Production of 2,3-butanediol by *Bacillus stearothermophilus*: fermentation and metabolic pathway

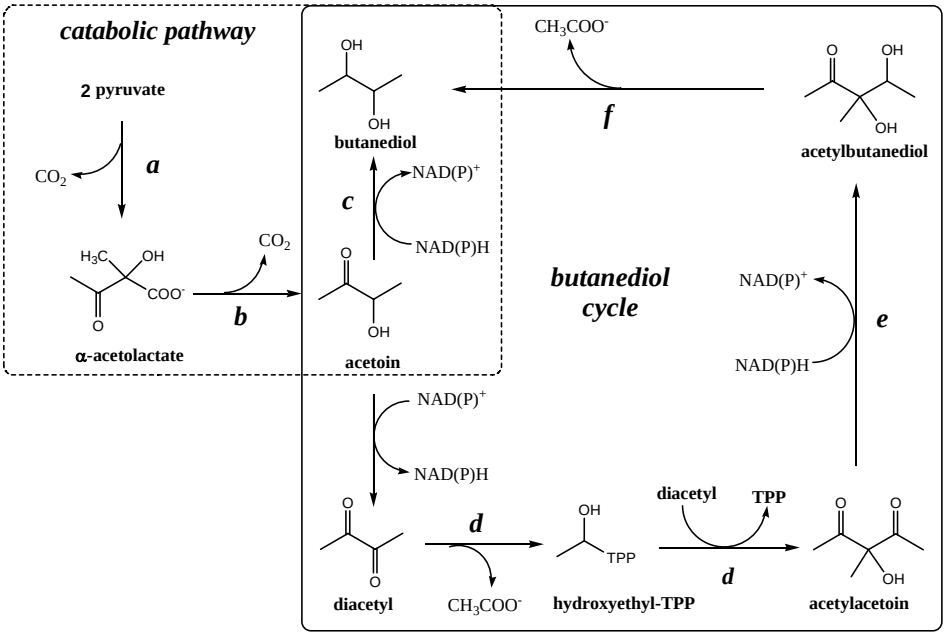
Pier Paolo Giovannini, Matteo Mantovani, Alessandro Medici and Paola Pedrini
Dipartimento di Biologia ed Evoluzione, Università di Ferrara
C.so Ercole I d'Este 32, 44100 Ferrara, Italy

The fermentation of different sugars by *Bacillus stearothermophilus* ATCC 2027 to obtain 2,3-butanediol is described. Fermentation tests in shake flasks show the complete conversion of sucrose to butanediol (91% yield) and acetoin (3-hydroxy-2-butanone) using a starting sugar concentration of 30 g/L. A similar result is obtained with sugar cane molasses, whereas glucose and fructose afford lower yields. Other disaccharides and monosaccharides are also tested without appreciable results. *B. stearothermophilus* forms a mixture of 2*R*,3*R*-butanediol and the *meso* isomer derived both from reduction of *R*-acetoin and from the butanediol cycle as demonstrated by the presence of acetoin reductase and acetylacetoin synthase.

Introduction

2,3–Butanediol (BD) is a colorless and odorless liquid with a large number of industrial applications. Actually by dehydration it can be converted to methyl ethyl ketone, a fuel additive, or to 2,3-butadiene, an important monomer for the manufacture of synthetic rubber. It's a potential fuel additive too, having a heating value comparable to ethanol and methanol (Perego et al., 2003). Furthermore it can be easily converted to diacetyl, a flavouring agent used in food industry and its *levo*-form is used as antifreeze agent. Recently, interest for its microbial production is increasing. Many bacteria produce BD but among them only *Bacillus polymyxa* and *Klebsiella pneumoniae* are used for fermentation on industrial scale (Syu, 2001). BD exists in three stereoisomeric forms (i.e. (-)-(2*R*,3*R*)-BD, (+)-(2*S*,3*S*)-BD, and the *meso*-form) and microrganisms generally give a mixture of *meso*-BD and one of the two enantiomer. Acetoin (AC) is the main precursor of BD and is formed in bacteria from pyruvate by action of the two enzymes α-acetolactate synthase, that catalyzes the condensation of two pyruvate molecules with a single decarboxylation to afford α-acetolactate, and α-acetolactate decarboxylase that decarboxylates this last one to acetoin (Juni, 1952) (catabolic pathway), (Fig.1). The different isomeric forms of BD can be produced by AC-reduction with various AC-reductase with different stereospecificity (Ui et al., 1984) or by a cyclic pathway called "butanediol cycle" which existence has been reported in different bacteria (Ui et al., 1994) (Fig 1). Using *Bacillus stearothemophilus* ATCC 2027 as biocatalyst for the synthesis of optically active secondary alcohols and *vic*-diols (Bortolini et al., 1997), we found high concentration of BD and AC in the culture broth when sucrose was used as carbon source.

