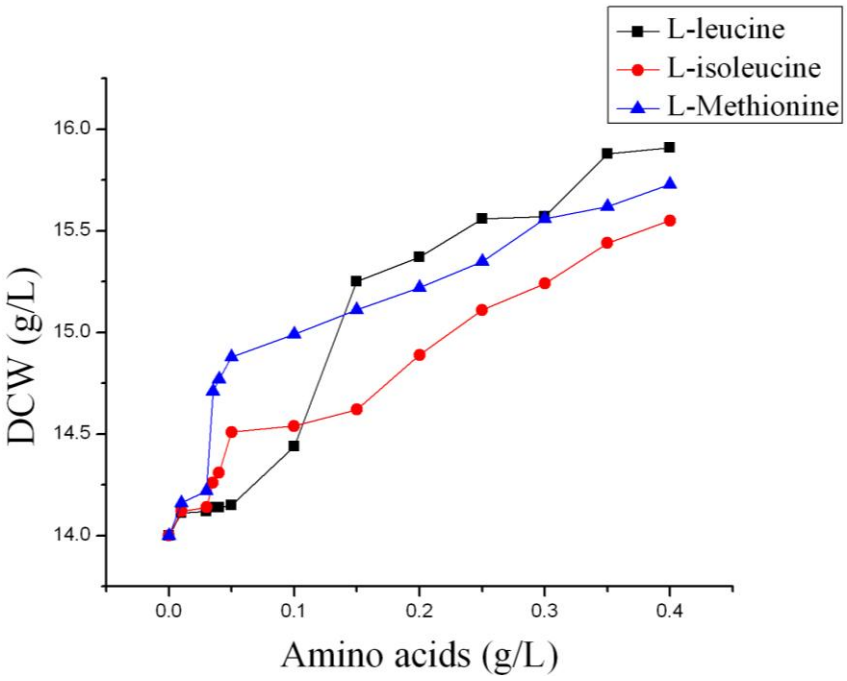

Figure 3. Effect of amino acids (L-leucine, L-methionine, and L-isoleucine) on DCW of XQ-6. Fermentation medium: glucose 12.5, $(NH_4)_2SO_4$ 4, KH_2PO_4 0.1, $MgSO_4 \cdot 7H_2O$ 0.05, corn-steep (65% dry wt.) 0.5, pH = 7.0.

Table 2. Effect of various organic acids on L-valine production by XQ-6.

Addition of organic acids	1.0 g/L	1.5 g/L	3.0 g/L
	L-valine produced (g/L)		
Citrate	58.2	58.6	56.7
Succinate	57.7	58.1	58.3
Fumarate	57.9	58.6	57.8
Tartaric acid	57.8	57.8	56.3
Oxalic acid	58.4	58.2	58.8
Lactic acid	57.9	58.1	58.2
Gluconic acid	58.1	58.2	58.2
Sorbic acid	57.7	58.1	58.0
Malonic acid	58.1	58.3	57.8
Malic acid	58.3	58.2	58.3
Not added	58		

Fermentation medium: glucose 12.5, $(NH_4)_2SO_4$ 4, KH_2PO_4 0.1, $MgSO_4 \cdot 7H_2O$ 0.05, corn-steep (65% dry wt.) 0.5, and pH = 7.0.

Table 2. Based on the data in Table 2, the addition of organic acids at three levels had little effect on the L-valine production by *B. flavum* XQ-6. Six kinds of alcohols (methanol, alcohol, n-propyl alcohol, n-butyl alcohol, propanetriol and ethylene glycol) at 0.1% (w/v) level were added into the fermentation medium, respectively. The results are shown in Table 3. Based on the data in Table 3, the addition of alcohols at 0.1 g/L had little effect on the L-valine production by *B. flavum* XQ-6. Six kinds of organic nourishment (yeast extract, hydrolyzate of soya bean cake, potato juice, peptone, beef extract and hydrolyzate of casein) at three different levels were added into the fermentation medium, respectively. The results were indicated in Table 4. Based on the data in Table 4, the addition of organic nourishment had little effect on the L-valine production by *B. flavum* XQ-6.

