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Full Length Research Paper

Development of a mutant strain of *Bacillus subtilis* showing enhanced production of acetoin

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This paper described the screening process of a mutant strain of *Bacillus subtilis* TH-49 showing specific characteristic and enhanced production of acetoin. The mutant was obtained by treating *B. subtilis* N-12 with ultraviolet ray (ultraviolet ray (UV)) or nitrosoguanidine (NTG) or compound mutation and subjecting it to a shake flask fermentation selection procedure. The acetoin production rate reached the highest value of 43.8 and 46.9 g/l in flask and 10-l fermenter fermentations, respectively. It was almost 4 times higher than that of the starting strain *B. subtilis* N-12. Through fermentation experiments, it was confirmed that the mutant strain, TH-49, was not capable of using acetoin accumulated in broth as its energy sources for growth after glucose was consumed. This phenomenon was inconsistent with that the majorities of bacteria accumulate acetoin as stored energy sources and could continue to use acetoin as energy sources after the exhaustion of glucose. By gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analysis, it was proved that the main metabolite produced by mutant strain TH-49 was acetoin and there were no other metabolites commonly found as byproducts of acetoin fermentation, such as 2,3-butanediol and 2,3-butanedione. The above characteristic of the mutant strain, TH-49, may be related to its specific composition of acetoin metabolism-related enzymes system. These results indicated that, the mutant strain TH-49 is conspicuously different from other acetoin producing strains reported previously in metabolic mechanism of acetoin.

Key words: *Bacillus subtilis*, acetoin, mutation, mutant strain.

INTRODUCTION

Acetoin (3-hydroxy-2-butanone) is an important flavor compound, widely existing in dairy products and some fruits. Because of its unique butter flavor, it can be used as flavor enhancer of butter, cheese, coffee and nut. The sensory characteristics of beer and wine are also related to acetoin (Romano et al., 1995, 1996). In addition, acetoin is also a platform chemical widely used in many other fields. It was classified as one of the 30 platform chemicals which were given the priority to their development and utilization by the U.S. Department of Energy (Werpy and Petersen, 2004). In recent years, the demand of acetoin has been growing, so its production methods and application research have attracted people's attention.

Presently, the production methods of acetoin are mainly based on chemical synthetic methods, such as the partly hydrogenation of diacetyl, the selective oxidation of 2,3-butanediol and the chloride hydrolysis of butanone. All the three methods have disadvantages of low conversion rate, severe environmental pollution, low safety of food, restrictions on raw materials and so on. So the application in production of acetoin by chemical synthetic methods is limited (Teixeira et al., 2002; Xiao et al., 2007). A lot of efforts have been made to develop natural acetoin production by biological methods including enzyme conversion and microbial fermentation. Hummel et al. (1992) used the diacetyl reductase obtained from lactic acid bacteria or yeast and the NAPH-CoA on catalytic conversion of diacetyl to generate acetoin at pH 5.0 and 70°C. Its conversion rate reached the highest

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value of 100%. De Faveri et al. (2003) used alcohol dehydrogenase of whole cells of *Acetobacter hansenii* MIM 2000/5 on the bio-oxidation of 2,3-butanediol to acetoin. The highest concentration of acetoin was 8.93 g/l. 2,3-butanedione and 2,3-butanediol are also used as raw materials on the production of acetoin by enzyme conversion method which is similar to chemical synthetic methods, but it is very difficult to obtain large quantity of specific enzymes and raw materials to produce acetoin by enzymatic conversion. Therefore, the scale production of acetoin by enzyme conversion method is also impossible. Compared to chemical synthetic and enzyme conversion methods, the microbial fermentation is the most competitive method in acetoin scale production because it has advantages of rich source of raw materials, mild process conditions, environmental-friendliness, high safety of food and so on.

Acetoin is an important physiological metabolite excreted by many microorganisms, such as *Bacillus subtilis* (Liu et al., 2009; Xiao et al., 2007), *Lactococcus lactis* (Bassit et al., 1993, 1995), *Leuconostoc mesenteroides* (Schmitt et al., 1997; Canas and owens, 1999) and *Hanseniaspora guilliermondii* (Teixeira et al., 2002) e.t.c. The previous study was mainly on the metabolic mechanism and regulation of acetoin in organism; and the study on production of acetoin by fermentation is seldom. As regard to a little study on accumulation of acetoin, acetoin exists only as the byproduct of 2,3-butanediol or 2,3-butanedione (Xiao and Xu, 2007). To our knowledge, there is still no large-scale application in production of acetoin from sugar or starch by microbial fermentation. The basic reason is lack of acetoin high-yielding strains applied in industrialized production.

In this study, a mutant strain of *B. subtilis* TH-49 showing specific characteristic and enhanced production of acetoin was obtained by random mutagenesis and shake flask fermentation selection procedure. The main metabolite produced by the mutant strain TH-49 utilizing glucose was analyzed and identified by gas chromatography (GC) and GC-mass spectrometry (MS).

