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Full Length Research Paper

Acetoin production associated with the increase of cell biomass in *Bacillus pumilus* ATCC 14884

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Some of *Bacillus* strains, especially *Bacillus pumilus*, were the best acetoin producers. This study attempts to disclose the mechanisms of vigorous acetoin excretion using *B. pumilus* ATCC 14884 as a model strain. The production of acetoin by this strain has been studied under a variety of conditions. Glucose was found to be a much better substrate than citrate, pyruvate, succinate, or fumarate for acetoin production. Malonate was used to slow down the Krebs cycle and the addition of 0.10 M malonate was found to inhibit cell growth slightly, but reduce the production of acetoin by 13%. The addition of 100 g/L NaCl strongly reduced cell growth and acetoin formation. On the other hand, 200 g/L glucose reduced the rate of cell growth, but led to the largest final acetoin production of all the conditions studied. Linear relationships between acetoin accumulation and cell biomass, which increased in the lag and arithmetic growth phases were found when glucose was used as the carbon source, which corroborated acetoin as a primary metabolite. Metabolite study indicated that there were few minor pathways for by-products. Acetoin was utilized as a preferential carbon source without the catabolite repression of glucose, suggesting that the main physiological function of acetoin excretion is an energy-storing strategy in this species.

Key words: Acetoin, biomass, association, metabolism, *Bacillus pumilus*.

INTRODUCTION

Acetoin (3-hydroxy-2-butanone) is a volatile compound that naturally occurs in a wide variety of foods and has multiple usages not only in foods, but in cosmetics, pharmacy and chemical synthesis, and its production from renewable resources is becoming increasingly interesting (Xiao et al., 2010; Xiao et al., 2009). It can be excreted by various microorganisms and the Voges Proskauer test is a routine procedure for diagnosing this ability and classifying the microbes.

The widely accepted pathway of acetoin biosynthesis during growth of bacteria on glucose (Xiao and Xu, 2007) involves two key enzymes: -acetolactate synthase (ALS) and - acetolactate decarboxylase (ALDC) (Renna et al., 1993). Glucose or other fermentable carbon sources are usually degraded via the Embden-Meyerhof (EM) pathway, which resulting in the formation of pyruvate. Thereafter, ALS condenses two molecules of pyruvate to form one -acetolactate, which is unstable and can be easily be converted to one molecule of acetoin by ALDC (Huang et al., 1999; Lopez et al., 1975). Therefore, one molecule of acetoin can be transformed from one molecule of glucose in bacteria. In other words, 1.00 g/L of acetoin requires at least 2.04 g/L of glucose. This is because there are other metabolic fluxes leading to the formation of non-acetoin products *in vivo*, the conversion ratios varies greatly according to bacterial species and fermentation conditions.

Effective screening and strain development, as well as medium optimization are essential to improve acetoin

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production. Acetoin can be reduced *in vivo* to 2,3-butanediol by 2,3-butanediol dehydrogenase and appears as a minor by- product of 2,3-butanediol at a concentration of 10 to 20 g/L in *Klebsiella pneumoniae* NRRL B-199 (Yu and Saddler, 1983), *Enterobacter cloacae* ATCC 27613 (Gupta et al., 1978), *K. pneumoniae* CICC 10011 (Qin et al., 2006), and *Enterobacter aerogenes* DSM 30053 (Zeng et al., 1991). Many common species and strains are even less productive. However, *Bacillus subtilis* CICC 10025 has been found to produce 37.9 g/L acetoin using an optimized molasses medium (Xiao et al., 2007), demonstrating the importance of medium optimization. *B. pumilus* DSM 16187, a selected strain following chemical mutation, produced 63.0 g/L acetoin from 200 g/L glucose (Xu et al., 2009), which set a new record on microbial acetoin production.