It seems that all the things tested in the fermentation media had little effect on the L-valine production by *B.*

Table 3. Effect of various alcohols on L-valine production by XQ-6.

Addition of alcohols 0.1 g/L	L-valine produced (g/L)
Methanol	57.7
Alcohol	58.2
N-propyl alcohol	57.5
N-butyl alcohol	58.1
Propanetriol	57.9
Ethylene glycol	58.2
Not added	58

Fermentation medium: glucose 12.5, $(NH_4)_2SO_4$ 4, KH_2PO_4 0.1, $MgSO_4 \cdot 7H_2O$ 0.05, corn-steep (65% dry wt.) 0.5, and pH = 7.0.

Table 4. Effect of various organic nutrients on L-valine production by XQ-6.

Addition of organic nutrients	0.1 g/L	0.5 g/L	1.0 g/L
	L-valine produced (g/L)		
yeast extract	58.6	57.8	58.4
hydrolyzate of soya bean cake	58.1	58.4	57.9
potato juice	58.3	57.9	58.3
Peptone	58.3	58.5	57.6
beef extract	58.2	58.2	57.3
hydrolyzate of casein	56.9	58.2	57.3
Not added	58		

Fermentation medium: glucose 12.5,$(NH_4)_2SO_4$ 4, KH_2PO_4 0.1,$MgSO_4 \cdot 7H_2O$ 0.05, corn-steep (65% dry wt.) 0.5, and pH = 7.0.

Table 5. Effect of various bases on L-valine production by XQ-6.

Addition of Bases	0.05 g/L	0.5 g/L	1.0 g/L
	L-valine produced (g/L)		
Uracil	58.2	57.9	58.4
Cytosine	58.3	58.3	58.5
Thymine	58.7	58.9	57.7
adenine	57.6	56.5	52.4
guanine	56.6	57.4	57.1
xanthine	58.6	58.1	58.2
hypoxanthine	58.1	58.5	58.4
Not added	58		

Fermentation medium: glucose 12.5, $(NH_4)_2SO_4$ 4, KH_2PO_4 0.1, $MgSO_4 \cdot 7H_2O$ 0.05, corn-steep (65% dry wt.) 0.5, and pH = 7.0.

flavum XQ-6 when the media contained 0.5% (w/v) corn-steep. It is obvious that the corn-steep is an effective nutrient to promote the L-valine production by *B. flavum* XQ-6.

Effect of the addition of various vitamins and bases on L-valine production by *B. flavum* XQ-6

XQ-6 was cultivated in the fermentation medium containing various bases or vitamins. As Table 5 shows, with the addition of seven kinds of bases, L-valine production was not clearly affected except adenine. The addition of adenine into fermentation medium decreased L-valine production slightly.

As Table 6 shows, with the addition of various vitamins, L-valine production was not affected significantly except biotin or thiamine.

To get detailed description of the effect on the regulation

Table 6. Effect of various vitamins on L-valine production by XQ-6.

Addition of vitamins	25 µg/L	50 µg/L	100 µg/L
	L-valine produced (g/L)		
Vitamin C	57.9	58.2	57.7
p-aminobenzoic acid	56.8	57.7	58.4
Folate	57.3	58.4	58.3
pantothenate	57.9	57.8	58.4
nicotinate	58.4	58.5	58.2
Biotin	59	61	60.5
Pyridoxine	58.1	57.9	58.4
Riboflavin	56.9	57.1	57.9
Thiamine	58.1	58.6	59.3
Vitamin B12	56.7	57.2	57.8
Not added	58		

Fermentation medium: glucose 12.5, $(NH_4)_2SO_4$ 4, KH_2PO_4 0.1,$MgSO_4 \cdot 7H_2O$ 0.05, corn-steep (65% dry wt.) 0.5, and pH = 7.0.