MATERIALS AND METHODS

Organism and culture conditions

B. subtilis N-12 isolated from fermented beans was used in the present study. It was identified to be closely related to *Bacillus* sp.CICC10073 strain on the basis of 16S rRNA homology. It was maintained on the following slant medium (g/l): glucose 20, peptone 10, yeast extract 5.0, corn steep liquor 5.0, sodium chloride 5.0, manganese sulfate 0.05 and agar 20 at pH 7.0. The seed culture was prepared by growing the bacterium in 30 ml of the seed medium in a 250 ml shake flask for 16 h with agitation at 180 rpm and 37°C. The composition of the seed medium (g/l) was as follows: glucose 60, yeast extract 5.0, corn steep liquor 20, ammonium sulfate 5.0 and potassium dihydrogen phosphate 1.0 at pH 7.0. Before autoclaving, the pH of the fermentation medium was adjusted to 7.0. The prepared seed culture was inoculated (1%, v/v) into the fermentation medium (g/l) (50 ml medium in a 500 ml Erlenmeyer flask) and incubated on a rotary vibrator at 180 rpm and 37°C for 72 h. The composition of the fermentation medium (g/l) was as follows: glucose 100, yeast extract 5.0, corn steep liquor 15, ammonium sulfate 5.0, and manganese sulfate 0.05.

Ultraviolet ray (UV) irradiation

4 ml of cell suspension (10^8cells/ml) of the starting strain contained in a Petri dish was placed under an ultraviolet lamp (15 W, 2537 A) with a distance of 30 cm and was irradiated for different time intervals between 20 and 180 s. The cell suspension was mixed by a magnetic stirrer during irradiation. Treated and untreated cells were diluted in sterile physiological saline and 1 ml of cell suspension was spread on to the single colony isolation medium (its composition is same to slant medium) to calculate the lethality rate. Samples with a lethality rate of 70 to 80% were subjected to subsequent isolation. The plates were incubated in dark at 37°C for 2 days and the mutants were selected on the basis of the production rate of acetoin through flask fermentation.

Nitrosoguanidine (NTG) mutagenesis treatment

1 ml of NTG solution (100 mg/ml, 0.2 M phosphate buffer pH 6.0) was added to 1 ml of cell suspension of the starting strain (10^8 cells/ml). After incubation at 30°C on a rotary shaker of 120 rpm for different time intervals between 20 and 80 min, the mixture was diluted 1,000 times with sterile distilled water immediately to terminate the reaction. Treated and untreated cells were diluted in sterile physiological saline and 1 ml of cell suspension was spread on to the single colony isolation medium to calculate the lethality rate. Samples with a lethality rate of 80% were subjected to subsequent isolation. The plates were incubated at 37°C for 3 days and the mutants were selected on the basis of the production rate of acetoin through flask fermentation.

Compound treatment

The starting strain cell suspension (10^8cells/ml) was firstly irradiated for 40 s by UV and then was treated for 50 min by NTG as described earlier. Treated cells were suitably diluted in sterile physiological saline and were spread on the isolation medium. Then, the acetoin high-yielding mutants were selected according to the production rate of acetoin.

Batch fermentation in a 10-l fermenter

To investigate the behavior of acetoin accumulation, batch fermen-tations were carried out in a 10-l fermenter. The prepared seed culture was inoculated (3%, v/v) into the fermentation medium with an initial pH 7.0. The fermenter was operated with temperature at 37°C, stirring at 450 rpm, airflow rate at 1.0 vvm.

Determination of acetoin

Fermentation broth was recovered and centrifuged at 1500 g for 10 min, then the supernatant was suitably diluted. Acetoin was determined by the modified Voges-Proskauer (VP) reaction of Westerfeld (Westerfeld, 1945). Acetoin with creatine and a-naphthol formed a pink complex in alkaline conditions. The color intensity of the complex was determined by measuring the absorbance at 530 nm using UV-Visible spectrophotometer and calculated concentration of acetoin.

