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Living cell reaction process for L-isoleucine and L-valine production

Masato Terasawa, Masayuki Inui, Makoto Goto, Kazumichi Shikata[a], Makoto Imanari and Hideaki Yukawa

Tsukuba Research Centre, Mitsubishi Petrochemical Co. Ltd., Ibaraki and [a]*Biobusiness Development Department, Mitsubishi Petrochemical Co. Ltd., Tokyo, Japan*

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SUMMARY

A new process (Living Cell Reaction Process) for L-isoleucine production using viable, non-growing cells of *Brevibacterium flavum* AB-07 was optimised using ethanol as the energy source and α-ketobutyric acid (α-KB) as precursor. L-valine also could be produced from glucose at high yield by this process. This process differs from the usual fermentation method in that non-growing cells are used, and the production of L-isoleucine and L-valine were carried out under conditions of repressed cell division and growth. Minimal medium missing the essential growth factor, biotin was employed as the reaction mixture for the production of L-isoleucine and L-valine. The productivity of L-isoleucine and L-valine were 200 mmol $\cdot$ l^{-1} $\cdot$ day^{-1} (molecular yield to α-KB: 95%) and 300 mmol $\cdot$ l^{-1} $\cdot$ day^{-1} (molecular yield to glucose: 80%) respectively. The content of L-isoleucine and L-valine in total amino acids produced in the each mixture were 97% and 96% respectively.

INTRODUCTION

There have been a number of reports regarding the production of L-isoleucine [1–5] and L-valine [6–9] by growing cells. However, these direct fermentations have some problems because of: large consumption of carbon source during growth, low conversion rate of carbon source to product, and high concentration of by-products. The living cell reaction (LCR) process was examined in an effort to reduce the above problems.

When cell division was repressed by eliminating

Correspondence: Hideaki Yukawa, Tsukuba Research Centre, Mitsubishi Petrochemical Co. Ltd., 8-3-1, Inashiki, Ibaraki, 300-03, Japan.

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an essential growth factor (biotin) from a minimal medium, viable, non-growing cells of *B. flavum* MJ-233 were effective in producting L-isoleucine or L-valine.

In this paper, the advantages of the LCR process for the potential to produce L-isoleucine or L-valine at high productivity are described.

MATERIALS AND METHODS

Materials

Amino acids and organic acids were obtained from Wako Pure Chemical Industries Co. Ltd., Osaka, Japan, or Sigma Chemical Co. Inc., St. Louis, U.S.A.

Yeast extract and casamino acids were obtained from DIFCO Laboratories, Michigan, U.S.A. Corn steep liquor was obtained from Nihon Shokuhin Kako Co. Ltd., Tokyo, Japan. Other materials were obtained from Wako Pure Chemical Industries Co. Ltd., Osaka, Japan.

Micro-organisms

The α-aminobutyric acid resistant mutant (AB-07) derived from *Brevibacterium flavum* MJ-233 [5] was employed throughout this study.

Media and reaction mixture

The compositions of the various media employed were as follows:

Seed culture medium: urea, 4 g; $(NH_4)_2SO_4$, 23 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; yeast extract, 1 g; casamino acids, 1 g; biotin, 200 μg; thiamine-HCl, 100 μg; $FeSO_4 \cdot 7H_2O$, 6 mg; $MnSO_4 \cdot 4\text{–}6H_2O$, 6 mg; deionized water, 1000 ml; ethanol, 20 ml (initial concentration); pH 7.6.

Main culture medium: $(NH_4)_2SO_4$, 23 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; corn steep liquor, 20 g; biotin, 200 μg; thiamine-HCl, 100 μg; $FeSO_4 \cdot 7H_2O$, 20 mg; $MnSO_4 \cdot 4\text{–}6H_2O$, 20 mg; deionized water, 1000 ml; ethanol, 20 ml; pH 7.6.

Basal medium (for production of L-isoleucine or L-valine) (BM): $(NH_4)_2SO_4$, 23 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 5 g; deionized water, 1000 ml; pH 7.6.

Ethanol and α-KB were intermittently added to the above mixture for L-isoleucine production, and glucose was added to the above mixture for L-valine production.

Fermenter

The fermenter consisted of a glass stirred vessel, with a total volume of three litres (ABLE Co. Ltd., Type MBC, Tokyo, Japan), H/D = 1.6.

Optimisation of L-isoleucine production

Exponentially growing cells were harvested by centrifugation and washed twice with Basal Medium (BM). One gram of wet cells were transfered into 50 ml of BM and incubated at 33°C for one hour with shaking.