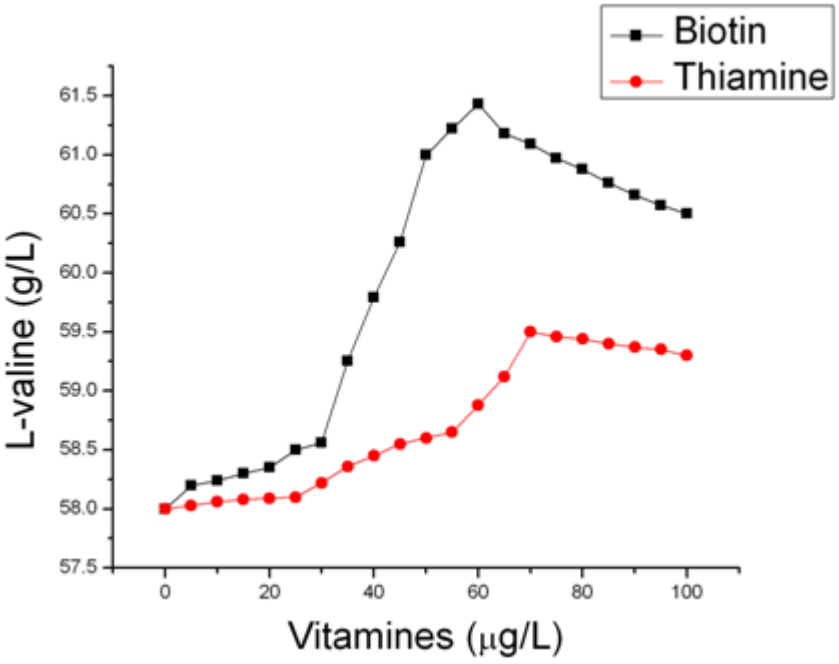

Figure 4. Effect of vitamines (Biotin and Thiamine) on L-valine concentration by XQ-6. Fermentation medium: glucose 12.5, $(NH_4)_2SO_4$ 4, KH_2PO_4 0.1, $MgSO_4 \cdot 7H_2O$ 0.05, corn-steep (65% dry wt.) 0.5, and pH = 7.0.

of the L-valine formation by the addition of different amounts of vitamins to the broth, biotin and thiamine were chosen for tests at sequential concentration. The results were illustrated in Figure 4. XQ-6 had the highest L-valine production, when about 57.5µg/L biotin was added into the broth. Thiamine promoted L-valine production by XQ-6 slightly, when addition of thiamine was between 50/L and 100µg/L.

The amount of biotin in the medium affected L-valine production by XQ-6 significantly. Biotin plays an important role in energy metabolism, acting as a coenzyme in chemical reactions that produce energy (Stephanopoulos et al., 1998). Biotin functions in this process as a carrier of carbon dioxide.

Effect of the addition of hydrogen peroxide on L-valine production by *B. flavum* XQ-6