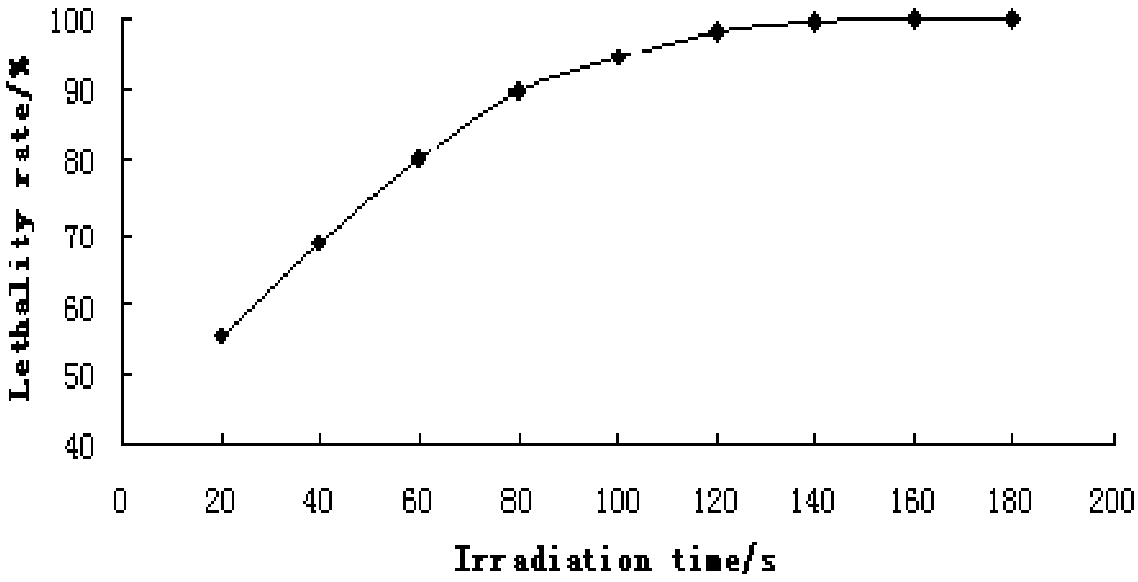

Figure 1. Influence of ultraviolet ray (UV) treatment time on *B. subtilis* N-12.

Table 1. Production rate of ultraviolet ray (UV)-mutants.

Irradiation time (s)	Mutant no.	Acetoin yield (g/l)*	Irradiation time (s)	Mutant no.	Acetoin yield (g/l)*
20	NV-20-7	12.3	60	NV-60-29	10.7
	NV-20-19	11.5		NV-60-44	14.4
40	NV-40-2	10.6		NV-60-71	9.6
	NV-40-32	13.7		NV-60-73	12.4
	NV-40-36	12.6	80	NV-80-53	11.2
	NV-40-49	10.1		NV-80-67	11.5
	NV-40-50	15.4	100	NV-100-5	11.3
	NV-40-64	12.6	Control strain	N-12	8.9

Composition of Shake-flask fermentation medium (g/l): glucose 60, yeast extracts 5.0, corn steep liquor 15, ammonium sulfate 5.0, manganese sulfate 0.05. The prepared seed culture was inoculated (1%, v/v) into the fermentation medium (g/l) (50 ml medium in a 500-ml erlenmeyer flask) and incubated on a rotary vibrator at 180 rpm and 37°C for 72 h. The result in Table 1 is the mean of triplicate tests.

Gas chromatography (GC) analysis of acetoin

Acetoin was assayed by injection of 1 µl of fraction of fermentation broth into a glass column (GEP 20 m, 180 cm × 2 mm). The carrier gas was helium at a flow rate of 0.9 ml min^{-1}. Injector temperature was 250°C. A standard solution containing 100 mg /l of acetoin was used to calibrate the column and recorder.

GC-MS analysis of acetoin

Acetoin was assayed by injection of 1µl of fraction of fermentation broth in the GC/MS system using a chiral capillary column (HP-INNOW-AX, 30 m × 0.25 mm i.d × 0.25 µm d.f). GC conditions: oven temperature increased from 40 to 200 at 3°C min^{-1}, after an initial hold at 40°C for 5 min; injector temperature: 250°C; helium gas flow: 0.9 ml min^{-1}; split ratio: 5:1. MS conditions: ion source temperature: 230°C; transmission line on temperature: 280°C; electron impact: 70 eV.

RESULTS

Selection of mutant strain induced by UV

Mutants were induced by treating cells of *B. subtilis* N-12 with a dose of UV light that produced different lethality rate by controlling the irradiation time (Figure 1). 712 survivors were isolated. A total of 15 improved strains, representing 2.1% of the survivors were obtained through flask fermentation as shown in Table 1. This result indicated that, improved strains were mainly obtained with irradiation time of 40 and 60 s, while UV light produces 70 to 80% lethality rate. The mutant strain NV 40 to 50 with

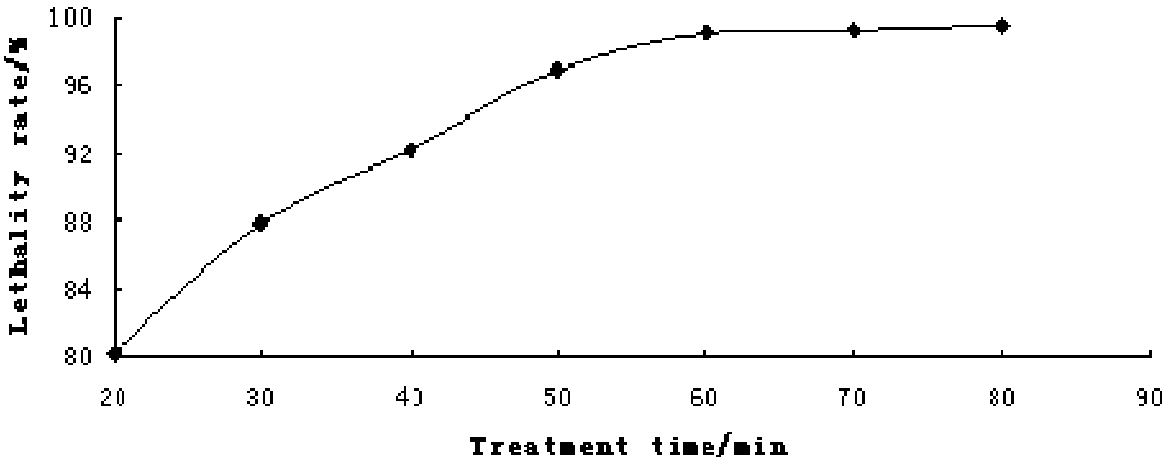

Figure 2. Influence of NTG treatment time on mutant NV 40 to 50.

