

ENZYMOLOGY OF ACETONE-BUTANOL-ISOPROPANOL FORMATION

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## I. Introduction

Several species of anaerobic bacteria within the genus *Clostridium* produce acetone, *n*-butanol, and isopropanol (solvents), which are important industrial chemicals and fuel additives. Commercial production of solvents by the clostridia is a classical example of large-scale chemical production by bacterial fermentation. Although the fermentation has been in use for decades, it still faces problems that include strain degeneration, a relatively low final product concentration due to butanol toxicity, and a need to fine-tune the growth conditions to achieve a high yield. A rational search for solutions to these problems became possible after the late 1970s because of the development of basic knowledge and molecular tools for examining the biochemical and genetic properties of the solvent-producing clostridia.

The long-term goal of this project was to understand the fundamental properties of bacterial solvent production for the purpose of achieving a positive control on the metabolic switch leading to solvent production and on the proportion of useful products formed as well as of developing strategies for preventing the degeneration of producing strains.

The objectives for the project included those approved in 1985 for the initial project period and those approved in 1988, 1991, and 1994 when the project was renewed. The objectives for the entire project period may be summarized as

- (1) To purify and characterize the enzymes that are specifically required for the formation of acetone, butanol, and isopropanol by the clostridia,
- (2) To clone and characterize the genes that encode enzymes or regulatory proteins for the production of solvents, and the emphasis was to determine the control mechanism for the transcription of the solvent-production genes,
- (3) To characterize the onset of solvent production and the intra- and extra-cellular parameters surrounding the metabolic switch to solvent production, and
- (4) To determine the genetic identity of the strains of solvent-producing clostridia that are currently in use by investigators around the world.

Significant progress toward these objectives was made during the project period, and important results have been published as journal articles, book chapters, abstracts, and thesis as referenced below.

## II. Summary of Scientific Progress

The approaches used in our research were to identify first the important solvent-producing clostridia. We selected *Clostridium beijerinckii* as the main organism for our study because this species includes strains that produce isopropanol as a major product and because commercial solvent production after the 1930s used organisms similar to *C. beijerinckii*, instead of *Clostridium acetobutylicum*. This was followed by molecular characterization of the solvent-forming enzymes and their genes and then by studying the control mechanisms to develop strategies for regulating the duration of active solvent production and the proportion of useful products formed.

### (1) Enzymology of solvent production.

A proposed metabolic pathway for solvent production was available at the beginning of the project, but except for acetoacetate decarboxylase (responsible for the final step in acetone formation), none of the other proposed solvent-forming enzymes was isolated. During the project period, we purified from *C. beijerinckii* the following acid- and solvent-forming enzymes: 3-hydroxybutyryl-CoA dehydrogenase (Colby and Chen, 1992), phosphotransbutyrylase (Thompson and Chen, 1990), two CoA-acylating aldehyde dehydrogenase (Yan and Chen, 1990; Ismaiel and Chen, unpublished), a primary-secondary alcohol dehydrogenase (ADH) (Ismaiel et al., 1993), three isozymes of a primary ADH (Yan, Ph.D. thesis; Chen, 1995), and acetoacetate:acetate/butyrate CoA-transferase (Colby, Ph.D. thesis). The kinetic properties and the N-terminal amino acid sequences of these enzymes have been determined. The results of the study of the enzymes have been reviewed (Chen, 1993, 1995).

Among the solvent-forming enzymes, the aldehyde and alcohol dehydrogenases are essential for the formation of butanol, but these dehydrogenases are not yet well understood because of the presence of multiple forms (Chen, 1995). The highlights in this area of research include

- (i) The discovery of the primary-secondary alcohol dehydrogenase in strains of isopropanol-producing *C. beijerinckii* as this ADH is responsible for the production of both *n*-butanol and isopropanol.
- (ii) The discovery, by other investigators, of the presence of a gene (*adhE/aad*) in *C. acetobutylicum*, which appears to encode a bifunctional aldehyde-alcohol dehydrogenase. We have purified the enzyme and measured its aldehyde and alcohol dehydrogenase activities (Ismaiel and Chen, unpublished). This enzyme is primarily an aldehyde dehydrogenase.
- (iii) The purification of an aldehyde dehydrogenase from two strains of *C. beijerinckii*. This aldehyde dehydrogenase is distinct from the bifunctional aldehyde-alcohol dehydrogenase found in *C. acetobutylicum*. *C. acetobutylicum* probably has a separate aldehyde dehydrogenase which is yet to be isolated.

### (2) Genes for solvent production.

Using oligonucleotide primers based on the N-terminal amino acid sequences, we have cloned and sequenced the *adh* gene encoding the novel primary-secondary ADH (Peretz et al., 1997) and the *ctfA* and *ctfB* genes for the CoA-transferase (Toth and Chen, 1997 abstract) from *C. beijerinckii* NRRL B593. Following the sequencing of the *ctfA* and *ctfB* genes, we have now sequenced and identified the genes encoding acetoacetate decarboxylase (*adc*) and aldehyde dehydrogenase (*ald*) (Toth and Chen, unpublished). Therefore, we have now established the organization of the solvent-production genes in *C. beijerinckii* NRRL B593. Our knowledge of the structure and organization of the solvent-production genes will allow us to elucidate the regulation of transcription of these genes, which is the focus of our ensuing studies.