To increase L-valine production, blocking or weakening

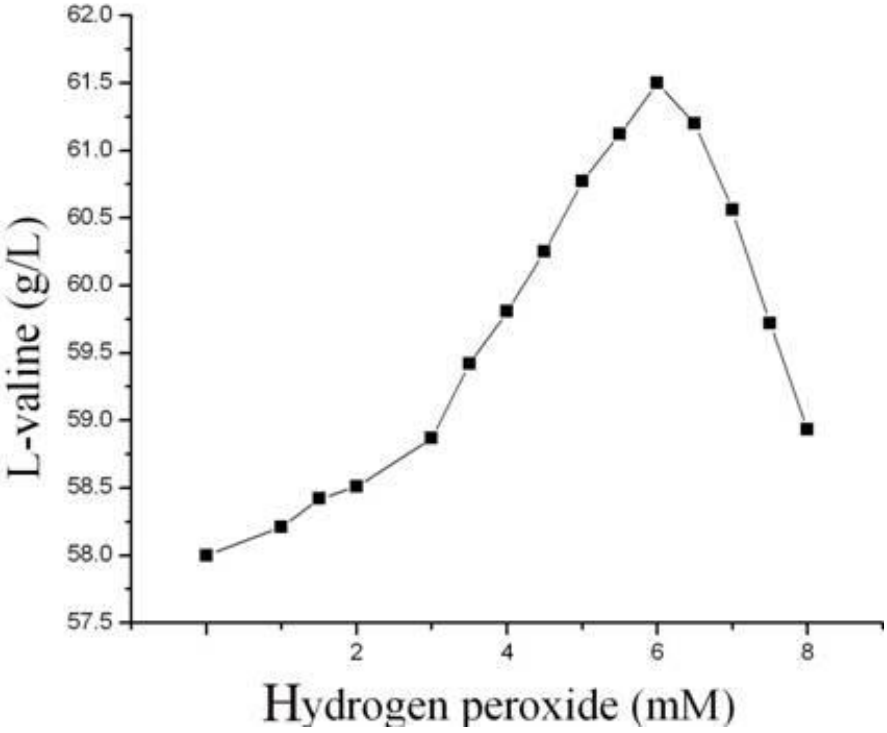

Figure 5. Effect of hydrogen peroxide on L-valine concentration by XQ-6.Fermentation medium: glucose 12.5,$(NH_4)_2SO_4$ 4, KH_2PO_4 0.1, $MgSO_4 \cdot 7H_2O$ 0.05, corn-steep (65% dry wt.) 0.5, pH=7.0.

the downstream carbon flux in TCA by inhibiting KGDH (α-ketoglutarate dehydrogenase complex) activity, might be an effective way to achieve the target. To verify the effectiveness of the strategy, hydrogen peroxide, a specific inhibitor of KGDH, was fed to the broth. As illustrated in Figure 5, with the increase of H_2O_2 concentration in the broth (0 - 8mM), the valine concentration increased. The highest final L-valine (61.7 g/L) were achieved when 5.6 mM H_2O_2 was added.

Metabolic flux analysis of L-valine fermentation in *B. flavum* XQ-6 by the combinational optimization strategy

In order to have a higher L-valine production by XQ-6, a combinational optimization strategy was adopted by adding some amount of amino acids (L-leucine, L-isoleucine and L-methionie), biotin and hydrogen peroxide. The strategy was implemented by using mutant XQ-6 for the fermentation and addition of 0.03 g/L L-leucine, 0.05 g/L L-isoleucine, 57.5 µg/L biotin and 5.6 mM H_2O_2 on the basis of fermentation medium mentioned above. With this strategy, the final L-valine was accumulated to a high level of 67.7 g/L.

The distribution ratio of carbon fluxes into EMP (Embden-Meyerhof-Parnas) pathway to carbon fluxes into HMP (hexose monophosphate) pathway at glucose-6-phosphate node was changed from 16.64:83.36 to 24.35:75.65 (Figure. 6). During the process of L-valine fermentation, NADPH is mainly supplied by the HMP. NADPH is very important for enhancing L-valine production. However, our results show that it is essential to promote carbon flux by EMP pathway in XQ-6. Maybe the amount of NADPH supplied by HMP pathway was enough for the demand of XQ-6 before the optimization.

The distribution ratio of carbon fluxes into L-valine biosynthesis pathway to carbon fluxes into other pathways at pyruvate node was changed from 66.18:33.82 to 76.69: 23.31 (Figure 7). Based on the ratio mentioned above, we can see that more carbon fluxes were directed into the L-valine biosynthesis pathway after combinational optimization strategy.

Conclusion

In this study, we demonstrated that: (1) A newly screened L-valine producing strain was obtained, which is not only a L-valine hyper-producer, but also can keep very steady on a large scale in the plant. (2) The addition of many kinds of amino acids at 0 and 0.4% levels had little effect on the L-valine production by XQ-6 except for L-leucine, L-methionine, or L-isoleucine. (3) Organic acids, alcohols or other organic nutrients did not affect L-valine production by XQ-6 when the medium contained 0.5% (w/v) corn-

