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Regulation of branched-chain amino acid biosynthesis by α-acetolactate decarboxylase in *Streptococcus thermophilus*

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Abbreviated running Headline : α-Acetolactate decarboxylase

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SUMMARY

Aims: To demonstrate the presence of an active α-acetolactate decarboxylase in *Streptococcus thermophilus* and to investigate its physiological function.

5 **Methods and results:** *Strep. thermophilus* CNRZ385 contains a gene encoding an α-acetolactate decarboxylase. Comparison of the production of α-acetolactate and its decarboxylation products, by the parent strain and an α-acetolactate decarboxylase-deficient mutant, demonstrated the presence of a control of the pool of α-acetolactate by valine, leucine and isoleucine. This control occurs via an allosteric activation of the α-acetolactate

10 decarboxylase. Cell-free extracts of *Strep. thermophilus* were not able to decarboxylate the isoleucine precursor α-acetohydroxybutyrate.

Conclusions: These results strongly suggest that one of the physiological functions of the α-acetolactate decarboxylase in *Strep. thermophilus* is to regulate leucine and valine biosynthesis by diverting the flux of α-acetolactate towards acetoin when the branched-chain

15 amino acids are present at a high concentration.

Significance and impact of the study: Regulation of branched-chain amino acid biosynthesis by α-acetolactate decarboxylase may occur in several other microorganisms and explain some of their growth properties.

20 Keywords : lactic acid bacteria, diacetyl, acetoin, α-acetohydroxybutyrate, leucine, valine

INTRODUCTION

Several species of lactic acid bacteria possess an α-acetolactate decarboxylase (Godtfredsen *et al.* 1984). This enzyme converts α-acetolactate, which is produced from pyruvate by the α-acetolactate synthase, into acetoin and CO_2. As acetoin is a neutral compound, whereas pyruvate is acidic, the conversion of pyruvate into acetoin helps to maintain pH homeostasis. In *Lactococcus lactis* strains isolated from non-dairy environments, α-acetolactate decarboxylase is also involved in the regulation of leucine and valine biosynthesis (Goupil-Feuillerat *et al.* 1997). Indeed, α-acetolactate is a precursor of these branched-chain amino acids, and when they are present in excess, diversion of the flux of α-acetolactate towards acetoin could stop their synthesis. It has been shown that the α-acetolactate decarboxylase of *Lactococcus lactis* is activated by the branched-chain amino acids (Phalip *et al.* 1994), and that the production of this enzyme is limited under branched-chain amino acid starvation (Goupil-Feuillerat *et al.* 2000). However, diversion of the flux of α-acetolactate towards acetoin in the presence of branched-chain amino acids has not yet been demonstrated *in vivo*. The main difficulty is due to the fact that α-acetolactate is an unstable compound, that can be spontaneously decarboxylated into acetoin or diacetyl. It is thus complicated to distinguish the enzymatic reaction from the chemical reaction. α-Acetohydroxybutyrate is a precursor of the branched-chain amino acid isoleucine. It has a structural similarity with α-acetolactate and it has been shown that the α-acetolactate decarboxylase from *Aerobacter aerogenes* is able to decarboxylate this compound (Løken and Størmer 1970). The possible involvement of the α-acetolactate decarboxylase, via the decarboxylation of α-acetohydroxybutyrate, in the regulation of isoleucine biosynthesis has not yet been investigated.

To our knowledge, there is no publication describing a significant production of acetoin by growing cultures of *Strep. thermophilus*. However, Teraguchi and coworkers

(1987) observed that some strains could produce some acetoin in resting-cell experiments. Tinson and coworkers (1982) attributed the production of acetoin by *Strep. thermophilus* resting-cells to the non-enzymatic decarboxylation of α-acetolactate and in another study, no α-acetolactate decarboxylase activity could be detected in cell-free extracts (Godtfredsen *et al.* 1984). The objective of this study was to demonstrate the presence of an active α-acetolactate decarboxylase in *Strep. thermophilus*, and to investigate its physiological function.