Bacillus, including *B. subtilis* as a representative, is one of the mostly studied Gram -positive bacteria species (Tjalsma et al., 1999). There have been many reports concerning acetoin metabolism in *B. subtilis*, but only about two of *B. pumilus* are most studied till date. One is the above patent by Xu et al. (2009) for *B. pumilus* DSM 16187, and the other is about *B. pumilus* ATCC 14884, which was found to be capable of growth in a chemically defined medium with acetoin as the sole carbon and energy source (Xiao et al., 2009). Fortunately, the genome of two *B. pumilus* strains (NC_009848, *B. pumilus* SAFR- 032 complete genome; NZ_ABRX00000000, *B. pumilus* ATCC 7061 whole genome shotgun sequencing project) became available in recent years. By comparison the two genomes and those from *B. subtilis* species, one can find that the two species shares remarkable similarities in most important metabolic pathways including the EM pathway, the Krebs cycle, and the acetoin formation pathway as well.

The Krebs cycle is a confessed central metabolic pathway in aerobic bacteria. Are there relations between this central pathway and the acetoin formation pathway? Succinate dehydrogenase is one of the key enzymes in the Krebs cycle. Malonate, a structural analog to succinate, inhibits succinate dehydrogenase by competing with succinate for this enzyme (Greene and Greenamyre, 1995). Thus, malonate could be a useful tool to inhibit the Krebs cycle and can be easily applied to test its effects on acetoin production. Because *B. pumilus* is one of the best acetoin producers (Xu et al., 2009), this paper explores different carbon sources, inhibition of the Krebs cycle, hypertonia, the effects of metabolites and a pathway analysis using *B. pumilus* ATCC 14884 as a model strain. The results would be important for better microbial acetoin production.

MATERIALS AND METHODS

Organism and growth

B. pumilus ATCC 14884 has been widely used in the assay of antibiotics (British Pharmacopoeia Commission, 2003; European Pharmacopoeia Commission, 1997). The spore strips of this bacterium can also be used to validate and monitor the radiation sterilization process (British Standards Institution, 2000). Briefly speaking, Test strips should be introduced into the radiation sterilization chamber along with the material to be sterilized. At the end of the sterilization process, the strips should be aseptically removed from the chamber and transferred to a rich recovery media and then incubated aerobically at about 35˚C. No bacterial growth indicates good sterilization process and vice versa.

The seed culture of this strain, prepared as described previously (Xiao et al., 2007), was washed with sterilized saline before inoculating (with starting cell concentration of 0.15±0.02 g/L cell dry weight (CDW)) into fermentation media in 500- ml Erlenmeyer flasks (50 ml medium in each flask) on a reciprocal shaker (100 rpm) at 37˚C. Luria-Bertani broth (LB) (Sezonov et al., 2007), or LB plus glucose, citrate, pyruvate, succinate, fumarate, malonate, high concentrations of sodium chloride or glucose, or acetoin were chosen, respectively as cultivation media depending on the experiment being performed. Before autoclaving, the pH of each kind of fermentation medium was adjusted to 7.0. The chemicals were purchased from Sinopharm (Beijing, China) and Sigma-Aldrich. The experiments in Table 1 were performed in triplicates and the standard deviations were calculated. The other experiments were carried out in duplicates and mean values were recorded.

Table 1. Comparison of different carbon sources on cell growth (CDW, cell dry weight) and acetoin production. LB was used as the control medium. The Glucose, Citrate, Pyruvate, Succinate, and Fumarate media were LB plus 3.60 g/L (0.02 M) glucose ($C_6H_{12}O_6$), 11.76 g/L (0.04 M) sodium citrate ($HOC(COONa)(CH_2COONa)_2 \cdot 2H_2O$), 4.40 g/L (0.04 M) sodium pyruvate ($CH_3COCOONa$), 6.48 g/L (0.04 M) sodium succinate dibasic ($NaOOCCH_2CH_2COONa$), or 6.40 g/L (0.04 M) sodium fumarate dibasic ($NaOOCCH=CHCOONa$), respectively.