Table 2. Production rate of NTG-mutants.

Processing time (min)	Mutant no.	Acetoin yield (g/l) *	Processing time (min)	Mutant no.	Acetoin yield (g/l) *
20	NT-20-46	19.5	60	NT-60-35	15.9
	NT-20-69	20.4		NT-60-39	27.4
30	NT-30-1	17.6		NT-60-45	23.5
	NT-30-13	20.2		NT-60-73	19.2
40	NT-40-14	25.7	70	NT-70-27	30.5
	NT-40-33	29.4		NT-70-51	23.6
	NT-40-75	19.7	80	NT-80-10	16.1
50	NT-50-12	32.2		NT-80-62	31.3
	NT-50-25	28.3		NT-80-64	20.9
	NT-50-43	16.7	Control strain	NV-40-36	15.4
	NT-50-44	35.6			
	NT-50-61	23.8			

In addition to 80 g/l of initial glucose, the other shake flask fermentation conditions and analytical methods are same with Table 1; the result in table 2 is the mean of triplicate tests.

acetoin production rate of 15.4 g/l was the most potential strain and was used in this study.

Selection of mutant strain induced by NTG

Mutant strain NV 40 to 50 was used as the starting strain for the NTG mutation. Mutants were induced by treating cells of mutant strain NV 40 to 50 with a dose of NTG that produced different lethality rate by controlling the intervals time between 20 and 80 min. The lethality rate after NTG treatment is shown in Figure 2. 682 survivors were isolated, while 21 strains with acetoin production rate higher than 15.4 g/l were obtained through flask fermentation. The results are summarized in Table 2.

The results in Figure 2 and Table 2 indicated that, NTG treatment had a significant effect on mutant strain NV 40 to 50. When the NTG treatment time was higher than 60 min, the lethality rate was more than 99%. Among these positive mutants, the mutant strain NT 50 to 44 with an acetoin production rate of 35.6 g/l was screened and was used for the compound treatment. Influence of NTG treatment time on mutant NV was 40 to 50.

Selection of mutant strain induced by compound treatment

Mutant strain NT 50 to 44 was treated by compound treatment. UV treatment time and NTG treatment time was 40 s and 50 min, respectively. 208 survivors were isolated. 7 strains with acetoin production rate higher

Table 3. Production rate of mutants treated by compound treatment.

Mutant no.	Acetoin yield (g/l)*
TH-3	37.5
TH-19	40.7
TH-49	43.8
TH-78	36.4
TH-99	35.7
TH-167	39.4
TH-201	41.7
NT-50-44	35.6

In addition to 100 g/l of initial glucose, the other shake flask fermentation conditions and analytical methods are same with Table 1; the result in Table 2 is the mean of triplicate test.

than 35.6 g/l were obtained through flask fermentation. The results are presented in Table 3.

Table 3 showed that, the mutant strain TH-49 with an acetoin production rate of 43.8 g/l was the best strain among these positive mutants of which acetoin production rate was 3.92 times higher than that of the starting strain, *B. subtilis* N-12. The stability of the mutant strain TH-49 was tested by flask fermentation as shown in Table 4.

Bench-scale fermentation of mutant strain TH-49

In order to investigate acetoin production, cell growth and sugar consumption, batch fermentations were carried out in a 10-l fermenter (Figure 3). In addition to 106 g/l l of initial glucose, the composition of the fermentation medium is same to shake flask fermentation medium.

As shown in Figure 3, the concentration of glucose kept on declining till the concentration almost reached zero at 64 h. Acetoin concentration, accompanied by the increasing optical density (OD), reached its maximum of 46.9 g/l at 64 h and remained constant thereafter until the termination of cultivation at 72 h. This phenomenon suggested that, acetoin production by mutant strain TH-49 was associated (or partially associated) with cell growth. And the results also confirmed that, the mutant strain TH-49 was not capable of using acetoin accumulated as its sole carbon source for growth after glucose was consumed. This phenomenon was inconsistent with that of the majority of bacteria that accumulate acetoin as stored energy sources and could continue to use acetoin as energy sources after the exhaustion of glucose (Huang et al., 1999). The yield of acetoin in the fermenter (46.9 g/l) was higher than that in the flask (43.8 g/l), probably mainly due to the improvement of aeration conditions. In other words, there is great potential for improvement of acetoin yield of mutant TH-49 by further optimizing the working conditions in future experiments.