MATERIALS AND METHODS

Culture media and conditions

Escherichia coli strains were grown in Luria-Bertani (LB) medium (Sambrook *et al.* 1989) at
5 37°C with shaking, and in the presence of erythromycin (150 µg ml^{-1}) or kanamycine (50 µg
ml^{-1}) when required. *Strep. thermophilus* strains were routinely grown in M17 broth (Terzaghi
and Sandine 1975).

The effect of branched-chain amino acids on the growth of *Strep. thermophilus* was
studied using the chemically defined medium (CDM) described by Neviani and coworkers
10 (1995), that was supplemented with $NiSO_4.7H_2O$ (10 mg l^{-1}) and $MnSO_4.1H_2O$ (10 mg l^{-1}),
and autoclaved for 15 min at 110°C. After two successive cultures in CDM at 37°C, cells of
Strep. thermophilus were recovered by centrifugation for 10 min at 5,000 x *g* and 4°C,
washed in a saline solution (9 g l^{-1} NaCl), and resuspended in the same solution at an
absorbance at 575 nm equivalent to 1.4. Cells were then inoculated at 0.5% (v/v) in CDM.
15 Cultures were performed at 37°C and in partial anaerobic conditions (incubation without
agitation and the headspace volume represented less than 2% of the culture volume).

Synthesis of α-acetolactate and α-acetohydroxybutyrate

α-Acetolactate was generated by hydrolyzing ethyl 2-acetoxy-2-methyl-acetoacetate (Aldrich,
20 Saint-Quentin-Fallavier, France) with two equivalents of NaOH (Veringa *et al.* 1984). The
reaction was carried out at 20°C for 30 min, and the resulting solution was used immediately
for the assay of α-acetolactate decarboxylase. α-Acetohydroxybutyrate was generated by
hydrolyzing ethyl-α-aceto-α-hydroxybutyrate (Crout and Rathbone 1989) with one equivalent
of NaOH.

25

Measurement of α-acetolactate decarboxylase activity

At the end of the exponential growth phase, *Strep. thermophilus* cells were harvested by centrifugation of 250 ml of culture for 15 min at 14,000 x *g* and 4°C. They were then washed with 250 ml of 50 mmol l^{-1} sodium phosphate buffer (pH 6), resuspended in 1.5 ml of the
5 same buffer, and placed in a 2 ml tube containing 600 mg of 0.1 mm diameter glass beads (PolyLabo, Strasbourg, France). They were then disrupted in a cell disruptor (FP120 FastPrep™; Savant Instruments Inc., Holbrook, USA) for 30 s at 4°C and at speed 6.5. After centrifugation for 5 min at 21,000 x *g* and 4°C, α-acetolactate decarboxylase activity was determined in the supernatant (Løken and Størmer 1970; Hugenholtz and Starrenburg 1992).
10 The reaction mixture contained 200 mmol l^{-1} sodium phosphate buffer (pH 6) and cell-free extract. Reaction was started by adding α-acetolactate or α-acetohydroxybutyrate. Concentration of the D-isomer of the substrates at the beginning of the assay was 60 mmol l^{-1}. Acetoin (hydroxypentanone) production from α-acetolactate (α-acetohydroxybutyrate) was determined with the method of Westerfeld (1945), which is based on a spectrophotometric
15 measurement at 530 nm. As α-acetolactate (α-acetohydroxybutyrate) undergoes a slow non-enzymatic decarboxylation into acetoin (hydroxypentanone) or diacetyl (2,3 pentanedione), compounds that all give rise to complexes absorbing at 530 nm, a control reaction was run without cell-free extract. One enzyme unit was defined as 1 μmol of acetoin (hydroxypentanone) produced per min. Protein concentration in cell-free extracts was
20 determined with the method of Bradford (1976) using bovine serum albumin as a protein standard.