Medium	CDW (g/L)	Acetoin (g/L)
Glucose	2.34 ± 0.01	0.50 ± 0.01
Citrate	2.61 ± 0.09	0.04 ± 0.00
Pyruvate	2.45 ± 0.04	0.07 ± 0.01
Succinate	2.41 ± 0.02	0.08 ± 0.00
Fumarate	2.04 ± 0.03	0.10 ± 0.00
Control	1.98 ± 0.10	< 0.01

Analytical methods

Cell growth was spectrophotometrically measured at a wavelength of 620 nm (OD_{620}). The linear relationship between OD_{620} and cell dry weight (CDW) concentration was observed. Then biomass concentration CDW was calculated from OD_{620} (1 OD_{620} = 0.31 g CDW per liter) (Xiao et al., 2006). Glucose was enzymatically measured using an YSI 2700 SELECT biochemical analyzer (Yellow Springs Instrument Co., USA) (Xiao et al., 2007). Acetoin was extracted and then quantified by gas chromatography (Varian 3800, Palo Alto, USA) (Xiao et al., 2007). For metabolite investigations, samples were analyzed using HPLC (Agilent 1100 series) equipped with a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) and a differential refractometer. 10 mM H_2SO_4 at a flow rate of 0.4 ml/min was used as the mobile phase. Agilent Chemstation software 9.01 was used for instrument control, data collection and analysis.

RESULTS

Different carbon sources

Cell growth and acetoin biosynthesis were monitored when strain ATCC 14884 was cultured in different media. To improve the comparative analysis between different culture media, flask experiments were carried out with identical inoculums, initial pH values and aerobic cultivation conditions. In the Glucose medium, 0.04 M pyruvate is generated from glycolysis of 0.02 M glucose from the EM pathway. In the Pyruvate medium, there was 0.04 M pyruvate as well. However, the Glucose medium yielded about 7 times as much acetoin as the Pyruvate medium (Table 1). Because the formation of 0.50 g/L acetoin requires 1.02 g/L glucose, 28% of the total 3.60 g/L glucose must be consumed to synthesize acetoin and the remaining 72% used for cell growth, energy generation etc. In comparing the statistical analysis of the data in Table 1, the three kinds of intermediates in the Krebs cycle including citrate, succinate and succinate are obviously worse than glucose in supporting acetoin production. The data in Table 1 also shows that citrate is better than succinate, and that succinate is better than fumarate in improving cell growth. However, in the three non-sugar-based media, the production of acetoin is generally in the opposite direction to cell growth. This tendency is quite different with the results of those in the sugar-based media in this study, where acetoin production is strictly in the same direction to cell growth. The main conclusion from Table 1 is that glucose is confirmed as a better substrate for acetoin production than citrate, pyruvate, succinate or fumarate.

Effects of malonate

However, since glucose is a favorable substrate for efficient acetoin fermentation, glucose was used as the carbon source to study the effects of malonate. Figure 1a shows the on time development of ATCC 14884 cell growth, acetoin and glucose in the LB plus 10 g/L glucose medium. Cell growth and acetoin accumulation occurred in parallel, while glucose rapidly decreased. At the point when all the glucose has been depleted, cell growth and acetoin production also stopped. Acetoin in the culture broth reaches a maximum of 3.86 g/L. Since 1 mol of glucose corresponds to 1 mol of acetoin, the maximum yield of acetoin is 0.49 g per g of glucose, corresponding to a conversion yield of 79% of the theoretical maximum. Metabolite analysis revealed only trace amounts of 2,3-butanediol, acetate and lactate as glycolysis by-products.

Malonate was used to weaken the Krebs cycle. We found that 0.01 M malonate in the culture medium has a negligible effect on cell growth or acetoin production and we therefore, used a concentration of 0.10 M. Figure 1b shows that the main effect of this concentration is to slow down the catabolic speed of acetoin (compared with