The reaction was stopped by immersion of the cells into a bath of boiling-water for 5 min. The cells were separated by centrifugation.

Conditions of L-isoleucine or L-valine production by LCR (Living Cell Reaction)

Exponentially growing cells in main culture medium were harvested by centrifugation. The cells (20 g wet weight) were washed twice with BM and then re-suspended in 1 l of BM. The production reaction was carried out in a three litre fermenter (1000 rpm, 1 l air per minute) at 33°C and pH 7.6 for 24 h .

At the end of the production phase cells were harvested by centrifugation and were reused for the production of L-valine.

Analytical methods

Growth was measured by optical density at 610 nm. L-Isoleucine was determined either by bioassay using *Leuconostoc mesenteroides* P-60 (ATCC 8042) or by HPLC according to the method of Seiler [10]. Ethanol and α-KB were estimated by GC and HPLC respectively.

RESULTS

Optimisation of L-isoleucine production

While growth of AB-07 did not occur to any mea-

surable extent in basal medium (lacking biotin), L-isoleucine was formed at high yields indicating that growth was not essential for the formation of L-isoleucine. The effect of α-KB concentration on formation rate of L-isoleucine was examined.

α-KB addition at concentrations up to 200 mM was found to have no effect on formation rate of L-isoleucine.

The effect of the growth phase was also examined (Fig. 1), and exponential-growth phase, cells were found to be the most active. Thereafter exponential-growth phase cells were harvested 24 h after cultivation, and were re-submitted to the reaction.

The addition of organic acids into the reaction mixture was examined for enhancing formation of L-isoleucine and found to be very effective (Fig. 2).

The pH and temperature optima for the formation of L-isoleucine were found to be at pH 7.6 and 33°C.

Production of L-isoleucine by LCR process

L-Isoleucine production by LCR process with respect to time was monitored under the optimum conditions previously described (Fig. 3). Under these conditions the rate of L-isoleucine production was approximately 200 mmol · l^{-1} · day^{-1} and the

Addition of organic acid	Relative reaction rate (%) 50 100 150
Not added	
Pyruvate	
Oxalacetate	
Malate	
Fumarate	

Fig. 2. Effect of organic acids on reaction rate. The reaction was carried out under the same conditions as shown in Fig. 1. The amount of L-isoleucine production in the reaction mixture obtained without organic acid was 8.3 mmol · l^{-1}. This value was taken as 100%.

molecular yield from α-KB was 95%. The content of L-isoleucine produced with respect to other amino acids produced in the reaction mixture was 97%.

When the production of L-isoleucine was carried out reusing living cells, L-isoleucine could be produced at a high yield from α-KB and ethanol without a decrease in the production rate over at least for several runs.

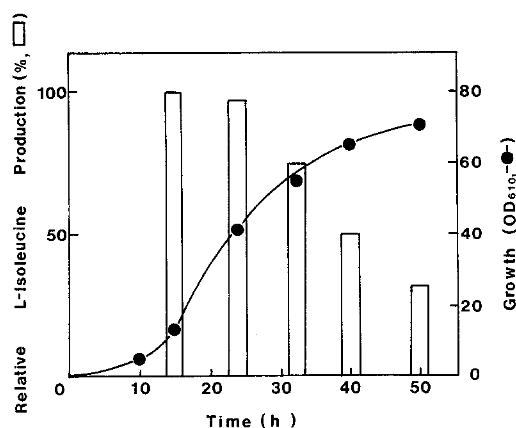

Fig. 1. Effect of growth phase on cell activity. The reaction was carried out with the basal mixture containing 50 mmol · l^{-1} of α-KB and 300 mmol · l^{-1} of ethanol. The amount of L-isoleucine production obtained using the cells harvested after 15 h cultivation was 8.3 mmol · l^{-1}. This value was taken as 100%.

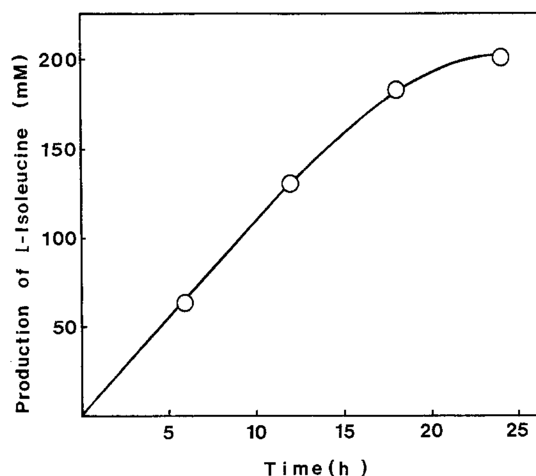

Fig. 3. Time course of L-isoleucine production. The reaction was carried out with the basal mixture containing 210 mmol · l^{-1} of α-KB and 100 mmol · l^{-1} of fumaric acid. Ethanol was added into the basal mixture intermittently and total amount of ethanol was 1000 mmol · l^{-1}.