Analytical methods

α-Acetolactate, α-acetohydroxybutyrate, diacetyl, 2,3-pentanedione, acetoin and
25 hydroxypentanone were measured using a method that was set up for the present study. In the

first procedure (A), 10 ml of a sample whose pH was adjusted to 0.5 with H_2SO_4, were steam-distilled in a Kjeltec System 1002 device (Tecator, Paris, France), and the first 50 ml distillate fraction was recovered. Diacetyl and 2,3-pentanedione were then assayed by mass spectroscopy gas chromatography (Landaud *et al.* 1998). Under these conditions, no significant amount of α-acetolactate (α-acetohydroxybutyrate) was converted to diacetyl (2,3-pentanedione), and the results thus reflect the true amounts of diacetyl (2,3-pentanedione). In the second procedure (B), 3 ml of sample were mixed with 7 ml of a buffer containing citric acid (150 mmol l^{-1}), Na_2HPO_4 (100 mmol l^{-1}) and $CuSO_4$ (2.14 mmol l^{-1}), and the first 50 ml distillate fraction was recovered. Under these conditions, α-acetolactate (α-acetohydroxybutyrate) was converted to diacetyl (2,3-pentanedione), and the results thus reflect the sum of diacetyl plus α-acetolactate (2,3-pentanedione plus α-acetohydroxybutyrate). In the third procedure (C), 10 ml of distillate recovered in procedure B were mixed with 1.4 ml of H_2SO_4 (5 mol l^{-1}) and 2.9 ml of a solution prepared by dissolving 10 g of $FeSO_4.7H_2O$ in 30 ml of $FeCl_3.6H_2O$ (500 g l^{-1}). The mixture was incubated for 40 min in a water bath at 80°C, and then cooled on ice. It was then steam distilled, and the first 25 ml fraction was recovered. Under these conditions, acetoin (hydroxypentanone) was converted to diacetyl (2,3-pentanedione), and the results thus reflect the sum of diacetyl plus α-acetolactate plus acetoin (2,3-pentanedione plus α-acetohydroxybutyrate plus hydroxypentanone).

DNA manipulation and analysis

Plasmid DNA manipulation and transformation of *E. coli* were performed using standard procedures (Sambrook *et al.* 1989). Total DNA from strain CNRZ385 (Institut National de la Recherche Agronomique, Jouy-en-Josas, France) was extracted as previously described (Pospiesh and Neumann 1995). PCR amplifications were performed with a Gene Amp 2400

PCR system (Perkin Elmer Corp., Norwalk, Conn., USA) using Taq polymerase (Appligene Oncor, Illkirch, France). A BigDye Terminator cycle sequencing Ready Reaction kit and a 310 Genetic Analyzer (Applied Biosystems, Foster City, Calif., USA) were used for DNA sequencing, and each strand was sequenced twice by using independent PCR products. The
5 DNA sequences were analysed with Genetics Computer Group sequence analysis software from the University of Winsconsin (Devereux *et al.* 1984) and Mail Fasta (National Center for Biotechnology Information).

Plasmid construction and knockout of the α-acetolactate decarboxylase gene

10 The nucleotide sequence of the putative α-acetolactate decarboxylase gene, named *aldC*, was obtained from the *Strep. thermophilus* LMG18311 genome sequence (P. Hols, unpublished data). The *aldC* gene was PCR-amplified using the two following two oligonucleotides: STHALDC1 (5' GAC ATC AAA CGC TTG GGC 3') and STHALDC2 (5' TGG TGC CTC ATC AGA AGC 3'). The resulting 1.9-kb PCR fragment was then cloned into the TOPO® XL
15 vector and transferred into *E. coli* TOP10 according to the manufacturer's instructions (Invitrogen, Groningen, The Netherlands). The resulting plasmid was named pTIL860. A deletion in the *aldC* gene was performed by double digestion of pTIL860 with *Cla*I and *Acc*I (Eurogentec, Seraing, Belgium), followed by treatment with T4 polymerase (Eurogentec) and ligation (fast-link DNA ligation kit; Epicentre Technologies, Madison, WI, USA). The
20 resulting plasmid, named pTIL861, was digested with *Hind*III and *Nae*I to release a 1.6 kb fragment containing the deleted *aldC* gene. This fragment was inserted in the plasmid pG^{+}host9::ISS1 (Maguin *et al.* 1996), previously digested with *Hind*III and *Sma*I. The resulting plasmid, named pTIL862, was obtained in *E. coli* TIL206 (TG1, which contains a chromosomal copy of the *repA* gene; kindly provided by P. Renault) and then introduced by
25 electroporation into *Strep. thermophilus* CNRZ385 using a previously described protocol

(Garault *et al.* 2000). The procedure for gene replacement described by Biswas and coworkers (1993) was then applied to the *aldC* gene to give the α-acetolactate decarboxylase-negative mutant TIL865. The nucleotide sequence of the *Strep. thermophilus* CNRZ385 *aldC* gene has been deposited in Genbank under the accession number AY072795.