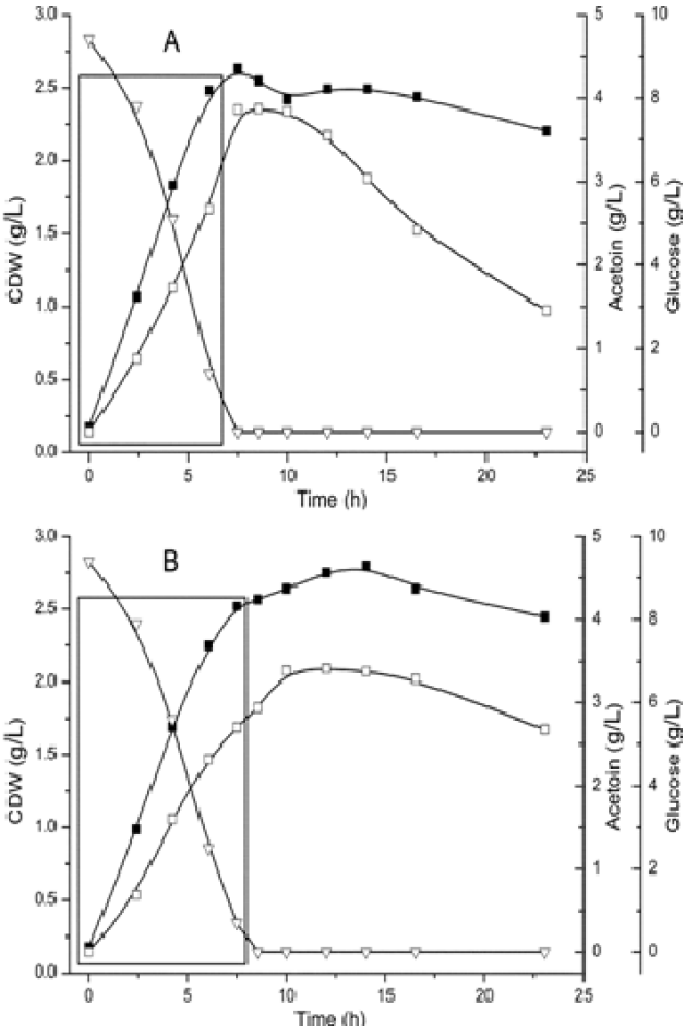

Figure 1. Effects of malonate on acetoin metabolism. Media used in A and B were LB plus 10 g/L glucose, LB plus 10 g/L glucose and 0.10 M malonate, respectively. Filled squares, CDW; open squares, acetoin; inverse open triangles, glucose. The values of CDW and acetoin in the marked rectangles were used to derive the corresponding plots in Figure 3.

Figure 1a), but it also reduces the production of acetoin by 13%.

Effects of hypertonic conditions

Furthermore, due to the fact *B. pumilus* DSM 16187 was found to be capable of growing in the presence of 100 g/L NaCl (Xu et al., 2009) (this characteristic may be connected to high acetoin production), 100 g/L NaCl was added into the LB medium. As shown in Figure 2a, the acetoin concentration increased slowly in parallel with cell growth, the bacterium experienced an obvious lag phase, and the yields of cell biomass and acetoin were much lower.