Table 4. Stability of the mutant of mutant TH-49.

Parameter	Values			
Generation	4	8	12	16
Acetoin yield (g/l)	43.6	43.2	44.1	43.8

Composition of shake-flask fermentation medium and analytical methods are same with Table 3; the result in table 4 is the mean of triplicate tests.

GC analysis of fermentation products of mutant strain TH-49

Fermentation broth was recovered and centrifuged at 1500 g for 10 min. The supernatant was distillated at atmospheric pressure. The fraction was assayed by GC. Standard acetoin was used as control (Figure 4). As shown in Figure 4, the main component in fraction was acetoin because both had identical retention time in the same chromatographic conditions. Furthermore, it also showed that there were very little volatile impurities in metabolites. A problem worthy to be pointed out is that 2,3-butanediol and butanedione which were commonly existing as the by-products in acetoin fermentation were not detected, that was beneficial for the separation of acetoin from the fermentation broth.

GC-MS analysis of fermentation products of mutant strain TH-49

The GC-MS method used enabled us to identify the main component metabolized by mutant TH-49 formally. The fermentation broth was treated as the method used in GC analysis. The results are shown in Figure 5. The identity of the compound was confirmed by GC-MS analysis to be acetoin.

DISCUSSION

Plate screening of acetoin producing mutant strains

It is a complex and time-consuming process to select high-yielding mutant after random mutagenesis. For a long time, people have done a lot of effort to simplify the procedure. There are many successful examples, such as, screening of organic acid producting strains on acid resistant agar plate and α-amylase producting strains on starch agar plate by transparent circle (Liu, 2003; Zhu and Wang, 1994). There are a few reports about screening of acetoin and a-acetolactate (precursor for acetoin) producing strains. Phalip et al. (1994) had established a simple method for screening citrate-utilizing and diacetyl/acetoin-producing strains that permits direct visualization on agar plate. This method is based on the ability of diacetyl and acetoin to form a red insoluble complex with

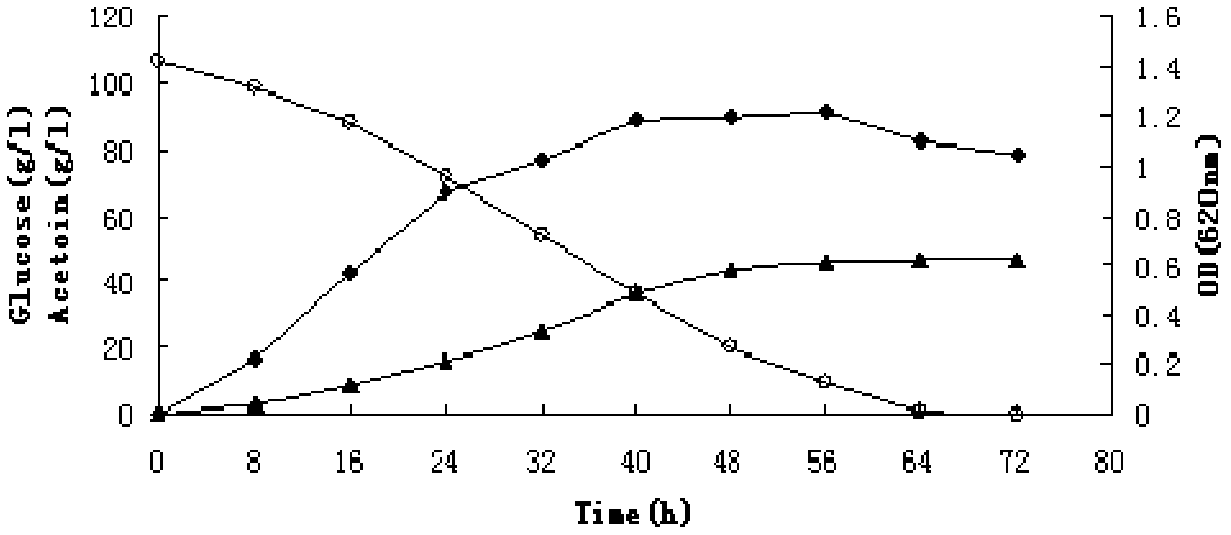

Figure 3. Typical time course of acetoin fermentation of mutant TH-49 in a 10-l fermenter.

α-naphthol in the presence of creatine. Addition of carboxymethyl-cellulose containing calcium citrate in the medium, allowed discrimination between citrate utilizing and non-utilizing bacteria. Monnet et al. (1997) developed a method to screen and isolate mutagenized *L. lactis* spp. *lactis* biovar diacetylactis strains accumulating a-acetolactate (Monnet et al., 1997). This compound is accumulated by a-acetolactate decarboxylase-deficient strains and undergoes spontaneous degradation into diacetyl on agar plates. The screening is based on specific detection of the production of diacetyl, which is converted into the intensely red-colored ammonoferrous dimethylglyoximate. The diacetyl produced is detected by a colorimetric reaction yielding a red halo around the colonies. Monnet et al. (1999) improved a screening method for screening a-acetolactate producing mutants. It was possible to screen up to 1000 colonies per agar plate, whereas the previous method allowed the screening of only 60 colonies per agar plate. The new screening method facilitates selection of a-acetolactate-deficient mutants.