5

RESULTS

Presence and allosteric control of an α-acetolactate decarboxylase in *Strep. thermophilus*

Using the assay conditions described in materials and methods, i. e. in the absence of
5 branched chain amino acids, it was impossible to detect any α-acetolactate or α-acetohydroxybutyrate enzymatic decarboxylation activity in cell-free extracts of *Strep. thermophilus* CNRZ385. However, these cell-free extracts displayed a significant α-acetolactate decarboxylase activity when branched-chain amino acids were added to the reaction mixture (Fig. 1). Valine was more stimulatory than leucine and isoleucine. No
10 enzymatic decarboxylation of α-acetohydroxybutyrate occurred when branched-chain amino acids where added to the reaction mixture.

Inactivation of the gene encoding an α-acetolactate decarboxylase in *Strep. thermophilus*

In order to investigate the physiological function of the α-acetolactate decarboxylase in *Strep.*
15 *thermophilus*, we decided to inactivate the gene encoding this enzyme in strain CNRZ385. Using oligonucleotides deduced form the genome sequence of *Strep. thermophilus* LMG18311 (P. Hols, unpublished data), a 1.9 kb fragment was PCR-amplified from strain CNRZ385. This fragment includes the whole *aldC* gene (1.6 kb) and part of the α-acetolactate synthase gene (*aldS*), located just upstream of *aldC*. Analysis of the 1.9 kb fragment revealed
20 the presence of a putative terminator (ΔGf = -31.4 kj mol^{-1}) 64 bp downstream of the *aldC* gene stop codon. The presence of a very short intergenic region (15 bp) and the absence of a putative terminator between *aldS* and *aldC* suggest that these two genes are co-transcribed. The protein encoded by the *aldC* gene from strain CNRZ385 showed high homology with the putative α-acetolactate decarboxylase from *Streptococcus pneumoniae* R6 (75% identity)
25 (Hoskins *et al.* 2001) and with the α-acetolactate decarboxylase from *Lactococcus lactis*

subsp. *lactis* NCDO2118 (61% identity) (Goupil *et al.* 1996). An α-acetolactate decarboxylase-negative mutant, named TIL865, was obtained by replacing the wild type gene with a deleted copy. The deletion covered a 133 bp region that starts 2 bp upstream of the ATG codon.

5

***In vivo* regulation of branched-chain amino acid biosynthesis by α-acetolactate decarboxylase**

The fact that, *in vitro*, the α-acetolactate decarboxylase was active only in the presence of branched-chain amino acids suggested that this enzyme may be involved in the regulation of
10 the biosynthesis of one or several of these amino acids. The growth rate of the parent strain was significantly higher than that of the mutant in the medium containing valine, leucine plus isoleucine, or leucine plus valine (Table 1). In the cultures of the parent strain, the proportion of α-acetolactate out of the total concentration of α-acetolactate plus diacetyl plus acetoin was higher in the absence of the three branched-chain amino acids, or in the presence of only
15 leucine or isoleucine, than in the other culture media. The same result was observed for the proportion of diacetyl, whereas the opposite was observed for the proportion of acetoin. These results suggest that in these three media, the α-acetolactate decarboxylase may be inactive, or at least less active than in the other media. Indeed, enzymatic decarboxylation of α-acetolactate results in the production of acetoin, whereas non-enzymatic decarboxylation
20 results in the production of diacetyl or acetoin (De Man 1959). In these three media, the non-enzymatic decarboxylation of α-acetolactate may thus be predominant. This is confirmed by the fact that the production of diacetyl, acetoin and α-acetolactate by the α-acetolactate decarboxylase-deficient mutant did not differ significantly from that of the parent strain in these three media. In the other culture media, the mutant always produced a higher proportion

of α-acetolactate and diacetyl, and a lower proportion of acetoin, compared to the parent strain.