The putative promoter region for the *adh* gene has the motif of σ54-dependent promoters. In *C. beijerinckii* NRRL B593, the *adh* gene is preceded by an ORF that encodes a

NtrC-like protein. Because NtrC represents the class of transcriptional activators that are required for the  $\sigma$ 54-dependent promoters, the ORF may play a role in the regulation of solvent-production genes, and this possibility is being investigated.

The primary-secondary ADH of *C. beijerinckii* NRRL B593 is not widespread in solvent-producing clostridia. We have constructed shuttle plasmids that contain this *adh* gene for use in *E. coli* and in clostridia that do not normally contain this gene (Li and Chen, 1995 abstract). The *adh* gene on the plasmid has been expressed in the new hosts, and we are using it as a reporter gene for the study of regulation of expression of solvent-production genes, which is a focus of our current research.

#### **(3) The onset of the solventogenic switch.**

We previously determined that an acidic pH, which is needed by *C. acetobutylicum*, is not necessary for *C. beijerinckii* to switch into the solvent-producing mode of metabolism. (Reviewed in Johnson and Chen, 1995) This physiological difference is not surprising in light of the significant genetic differences between the two species (see next section).

Using the appearance of specific enzyme activities as an indication for the metabolic switch into solvent production, we carefully characterized the onset of solvent production in *C. beijerinckii*. The results revealed that the onset was hours earlier than what would be detected by using product concentrations as the indicator, because of the higher sensitivity afforded by the enzyme activity assays (Yan et al., 1988). The precise determination of the onset point facilitates the identification of extracellular and intracellular parameters that are possible factor(s) triggering the solventogenic switch. With the cloning of the solvent-production genes, we can now use the appearance of the mRNAs for the solvent-production genes to monitor the onset of the solventogenic switch. This aspect of research is an integral part of our effort to elucidate the control mechanism for the transcription of the solvent-production genes in *C. beijerinckii*.

#### **(4) Taxonomy of the solvent-producing clostridia.**

Investigators studying solvent production by "*Clostridium acetobutylicum*" were plagued by conflicting results from different laboratories. We used the definitive DNA-DNA hybridization method to examine the identity of all available strains of solvent-producing clostridia, which were provided to us by investigators around the world (Johnson et al., 1997). The study revealed that strains previously labeled as "*C. acetobutylicum*" consisted of four species, with the genuine *C. acetobutylicum* and *C. beijerinckii* each constituting about half of the 39 cultures examined, and the other two species are each represented by one or two strains. *C. acetobutylicum* and *C. beijerinckii* are not closely related organisms because their percent relatedness based on the total genome is less than 20%. The finding has allowed interpretation of the conflicting results obtained with these mislabeled "*C. acetobutylicum*" strains and will ensure that future genetic studies of these organisms can be conducted with appropriate methodologies.

### III. Publications and thesis resulting from this project

#### A. Journal Articles

Hiu, S. F., C.-X. Zhu, R.-T. Yan, and J.-S. Chen. 1987. Butanol-ethanol dehydrogenase and butanol-ethanol-isopropanol dehydrogenase: different alcohol dehydrogenases in two strains of *Clostridium beijerinckii* (Syn. *Clostridium butylicum*). *Appl. Environ. Microbiol.* 53: 697-703.

Yan, R.-T., C.-X. Zhu, and J.-S. Chen. 1987. Oxidation product(s) in acetaldehyde reacts with NAD(P)H and interferes with assay of alcohol dehydrogenase. *Anal. Biochem.* 164: 362-366.

Yan, R.-T., C.-X. Zhu, C. Golemboski, and J.-S. Chen. 1988. Expression of solvent-forming enzymes and onset of solvent production in batch cultures of *Clostridium beijerinckii* (*Clostridium butylicum*). *Appl. Environ. Microbiol.* 54:642-648.

Thompson, D. K., and J.-S. Chen. 1990. Purification and properties of an acetoacetyl coenzyme A-reacting phosphotransbutyrylase from *Clostridium beijerinckii* ("*Clostridium butylicum*") NRRL B593. *Appl. Environ. Microbiol.* 56:607-613.

Yan, R.-T. and J.-S. Chen. 1990. Coenzyme A-acylating aldehyde dehydrogenase from *Clostridium beijerinckii* NRRL B592. *Appl. Environ. Microbiol.* 56:2591-2599.

Colby, G. D., and J.-S. Chen. 1992. Purification and properties of 3-hydroxybutyryl-coenzyme A dehydrogenase from *Clostridium beijerinckii* ("*Clostridium butylicum*") NRRL B593. *Appl. Environ. Microbiol.* 58:3297-3302.