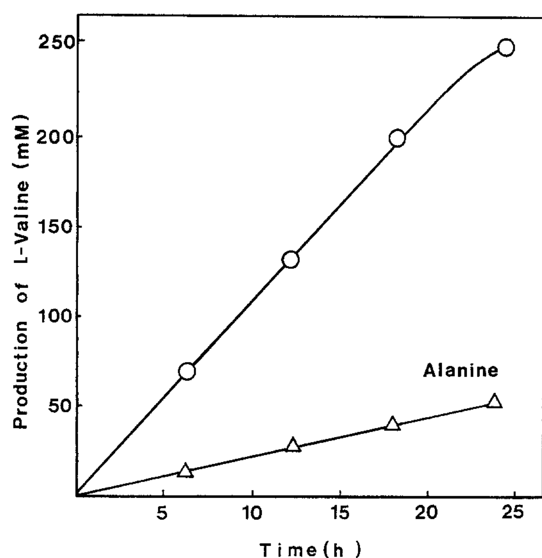

Fig. 4. Time course of L-valine production. The reaction was carried out with the basal mixture containing 380 mmol · l^{-1} of glucose.

Optimisation of L-valine production

L-Valine was produced at high yield from glucose by the LCR process (Fig. 4). The rate of production of L-valine was approximately 250 mmol · l^{-1} · day^{-1} and the molecular yield from glucose was 67%. Alanine was formed as a by-product during the production of valine from glucose. The amount of alanine produced was approximately 50 mmol · l^{-1}.

Repression of alanine formation

The effects of ammonium sulfate on alanine formation are shown in Table 1. Alanine formation was repressed markedly at concentrations ⩾4% (w/v) ammonium sulfate. When alanine formation was repressed the productivity of L-valine increased to approximately 300 mmol · l^{-1} · day^{-1} and the molecular yield from glucose was 80%. The content of L-valine produced with respect to other amino acids in the reaction mixture was 96%.

L-Valine also could be produced at high yield by reusing living cells for several runs.

Table 1

Effect of ammonium sulfate concentration on L-valine formation

$(NH_4)_2SO_4$ conc. (%)	Formation of valine (mM)	Formation of alanine (mM)
0.5	212	80
1.0	220	72
2.0	245	55
4.0	297	10
5.0	296	10

DISCUSSION

While investigating the industrial use of ethanol a mutant strain of *Brevibacterium* was isolated which was capable of producing L-isoleucine from α-KB and ethanol by cell reactions in minimal media lacking an essential growth factor.

The addition of TCA cycle organic acids (Fig. 2) was effective in enhancing the reaction rate. This result may indicate that pyruvic acid was supplied sufficiently since the strain used in this study could produce pyruvic acid efficiently from these acids (data not shown).

In the LCR process the productivity of L-isoleucine was 200 mmol · l^{-1} · day^{-1}, however, if α-KB was added to the medium intermittently after the middle phase of cultivation, cell growth was inhibited and L-isoleucine was not produced (data not shown).

The content of L-isoleucine with respect to total amino acids in the reaction mixture was very high compared with the direct fermentation. This is expected to result in a simplified purification process.

It was interesting that L-valine was produced at high productivity when the materials were converted from ethanol and α-KB to glucose. In this case, L-isoleucine as a by-product was below one mmol · l^{-1} and the content of L-valine in total amino acids was also very high.

Both L-isoleucine and L-valine could be formed at high yield for several runs by recycling cells. Contamination of the reaction mixture was not detected even after several runs.

In summary the characteristics of LCR process are as follows:

(1) Productivity is very high.
(2) Since living cells are directly used for the production of L-isoleucine or L-valine a complicated process such as immobilization is not necessary.
(3) Since living cells can be re-used several times, cost of the catalyst is low.
(4) Operational stability is very high.
(5) Since contamination is not an issue in a simple reaction mixture, the sterilization of reaction mixture is not necessary.
(6) Since the content of product is very high, the purification process is simple.

We believe that LCR process has a high potential for the industrial production of L-isoleucine or L-valine.

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