Figure 1. BD formation in bacteria: catabolic pathway and BD cycle.



a: α-acetolactate synthase; **b**: α-acetolactate decarboxylase; **c**: AC reductase; **d**: acetylacetoin synthase; **e**: acetylacetoin reductase; **f**: acetylbutanediol hydrolase

On the basis of this observation, a preliminary study to evaluate the potential of this bacterium in bioconversion of different mono- and disaccharides to BD was performed. Since a mixture of (2*R*,3*R*)-BD and *meso*-isomer was produced in a 1:1.5 ratio, we focused our attention also in the metabolic pathway mechanisms.

Results and discussion

Fermentation of various carbohydrates

Since *B. stearothermophilus* ATCC 2027 easily grows on sucrose producing interesting amounts of BD and AC, the fermentation was carried out in order to evaluate the effect of sucrose concentration (0, 10, 20, 30 and 40 g/L, respectively) on BD production. The best result of fermentation was obtained with 30 g/L sucrose in shake flasks at 40°C (Table 1). Although the 40 g/L sucrose fermentation afforded the highest BD concentration after 32 h (18.3 g/L), at this time unutilized sucrose was about 7 g/L. On the contrary, *B. stearothermophilus* fermentation of 30 g/L sucrose gave lower amount of BD after 28 h (14.4 g/L) but practically no sucrose was detected. It can be pointed out that the BD concentration increased till the sucrose consumption was fast, subsequently, when a low amount of sucrose was present, AC concentration increased due to the oxidation of BD as demonstrated by the trend of the sum of both metabolites (Fig. 2).

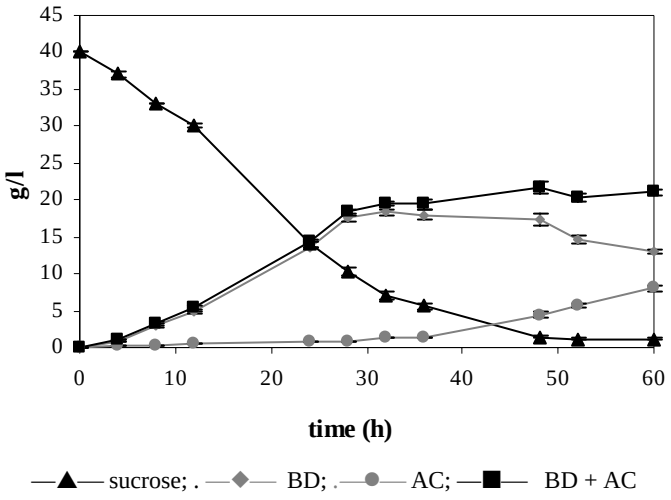
Table 1. Production of BD and AC with *B. stearothermophilus* using various sugars.

Sugar (g /L)	Time H	Residual sugar g/L	2,3-BDL[a] g/L (yield)	AC[b] g/L (yield %)	2,3-BDL + AC g/L (yield%)
Sucrose (40)	32	7.0	18.3 (87%)	1.3 (6%)	19.6 (93%)
Sucrose (30)	28	0.8	14.4 (91%)	1.7 (11%)	16.1 (102%)
Glucose (30)	32	16.4	8.7 (58%)	2.6 (18%)	11.3 (72%)
Fructose (30)	36	15.4	7.1 (47%)	4.5 (31%)	11,6 (78%)
Molasses(30)[c]	40	7.8[d]	12.0 (76%)	1.5 (10%)	13.5 (86%)

[a] The data are the total of 2*R*,3*R*-BD (ee ≥95%) and *meso*-BD in ratio 1:1.5. [b] *R*-AC is obtained (ee ≥ 95%). [c] The sugar concentration is the sum of sucrose (63%) and invert sugar (37%). [d] Only invert sugar.