To simplify screening procedure, we tried to establish a method to improve acetoin yield using random mutagenesis followed by screening of high-yielding acetoin colonies visually and quantitatively on agar plates. Facts show that it is very difficult. Pyrultraviolet ray (UV) ic acid is intermediately excreted by bacteria when they utilize glucose or other carbon sources via Embden-Meyerhof-pathway to produce acetoin. Acetoin producing strains can convert pyrultraviolet ray (UV) ic acid to acetoin. In our studies, we replaced the calcium citrate using the method of Phalip et al. (1994) by calcium pyrultraviolet ray (UV)ate and obtained a similar phenomena and results. However, we could only determine the mutant strain to show whether it secretes acetoin qualitatively as we could not exactly determine the production rate of acetoin. So a simple and available method for screening of high-yielding acetoin mutants on agar plates still needs further study.

Breeding strategy for acetoin high-yielding mutant

Acetoin is a product of fermentative metabolism in many microorganisms. Bacteria secrete specific metabolites that reflect their enzyme composition and their growth conditions. For 80 years it has been known that bacteria are capable of using acetoin as its energy sources for growth (Huang et al., 1999). The excretion of acetoin, which can be diagnosed by the Voges Proskauer test and serves as a microbial classification marker, has its vital physiological meanings to these microbes mainly including avoiding acidification, participating in the regulation of NAD/NADH ratio and storage carbon. Although, more attentions have been focused on anabolism and catabolism and great advances have been achieved, there are still different views (Tsau et al., 1992; Xiao and Xu, 2007). Presently, the main acknowledged metabolic pathways and enzymes about biosynthesis of acetoin are as follows: Catabolic α-acetolactate synthase (E1) condenses two molecules of pyrultraviolet ray (UV)ate to form one molecule of a-acetolactate, α-acetolactate can be converted to acetoin catalyzed by α-acetolactate decarboxylase (E2) or undergo spontaneous decarboxylation producing diacetyl. Diacetyl is converted to acetoin catalyzed by diacetyl reductase (E4) or 2,3 - butanediol dehydrogenase (E3). And acetoin can be reversibly transformed into 2,3-butanediol catalyzed by 2,3-butanediol dehydrogenase (E3). From the above statement, the ideal enzymes system for accumulation of acetoin may consist of high activity of E1 and E2 and inactivation or missing of E4, E3 and pyrultraviolet ray (UV) ate dehydrogenase system (PDHS). With the continuous