The fact that strain DSM 16187 was also found to be

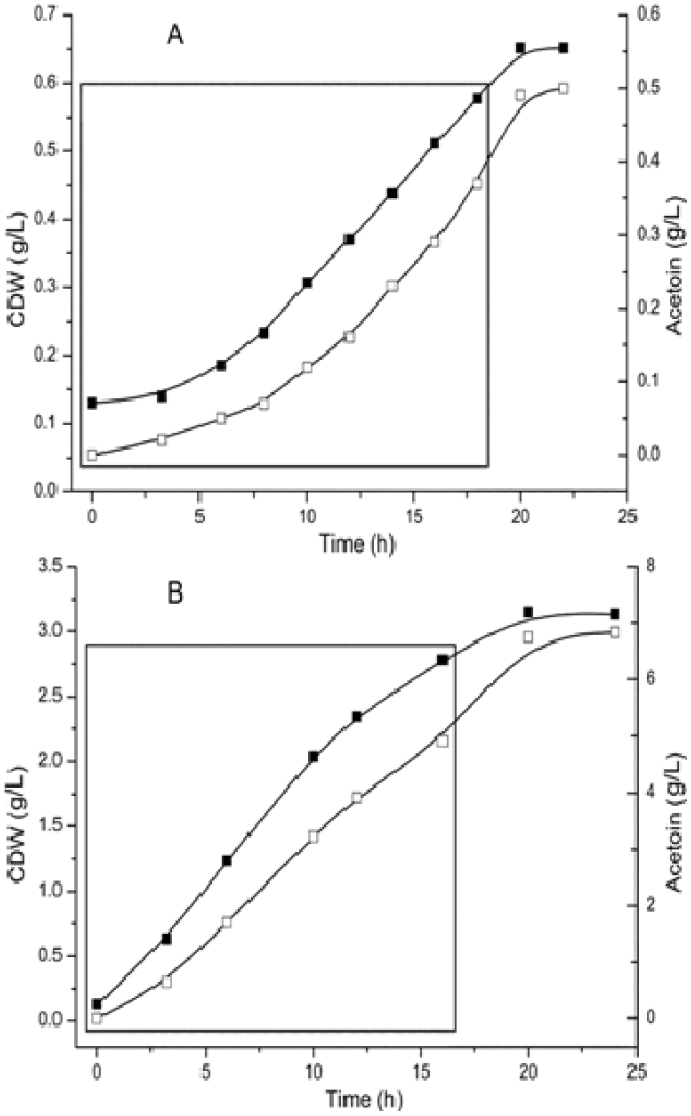

Figure 2. Effects of high concentrations of NaCl or glucose on acetoin production. Media used in A and B were LB plus 10 g/L glucose and 100 g/L NaCl, and LB plus 200 g/L glucose, respectively. Filled squares, CDW; open squares, acetoin. The values of CDW and acetoin in the marked rectangles were used to derive the corresponding plots in Figure 3.

capable of growing in high osmotic LB medium containing 200 g/L glucose (Xu et al., 2009), similar experiment was repeated here. Figure 2b shows that the addition of 200 g/L glucose strongly reduced the rate of cell growth and acetoin production, but the yields of cell biomass and acetoin were the highest of all the experiments in this study.

Biomass and acetoin relationship

Figure 3 shows good linear relationships between cell growth (CDW) and acetoin production in both bacterial lag and arithmetic growth phases when glucose was used as the carbon source. This is evidence that, in these experiments, there was a steady and large metabolic flux from pyruvate to acetoin during glycolysis in this bacterium. It also corroborates acetoin as a primary metabolite. The linear relationship between cell growth and acetoin production is in agreement with previous studies: The addition of yeast extract into culture media was found to give a simultaneous improvement of cell biomass and acetoin production (Garg and Jain, 1995; Yadav and Gupta, 1975).

Acetoin as a preferential carbon source

Figure 4 shows that yeast extract and peptone present in the LB medium provide sufficient amino acids, peptides, growth factors, etc. for proper cell multiplication of strain ATCC 14884. As a result of the fact that the content of carbohydrate is very low in LB, the amino acids act as both carbon and nitrogen sources. The addition of acetoin to the LB medium, inhibits cell growth in the lag phase, as can be seen from the two kinetic profiles in Figure 4. The influence of adding acetoin is to induce acetoin decomposing enzymes (acetoin dehydrogenase enzyme system, AoDH ES), which then function vigorously to catabolize acetoin as both carbon and energy source in order to support a rapid cell growth. This result suggests that acetoin catabolism can occur not only in the stationary phase and spore- forming period (Grundy et al., 1994; Yoshida et al., 2000), but also in the growth phase. More importantly, it implies that acetoin is a preferred carbon source in the absence of sugars.

DISCUSSION

Blocking of the Krebs cycle

Causey et al. (2004; 2003) disrupted the Krebs cycle, minimized ATP yield, reduced substrate loss to cell mass and CO_2, and knocked out some genes in by-product pathways to obtain a high yield of pyruvate or acetate using *Escherichia coli* engineering strains. Comparison with these results suggests that the Krebs cycle is probably also involved (indirectly) in the production of acetoin under aerobic conditions because it is essential for the generation of bacterial cell energy. However, there have been few reports on the effect of blocking or weakening the Krebs cycle on acetoin biosynthesis. Here, we have used malonate to slow down the Krebs cycle and shown that it reduces acetoin production.