Figure 2 shows the evolution, as a function of time, of the concentration of α-acetolactate and α-acetohydroxybutyrate in the medium containing the three branched-chain
5 amino acids and in the medium containing none of them. The parent strain and the mutant reached the stationary growth phase after approximately 6 hours of growth in the medium containing the three branched-chain amino acids, and after approximately 7 hours of growth in the medium containing none of them (results not shown). When the parent strain and the mutant were cultivated in the absence of branched-chain amino acids, α-acetolactate
10 concentration reached approximately 0.15 mmol l^{-1}, and decreased afterwards. The fact that the production was similar for the two strains confirms that the α-acetolactate decarboxylase is inactive in the absence of branched-chain amino acids. However, in contrast to the mutant, the parent strain did not accumulate significant amounts of α-acetolactate in the presence of the three branched-chain amino acids. This indicates that the α-acetolactate decarboxylase of
15 the parent strain is active in this medium.

Production of α-acetohydroxybutyrate by the parent strain and the mutant was very low compared to that of α-acetolactate. There is no evidence for *in vivo* enzymatic decarboxylation of α-acetohydroxybutyrate by α-acetolactate decarboxylase, as the production of this compound by the parent strain was similar to that of the mutant.

20

α-Acetolactate decarboxylase synthesis

Absence of *in vivo* enzymatic decarboxylation of α-acetolactate in the cultures of the parent strain when no branched-chain amino acids were present or when only leucine or isoleucine were present, may be explained by the allosteric properties of the α-acetolactate

decarboxylase. Another explanation would be an absence of synthesis of α-acetolactate
decarboxylase in these media. In order to address this question, we measured the α-
acetolactate decarboxylase activity in cell-free extracts that were prepared after growth of the
parent strain in the presence of different combinations of branched-chain amino acids. The *in*
5 *vitro* α-acetolactate decarboxylase activity measurements were performed in the presence of
100 mmol l^{-1} valine. All combinations of branched-chain amino acids resulted in cell-free
extracts that displayed a significant α-acetolactate decarboxylase activity, indicating that α-
acetolactate decarboxylase was synthesized in all cultures. The activity ranged from 0.270 (±
0.008) U . mg of protein^{-1} for the culture in the presence of leucine and isoleucine, to 0.472 (±
10 0.019) U . mg of protein^{-1} for the culture in the presence of leucine and valine.

DISCUSSION

This study demonstrates the presence of an α-acetolactate decarboxylase in *Strep. thermophilus*. *In vitro*, this enzyme is not able to decarboxylate α-acetohydroxybutyrate, and α-acetolactate is decarboxylated only in the presence of branched-chain amino acids. Activation of α-acetolactate decarboxylase by branched-chain amino acids had already been observed in *Lactococcus lactis* (Phalip *et al.* 1994), but the decarboxylase present in this microorganism is not fully inactive in the absence of branched-chain amino acids.

Comparison of the productions of α-acetolactate, diacetyl and acetoin by the parent strain with those of the α-acetolactate decarboxylase-deficient mutant, makes it posssible to establish whether the α-acetolactate decarboxylase is active, *in vivo*. This enzyme was inactive in the absence of branched-chain amino acids, or when only leucine or isoleucine were present. As α-acetolactate decarboxylase was always present in the cells of the parent strain, it may thus be hypothesised that in these media, the intracellular concentration of branched-chain amino acids was insufficient to enable an allosteric activation of this enzyme. These results strongly suggest that in *Strep. thermophilus*, the α-acetolactate decarboxylase regulates the biosynthesis of leucine and valine by diverting the flux of α-acetolactate towards acetoin when branched-chain amino acids are present at a high concentration. The presence of this enzyme may result in growth stimulation, depending on the culture medium (for example in the presence of valine). In contrast to *Lactococcus lactis*, the presence of an α-acetolactate decarboxylase is not responsible for an inhibition of growth when only leucine is present in the culture medium (Goupil *et al.* 1996). Regulation of branched-chain amino acid biosynthesis by the α-acetolactate decarboxylase in *Strep. thermophilus* appears thus to be different from that in *Lactococcus lactis*. Furthermore, as the supplementation of the medium with branched-chain amino acids did not significantly modify the α-acetolactate