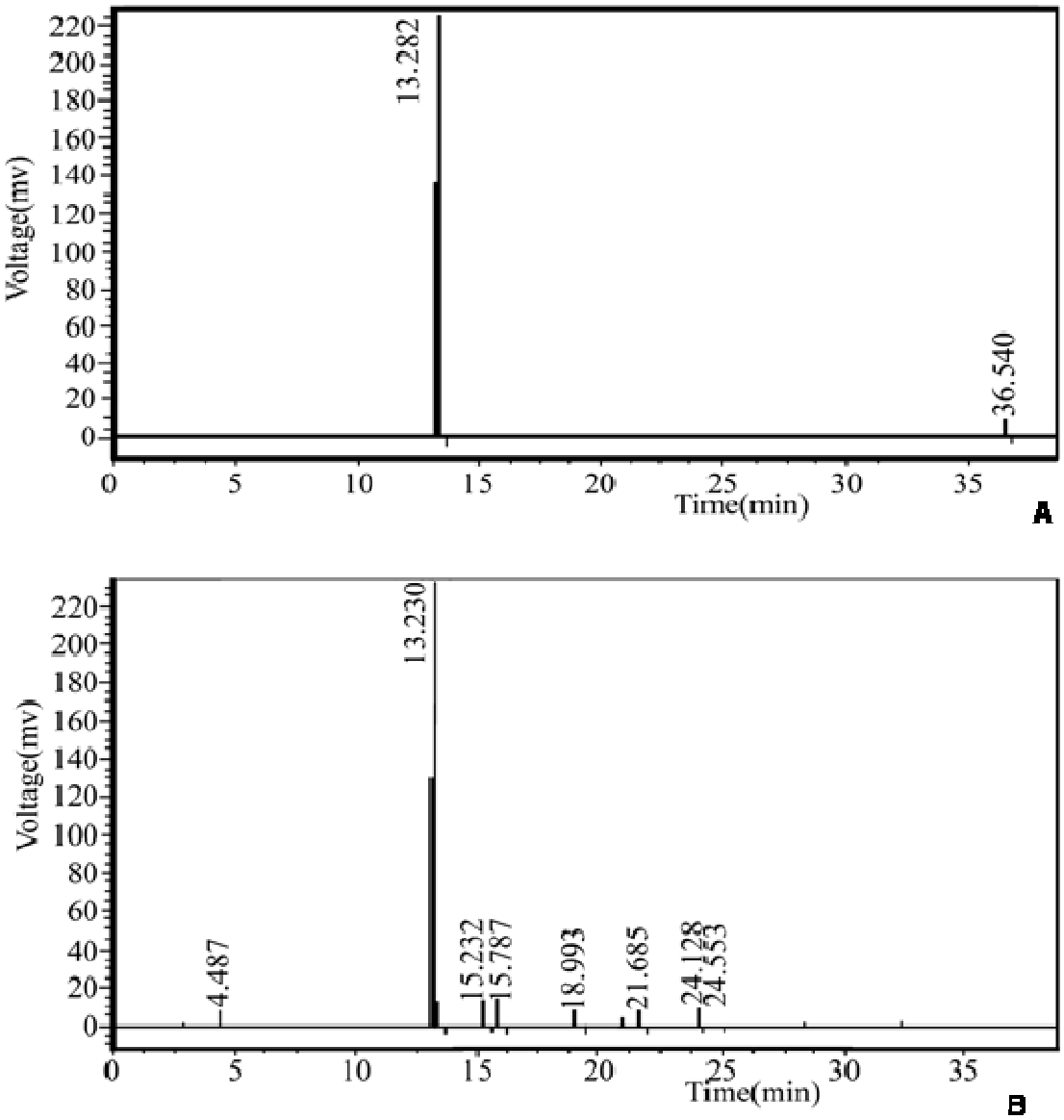

Figure 4. Gas chromatography of main component metabolized by mutant TH-49. A, standard sample of acetoin; B, sample.

progress of study on the encoding genes of enzymes system for accumulation of acetoin and their regulation mechanisms in microorganisms, it is possible to have the acetoin production of mutant strains by direct breeding through genetic engineering (Grundy et al., 1993, 1994; Huang et al., 1999, Silbersack et al., 2006). In addition, the increased production of acetoin as a result of metabolic engineering is also feasible to breeding, for instance, blocking of the branched metabolic pathway. As shown in Figure 6, the branched-chain amino acids, leucine and valine biosynthesis pathways share the same intermediate, that is, α-acetolactate with the acetoin pathway in bacteria. It has been confirm that there are two α-acetolactate forming enzymes, the anabolic α-acetolactate synthase (E5) and the catabolic one (E1) (Halpern and Even-ahoshan, 1967; Mallonee and Speckman, 1988). The E5 is involved in branched-chain amino acids synthesis. The presence of E1 and E5 provides a selection procedure for obtaining mutant microbial strains displaying constitutive production of E1. By eliminating E5, selection can be made for a mutant strain showing constitutive production of E1. By combining the knowledge on redirecting metabolic flux and the mechanism of acetoin production, a very effective strategy to enhance acetoin production by *B. subtilis* via metabolic engineering can be designed. Either disruption of PDHS and E5 or over expression of NADH oxidase and E1 in combination would result in a high diacetyl and acetoin