On the basis of these data the fermentability of other carbohydrates (30 g/L) was tested. Cellobiose, maltose and lactose were not fermented probably due to the lack of efficient glycosidases as confirmed by the high concentration of the corresponding disaccharides in the fermentation broth after 48 h. Among the tested monosaccharides (i.e. glucose, fructose, galactose and xylose) only glucose and fructose gave appreciable results even though showing lower yields in comparison with sucrose (Table 1). When the higher BD concentrations were reached (8.7 g/L with glucose and 7.1 g/L with fructose) about 16 g/L of unutilized monosaccharide were still present in the fermentation broth. Also in these cases the amount of BD decreased in favour of AC at longer time. Finally sugar cane molasses was tried too. In this case the broth was obtained simply diluting the molasses in order to have a final sugar concentration of 30 g/L (sucrose 63% and invert sugar 37%) without adding supplemental nutrient.

Figure 2. Time course of sucrose fermentation (30 g/L) by *B. stearothermphilus*



The trend of BD and AC production was similar to that reported for the 30 g/L sucrose fermentation affording, after 40 h, 12.0 g/L of BD and 1.5 g/L of AC together with 7 g/L of invert sugar. This data confirm that sucrose is the elective carbohydrate for BD production using *B. stearothermophilus* ATCC 2027 and that glucose and fructose are fermentable too but with lower efficiency.

Metabolic pathway and metabolite stereochemistry

All the main BD forming bacteria convert pyruvate to AC with *R* configuration (Ui et al., 1986) by action of the two enzymes α-acetolactate synthase and α-acetolactate decarboxylase (Figure 1. react. a and b). In order to confirm this behaviour in *B. stearothermophilus* ATCC 2027, we determined the configuration of the AC formed by adding freshly prepared cell free extract to a solution of sodium pyruvate in phosphate buffer at pH 6.0. After standing two hours at 30°C, the reaction mixture was extracted with ethyl acetate and analyzed by GC. Only one AC enantiomer was detected (e.e. >96%) and its configuration was deduced on the basis of the BD isomers formed by reduction with $NaBH_4$. This reduction afforded a mixture of 2*R*,3*R*- and *meso*-BD proving that AC produced was the *R*-enantiomer according to the other BD forming bacteria. In our previous work we described the purification and characterization of a diacetyl (acetoin) reductase from *B. stearothermophilus* (Giovannini et al., 1996). This NAD-dependent enzyme showed a *S*-enantioselectivity in the reversible reduction of AC so it could be responsible of the *meso*-BD formation from *R*-AC (Fig.1, react.c). In addition this strain has another NAD-dependent dehydrogenase able to catalyze the oxidation of 2*R*,3*R*-BD as demonstrated by formation of NADH when this substrate was added to a solution containing NAD^+ and cell free extract in phosphate buffer at pH 8.4. Unfortunately, this enzyme, until today, was not completely purified, so we can only suppose its activity in the reduction of *R*-acetoin to form 2*R*,3*R*-BD. Finally we verified the existence of BD cycle in *B. stearothermophilus* following a procedure described for *B. cereus* YUF-4 (Ui et al., 1994). After addition of diacetyl (10 g/L) to a culture grown on a medium containing AC (5 g/L) as carbon source, we observed a significant accumulation (about 2 g/L) of acetylacetoin. Its presence proves the existence of an acetoin-inducible acetylacetoin synthase (Fig 1, react. d), that has been proposed as a marker enzyme of BD-cycle (Ui et al., 2002).

Experimental

Material

All the reagents are commercially available. Sugar cane molasses was kindly furnished by prof. G. Vaccari from Ferrara University. For the GC analysis, a Carlo Erba 6000 chromatograph was used. ^{1}H-NMR spectra were acquired on a Varian Gemini 300 MHz spectrometer.

Fermentation tests

A culture of *Bacillus stearothermophilus* ATCC 2027 was grown 24 h at 38-39° C and 110 rpm in a medium containing sucrose (40 g/L), peptone (20 g/L), yeast extract 10 (g/L), $Na_2HPO_4 \cdot 6H_2O$ (6.8 g/L), K_2SO_4 (2.6 g/L), and $MgSO_4$ (0.3 g/L). Each

fermentation was started adding 5 ml of this culture to a medium (115 ml in 500 ml flask) with the same composition except for the carbon source (see Table 1). A similar inoculum was used to start fermentations with sugar cane molasses as unique medium component. The concentration and stereochemistry of BD, AC, and acetylacetoin were determined by gas chromatographic analyses. Each sample (2 mL) was centrifuged (6000 rpm, 10 min), added with NaCl (0.2 g) and extracted with ethyl-acetate (0.8 mL) containing 0.1% (v/v) of acetophenone as internal standard. Separations were performed with a fused capillary column Megadex 5 (25 m X 0.25 mm) containing dimethyl-*n*-pentyl-β-cyclodextrin on OV 1701 (from Mega snc): helium as carrier gas (68 kPa); temp. 90-200°C (2°C/min). Retention times (in min): (-)-3*R*-AC, 2.95; (+)-3*S*-AC, 3.10; (+)-2*S*,3*S*-BD, 5.90; (-)-2*R*,3*R*-BD, 6.20; *meso*-BDL, 6.60; acetophenone 11.30. Glucose, fructose, and sucrose concentrations were determined by enzymatic analysis (SIGMA kits).