The different influences of the concentration of malonate have two possible explanations. First, malonate is only a competitive inhibitor to succinate dehydrogenase and its inhibition may not be strong enough to have significant effects on the Krebs cycle when the concentration of malonate is low. Secondly, the

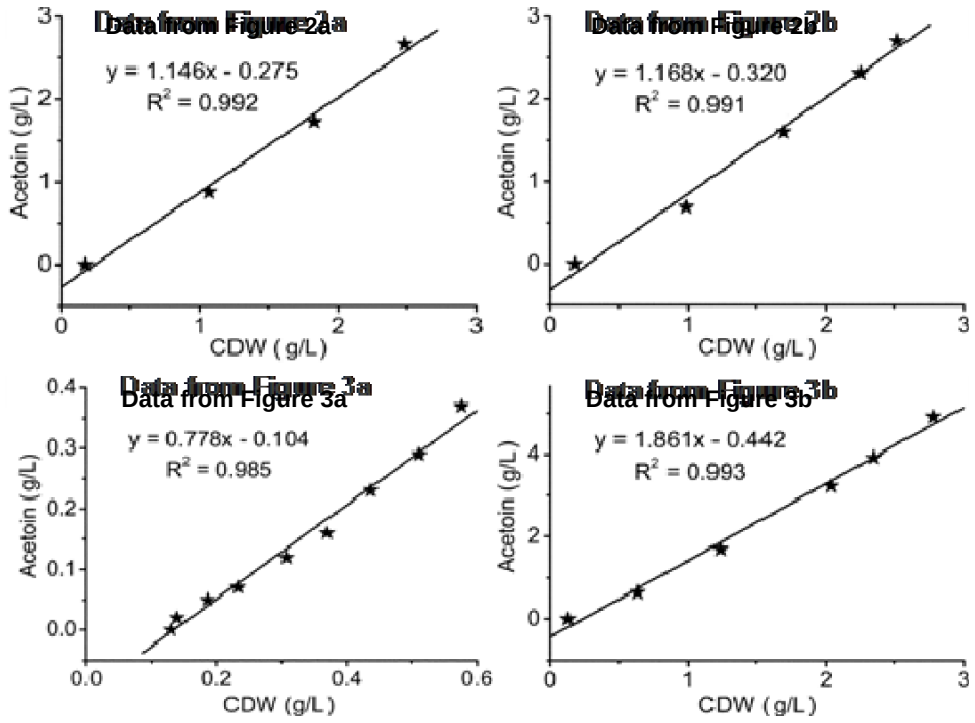

Figure 3. Linear relationships between CDW and acetoin production in lag and arithmetic growth phases of *B. pumilus* ATCC 14884 when glucose was used as the carbon source.

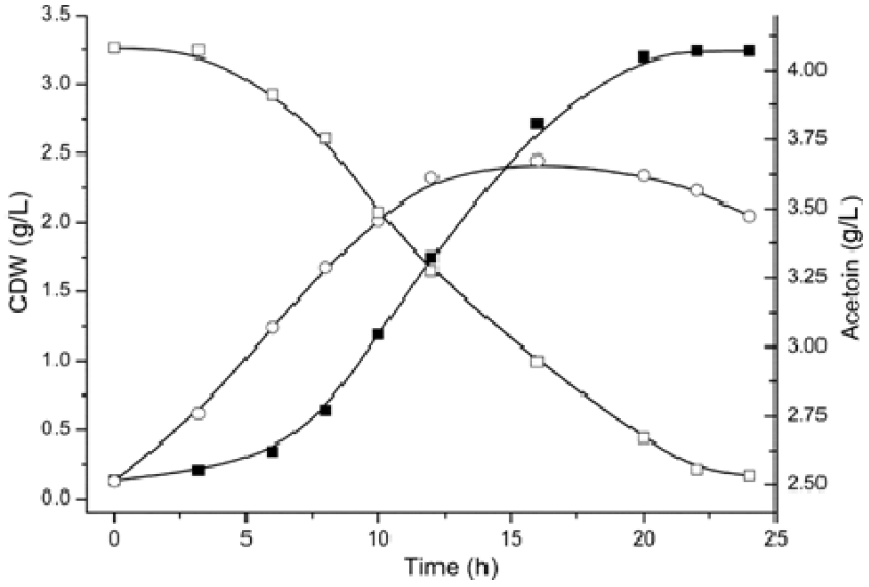

Figure 4. Growth of *B. pumilus* ATCC 14884 in the acetoin medium (LB plus 4.1 g/L acetoin). Filled squares and open circles represent CDW profiles in the acetoin medium and LB medium, respectively. Open squares are the time dependent acetoin degradation profile.