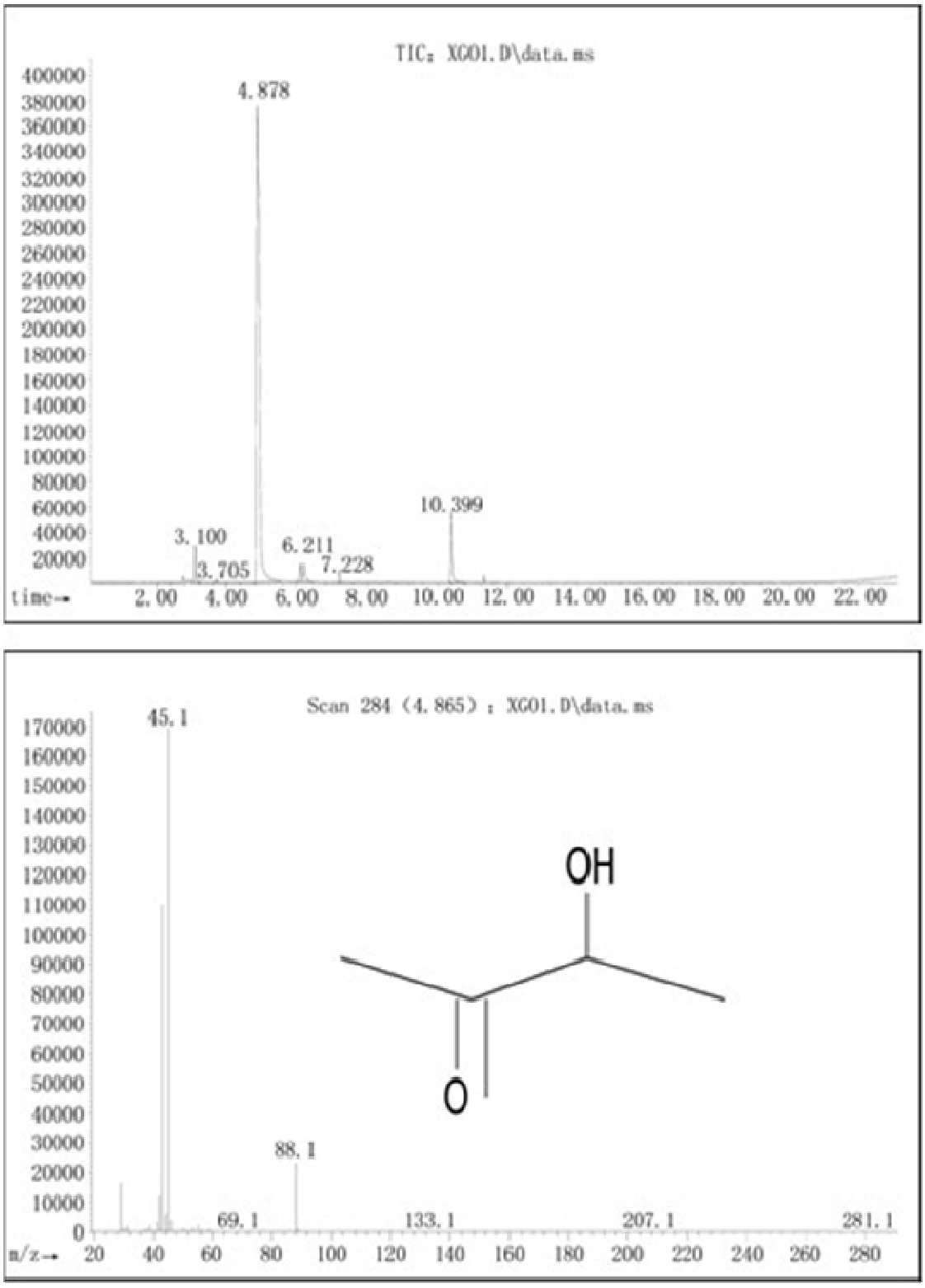

Figure 5. GC/MS chromatography of main component metabolized by mutant strain TH-49.

production.

The role of biotechnology in the synthesis of flavors is increasing. Synthetic flavorings derived from chemical or petrochemical industries have gradually been substituted for flavors of enzymatic or fermentative origin. Although, much attention has been focused on theories of meta-

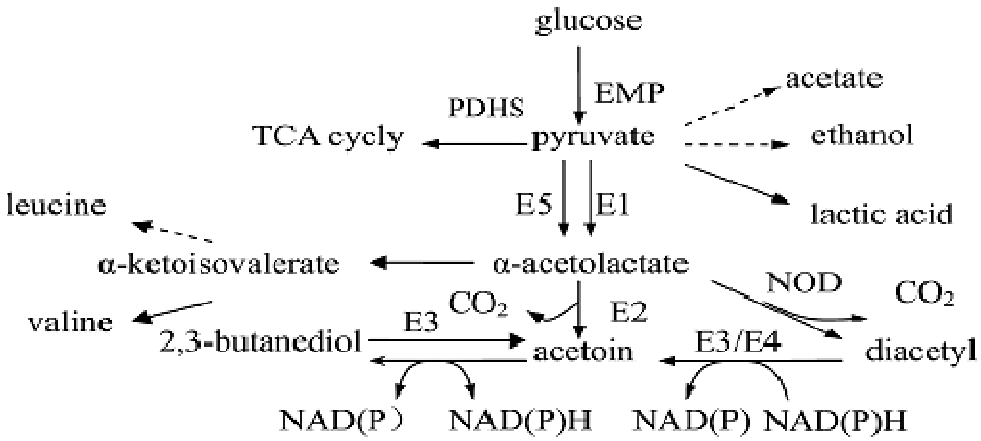

Figure 6. Biosynthetic pathway of acetoin in bacteria. EMP, Embden-Meyerhof-pathway; TCA, tricarboxylicacid cycle; NAD(P), oxidized coenzyme I (II); NAD (P) H, reduced coenzyme I (II); PDHS, pyrultraviolet ray (UV)ate dehydrogenase system; NOD, nonenzymatic oxidative decarboxylation; E1, catabolic α-Acetolactate synthase; E2, α-acetolactate decarboxylase; E3, 2,3 -butanediol dehydrogenase; E4,diacetyl reductase; E5, anabolic α-acetolactate synthase.

bolic pathway of acetoin and great advances have been achieved, studies on production of acetoin through microbial fermentation are few. Acetoin was reported only as a minor byproduct of 2,3-butanediol and diacetyl production and its accumulation usually needs complex and expensive growth factors. In this study, *B. subtilis* mutant strain TH-49 was obtained by random mutagenesis. The acetoin production rate reached the highest value of 43.8 and 46.9 g/l in flask and 10-l fermenter fermentations, respectively. In addition, it deserves attention because acetoin is the main product and there are no other byproducts such as 2,3-butanediol and 2,3-butanedione. This phenomenon may be related to the composition of acetoin metabolism-related enzymes of mutant strain TH-49. All the characteristics show that the strain TH-49 obtained has the potentials of been applied to industrial production. The studies are under way to optimize the production of the acetoin by the mutant strain TH-49.

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