decarboxylase activity that was measured *in vitro* (this measurement reflects the amount of functional enzyme present in the cell), one can conclude that, in contrast to *Lactococcus lactis*, the synthesis of this enzyme is not inhibited at the translational level under branched-chain amino acid starvation. Given that the α-acetolactate decarboxylase present in *Strep.*
5 *thermophilus* does not decarboxylate α-acetohydroxybutyrate, it is surprising that isoleucine, which is produced from α-acetohydroxybutyrate and not from α-acetolactate, is an allosteric activator of the α-acetolactate decarboxylase. This activation might be the consequence of the structural similarity of isoleucine with the two other branched-chain amino acids.

Another possible function of the α-acetolactate decarboxylase present in *Strep.*
10 *thermophilus* is its involvement in the catabolism of pyruvate to acetoin, which may help to maintain pH homeostasis and NAD/NADH balance. However, in the present study, we have confirmed that *Strep. thermophilus* does not produce large amounts of acetoin, which indicates that this function may be even less important than in citrate-utilizing lactococci.

The results of the present work also improve our comprehension of the biosynthesis of
15 diacetyl by *Strep. thermophilus*, which is an aroma impact compound of yoghurt flavour (Ott *et al.* 2000). Cultivation of *Strep. thermophilus* strains in conditions of branched-chain amino acid starvation, or disruption of the gene encoding for the α-acetolactate decarboxylase, results in a higher production of diacetyl. Interestingly, Ott and coworkers (2000) observed that adding valine decreased the production of diacetyl by mixed cultures of *Strep.*
20 *thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* in milk. This result may be explained by an allosteric activation of the α-acetolactate decarboxylase by valine.

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5

FIGURE LEGENDS

Fig. 1 Effect of leucine (●), valine (□) and isoleucine (△) concentration on α-acetolactate decarboxylase activity of cell-free extracts of *Strep. thermophilus* CNRZ385.

5

Fig. 2 Evolution of the concentrations of α-acetolactate and α-acetohydroxybutyrate in cultures of the wild-type strain CNRZ385 (full symbols) and the α-acetolactate decarboxylase-deficient mutant TIL865 (open symbols) in a chemically defined medium containing the three branched-chain amino acids (■,□) or containing none of them (●,❍).

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Table 1 Comparison of growth and production of α-acetolactate and its decarboxylation products, by wild-type strain *Strep. thermophilus* CNRZ385 and α-acetolactate decarboxylase-deficient mutant TIL865[†]

Presence of:			Growth rate (h^{-1}):		Compounds (mM) produced by: [‡]					
					CNRZ385			TIL865		
Leucine	Isoleucine	Valine	CNRZ385	TIL865	Acetolactate	Diacetyl	Acetoin	Acetolactate	Diacetyl	Acetoin
			1.11	1.14	0.082 (21)	0.079 (21)	0.222 (58)	0.073 (19)	0.100 (26)	0.220 (56)
+			0.67	0.61	0.088 (16)	0.101 (19)	0.352 (65)	0.104 (17)	0.142 (23)	0.375 (60)
	+		0.19	0.16	0.110 (18)	0.097 (16)	0.400 (66)	0.157 (25)	0.114 (18)	0.358 (57)
		+	1.11	0.68*	0.012 (3)	0.019 (4)	0.408 (93)	0.104* (29*)	0.075* (21*)	0.185* (51*)
+	+		0.78	0.52*	0.039 (7)	0.055 (10)	0.456 (83)	0.133* (26*)	0.092* (18*)	0.280* (55*)
+		+	1.14	0.70*	0.016 (3)	0.017 (3)	0.574 (95)	0.084* (15*)	0.119* (21*)	0.353* (64*)
	+	+	1.05	1.04	0.020 (5)	0.035 (9)	0.352 (86)	0.050* (14*)	0.067* (18*)	0.249* (68*)
+	+	+	1.71	1.45	0.013 (4)	0.014 (4)	0.289 (92)	0.048* (16*)	0.081* (27*)	0.169* (57*)

[†] The values are means of three experiments.