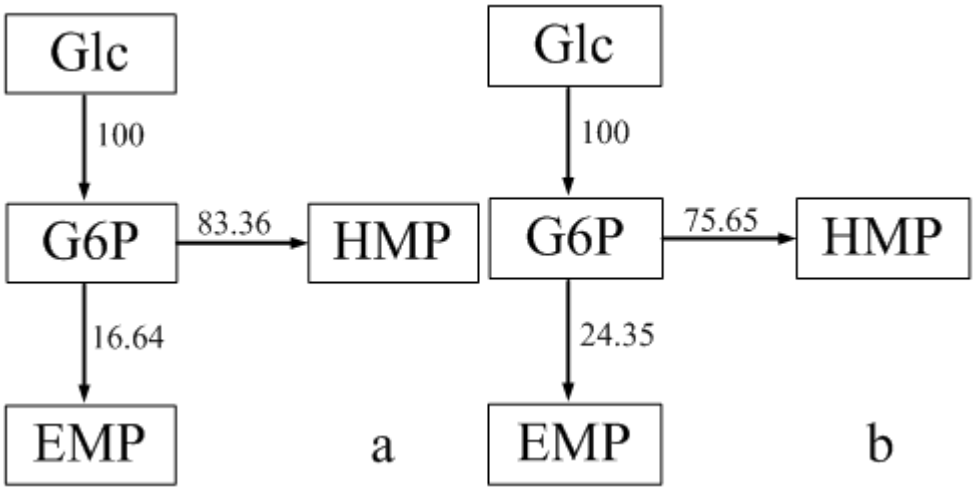

Figure 6. The distribution ratio of carbon fluxes between EMP pathway and HMP pathway at glucose-6-phosphate node. (a) Strain without any optimal strategy; (b) Strain by the combinational optimization strategy. Glc glucose, G9P glucose-6-phosphate, HMP Hexose Monophophate Pathway, EMP Embden-Meyerhof pathway.

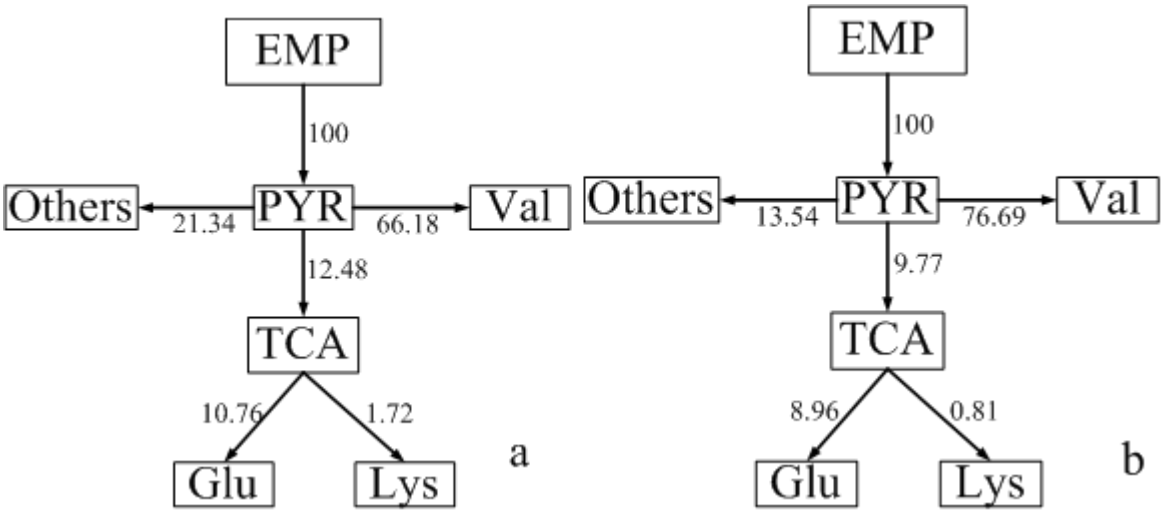

Figure 7. The distribution ratio of carbon fluxes between L-valine biosynthesis pathway and other pathways at pyrvate node. (a) Strain without any optimal strategy; (b) Strain by the combinational optimization strategy. EMP Embden-Meyerhof pathway, PYR pyruvate, Val valine, TCA tricarboxylic acid cycle, Glu glutamic acid, Lys lysine.

steep. (4) Biotin and H_2O_2 affected L-valine production by XQ-6 significantly. (5) A combinational optimization strategy was carried out and the final L-valine was accumulated to a high level of 67.7 g/L. The strategy may provide a potential and novel approach to enhance the aimed metabolite production in *B. flavum*.

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