Biosynthesis of AC from pyruvate

Sodium pyruvate (0.1 g, 0.9 mmol) was added to a solution containing 4.0 mL of cell free extract obtained as described (Bortolini, 1997) in phosphate buffer 50 mM, pH 6.0 (100 mL). After two hours at 30°C the reaction mixture was saturated with NaCl and extracted with ethyl-acetate (3 X 20 mL). The organic phase was dried over anhydrous sodium sulphate and analyzed by gas chromatography using the above reported conditions. A single peak at 2.95 min. was detected. Ethyl-acetate was removed under reduced pressure, and the residue was dissolved in diethyl-ether/methanol 5:1 (20 mL). The mixture was cooled (ice bath) and $NaBH_4$ (35 mg, 0.9 mmol) was added. After 30 min. the mixture was diluted with water (10 mL), the organic phase was separated, dried with anhydrous sodium sulphate and analyzed by GC. Two peaks were detected at 6.20 and 6.60 min corresponding to 2*R*,3*R*- and *meso*-BD.

Biosynthesis of acetylacetoin from diacetyl

Diacetyl (2.0 g, 23.3 mmol) was biotransformed as described by Ui et al. (1994) in a 200 mL culture of *B. stearothermophilus* grown on Bouillon medium containing AC (1.0 g, 11.4 mmol) as carbon source. Acetylacetoin (0.3 g, 10 %) was recovered and purified according to the same reference. It is a colorless oil, ^{1}H-NMR ($CDCl_3$): δ 4.70 (s, 1 H, OH); 2.25 (s, 6 H, 2 Ac); 1,60 (s, 3H, Me). Its concentration in the broth was determined by gas chromatographic analysis using acetophenone as internal standard as described for BD and AC (retention time 4.30 min).

Conclusions

B. stearothermophilus ATCC 2027 has demonstrated to convert efficiently sucrose to BD and although with lower yields, it ferment also glucose and fructose. Otherwise this bacterium can grows at temperature above 40°C, so we intend to study its potential in bioreactor to understand if it could be a candidate for industrial application. About the BD production routes, we will focus on the specificity of the AC- and acetylbutanediol reductases to make clear the influence of the catabolic pathway and BD cycle on the stereochemistry of the product.

References

Bortolini, O., G. Fantin, M. Fogagnolo, P. P. Giovannini, A. Guerrini, and A. Medici, 1997, An easy approach to the synthesis of optically active *vic*-diols: a new single-enzyme system. J. of Org. Chem. 62, 1854.

Giovannini, P. P., A. Medici, C. M. Bergamini, and M. Rippa, 1996, Properties of Diacetyl (Acetoin) Reductase from *Bacillus stearothermphilus*, Bioorg. and Med. Chem. 4, 1997.

Juni, E., 1952, Mechanisms of formation of acetoin by bacteria, J. Biol. Chem. 195, 715.

Perego P., A. Converti and M. Del Borghi, 2003, Effect of temperature, inoculum size and starch hydrolyzate concentration on butanediol production by *Bacillus licheniformis*, Bioresource Technology 89, 125.

Syu M. J., 2001, Biological production of 2,3-butanediol, Appl. Microbiol. Biotechnol. 55, 10.

Ui, S., T. Masuda, H. Masuda and H. Muraki, 1984, Mechanism for the formation of 2,3-butanediol stereoisomers in *Klebsiella pneumoniae*, J. Ferment. Technol. 62, 551.

Ui, S., K. Watanabe and T. Magaribuchi, 1994, Production of acetylacetoin by bacterial fermentation, Biosci. Biotech. Biochem. 58, 2271.

Ui, S., T. Masuda, H. Masuda, and H. Muraki, 1986, Mechanism for the formation of 2,3-butanediol stereoisomers in *Bacillus polymyxa*, J. Ferment. Technol. 64, 481.

Ui, S., T. Hosaka, K. Mizutani, T. Ohtsuki and A. Mimura, 2002, Acetylacetoin synthase as a marker Enzyme for detecting the 2,3-butanediol cycle, J. of Biosci. 93, 248.