5 [‡] Data in parentheses are expressed as a percentage of the total production of α-acetolactate plus diacetyl plus acetoin. Measurements were taken after 15 h of growth.

* Values of mutant strain differed from those of the parent strain grown in the same medium ($P < 0.05$).

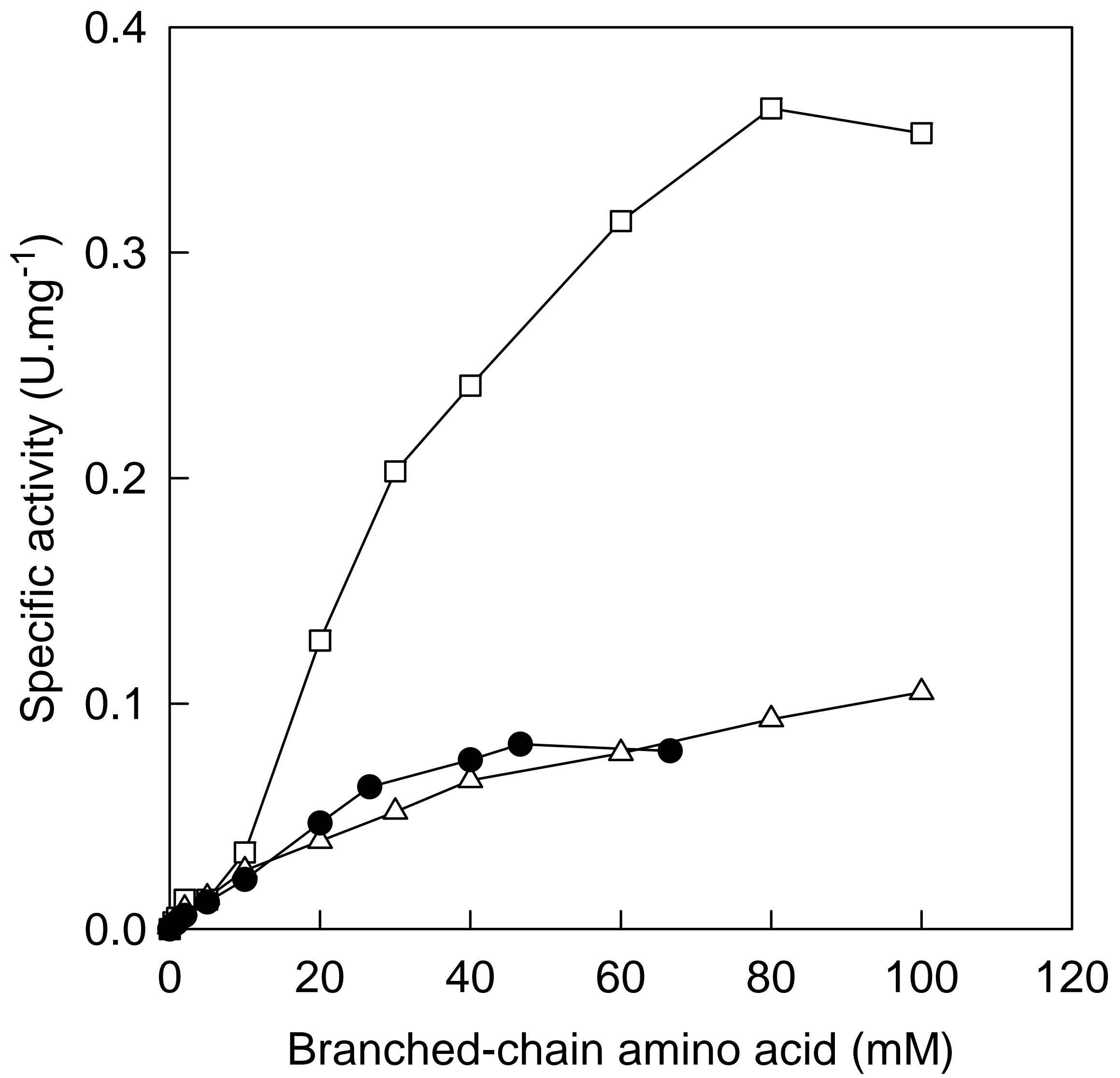

Fig. 1

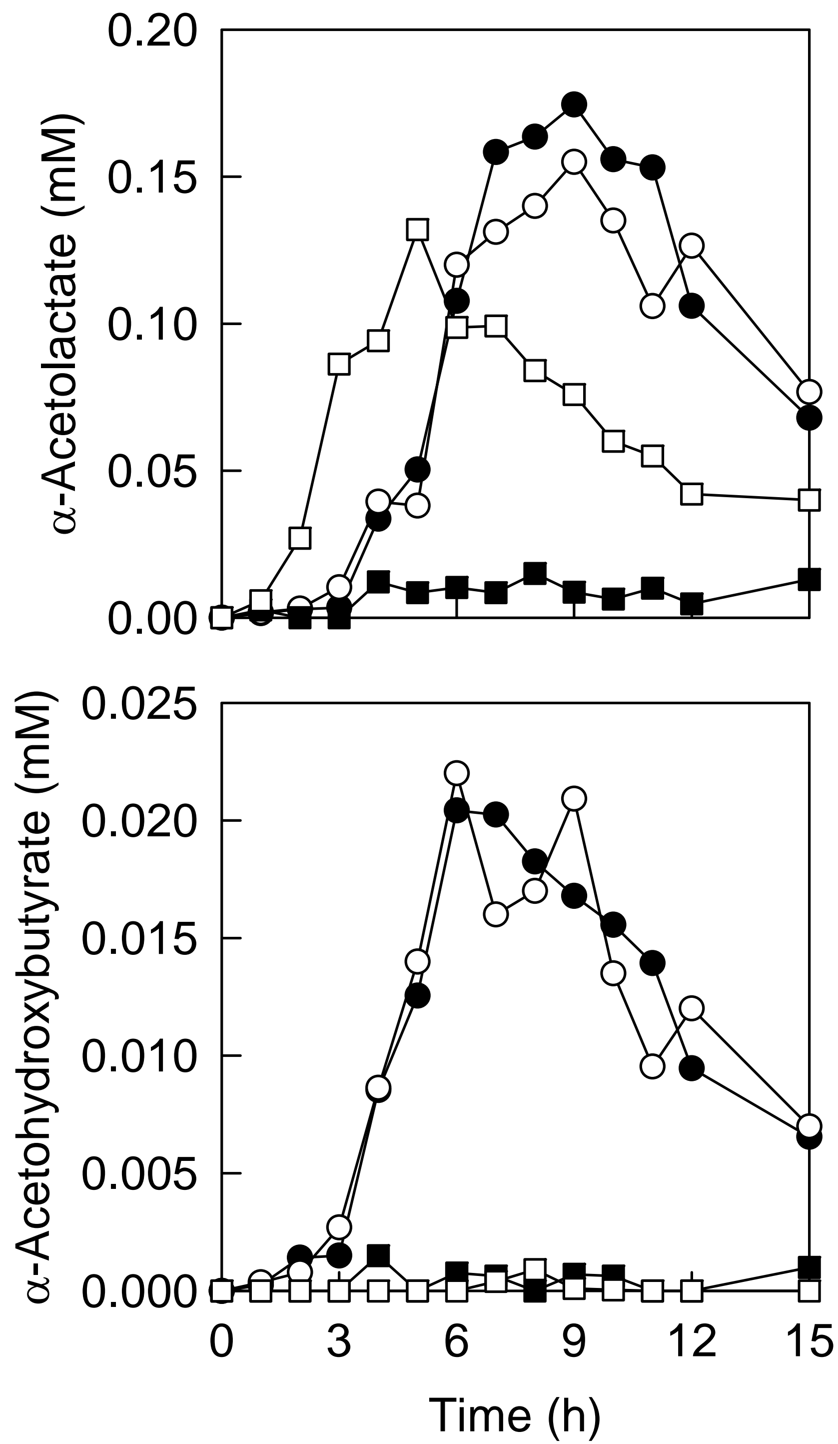

Fig. 2