glyoxylate cycle is expected to replenish energy generation and precursor compounds needed for biosynthesis when the Krebs cycle is slowed down (Lorenz and Fink, 2001). However, the efficiency of such anaplerotic reactions is limited. The degradation rate of acetoin was therefore much slower immidiately succinate dehydrogenase was inhibited (Figure 1b compared with Figure 1a) because the catabolic result of acetoin, that is,

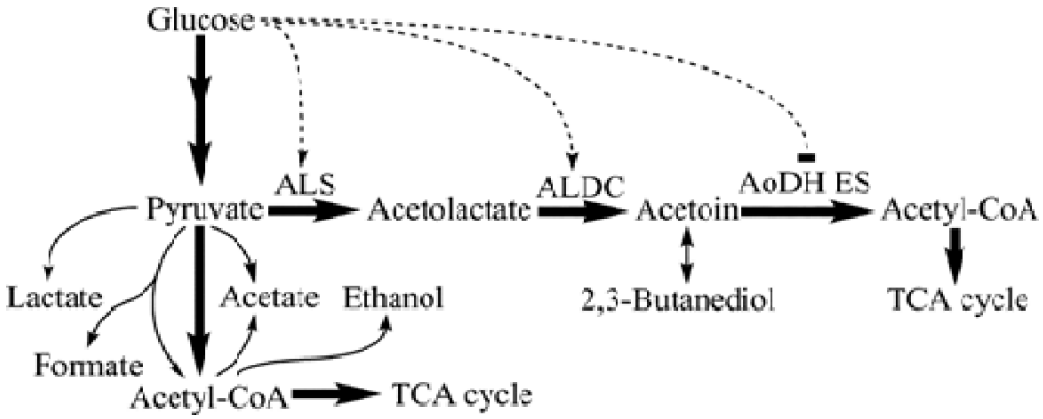

Figure 5. Interconnections of pyruvate metabolic pathways in *Bacillus pumilus*. Bold or plain arrows indicate the dominant or minor metabolic fluxes, respectively. Dotted lines with arrows or blunt ends indicate transcriptional activation or repression pathways, respectively. ALS, -acetolactate synthesase; ALDC, -acetolactate decarboxylase; AoDH ES, acetoin dehydrogenase enzyme system.

acetyl-CoA, needs to be introduced into central metabolism through the Krebs cycle.

Physiological meaning of the acetoin pathway

Generally speaking, the acetoin biosynthesis pathway has three main physiological functions in bacteria (Xiao and Xu, 2007): Avoidance of overacidification due to acetate excretion (Speck and Freese, 1973; Tsau et al., 1992), a carbon- storing strategy by acetoin reutilization (Johansen et al., 1975; Grundy et al., 1993; Mayer et al., 1995), and participation in regulation of the NAD/NADH ratio through the reversible conversion of acetoin and 2,3-butanediol (Johansen et al., 1975; Magee and Kosaric, 1987). The first could probably explain why acetate has profound effects on acetoin production in some microbes (Nakashimada et al., 2000; Romick and Fleming, 1998; Stomer, 1977).

For strain ATCC 14884, apart from large acetoin accumulation, only trace amounts of acetate and 2,3-butanediol were detected as glycolysis by-products and acetoin was reused as a preferential carbon source without glucose, both indicating that the physiological relevance of acetoin pathway is carbon-storing without overacidification. This attribute would be advantageous for the bacilli when competing with others in an environmental niche containing glucose or other fermentable carbohydrates, which are degraded via the Embden-Meyerhof pathway.

A map of pyruvate metabolic pathways in *B. pumilus* is presented in Figure 5 following the published work on acetoin metabolism in *Bacillus*, the results from the present study and the nucleotide sequence data on *B. pumilus* SAFR-032 (Gioia et al., 2007). This map helps to explain why *B. pumilus* has a superior performance in acetoin accumulation: Fewer and weaker by-product pathways, a steady and strong metabolic flux from glucose to acetoin, and vigorous growth activity in hypertonic sugar media.

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