Ismail, A. A., C.-X. Zhu, G. D. Colby, and J.-S. Chen. 1993. Purification and characterization of primary/secondary alcohol dehydrogenase from two strains of *Clostridium beijerinckii*. *J. Bacteriol.* 175:5097-5105.

Chen, J.-S. 1995. Alcohol dehydrogenase: multiplicity and relatedness in the solvent-producing clostridia. *FEMS Microbiol. Rev.* 17:263-273.

Johnson, J. L., and J.-S. Chen. 1995. Taxonomic relationships among strains of *Clostridium acetobutylicum* and other phenotypically similar organisms. *FEMS Microbiol. Rev.* 17:233-240.

Johnson, J. L., J. Toth, S. Santiwatanakul, and J.-S. Chen. 1997. Cultures of "*Clostridium acetobutylicum*" from various collections comprise *Clostridium acetobutylicum*, *Clostridium beijerinckii*, and two other distinct types based on DNA-DNA reassociation. *Int. J. Syst. Bacteriol.* 47:420-424.

Peretz, M., O. Bogin, S. Tel-Or, A. Cohen, Y. Burstein, G. Li, and J.-S. Chen. 1997. Molecular cloning, nucleotide sequencing, and expression of genes encoding alcohol dehydrogenases from the thermophile *Thermoanaerobacter brockii* and the mesophile *Clostridium beijerinckii*. *Anaerobe* 3:259-270.

#### B. Book Chapter

Chen, J.-S. 1993. Properties of acid- and solvent-forming enzymes of clostridia, p. 51-76. In D. R. Woods (ed.), *The clostridia and biotechnology*. Butterworth-Heinemann, Boston, MA.

#### C. Abstracts

Hiu, S. F., and J.-S. Chen. 1986. Alcohol dehydrogenase for butanol-isopropanol formation by *Clostridium beijerinckii* (syn. *C. butylicum*). *Abstr. Annu. Meet. Am. Soc. Microbiol.* K-182, p.223.

Yan, R.-T., C.-X. Zhu, C. Golemboski, and J.-S. Chen. 1987. The onset of solvent production in *Clostridium beijerinckii* (syn. *Clostridium butylicum*). *Abstr. Annu. Meet. Am. Soc. Microbiol.* O-58, p. 270.

Yan, R-T., and J.-S. Chen. 1987. A potassium-activated acetoacetyl CoA hydrolase in *Clostridium beijerinckii* (*C. butylicum*). *Federation Proc.* 46:1951.

Zhu, C.-X., and J.-S. Chen. 1988. Purification and characterization of a primary/secondary alcohol dehydrogenase from the anaerobe *Clostridium beijerinckii*. *FASEB J.* 2:A765.

Thompson, D. K., R.-T. Yan, M. B. Walker, and J.-S. Chen. 1989. Phosphotransacetylase, phosphotransbutyrylase, and thiolase from *Clostridium beijerinckii*. *Abstr. Annu. Meet. Am. Soc. Microbiol.* O-77, p. 317.

Rifaat, M., and J.-S. Chen. 1991. Cloning of a primary/secondary alcohol dehydrogenase gene (*adh*) from *Clostridium beijerinckii* NRRL B593. *Abstr. 91st Gen. Meet. Am. Soc. Microbiol.* O-48, p. 266.

Colby, G. D., and J.-S. Chen. 1992. Purification and characterization of 3-hydroxybutyryl-CoA dehydrogenase from *Clostridium beijerinckii* strain NRRL B593. *Abstr. 92nd Gen. Meet. Am. Soc. Microbiol.* K-76, p. 269.

Rifaat, M., and J.-S. Chen. 1992. A transcriptional regulator gene upstream from the *adh* gene of *Clostridium beijerinckii* NRRL B593: cloning and sequence analysis. *Abstr. 92nd Gen. Meet. Am. Soc. Microbiol.* O-66, p. 320.

Ismail, A., R.-T. Yan, and J.-S. Chen. 1993. Multiple primary alcohol dehydrogenases in *Clostridium beijerinckii* NRRL B592. *Abstr. 93rd Gen. Meet. Am. Soc. Microbiol.*, K-99, p. 277.

Colby, G. D., and J.-S. Chen. 1994. Purification and characterization of acetoacetate:butyrate coenzyme A-transferase from *Clostridium beijerinckii* NRRL B593. Abstr. 94th Gen. Meet. Am. Soc. Microbiol. K-75, p. 288.

Johnson, J. L., J. Toth, and J.-S. Chen. 1995. Taxonomy of solvent-producing clostridia from various culture collections. Abstr. 95th Gen. Meet. Am. Soc. Microbiol. H-29, p. 497.

Li, G.-S., and J.-S. Chen. 1995. Expression of the clostridial gene for a primary-secondary alcohol dehydrogenase in *Escherichia coli* and other bacteria. Abstr. 95th Gen. Meet. Am. Soc. Microbiol. K-60, p. 547.

Toth, J., and J.-S. Chen. 1997. Cloning and sequence analysis of the *ctfA* and *ctfB* genes encoding the acetoacetate:butyrate/acetate coenzyme A-transferase from *Clostridium beijerinckii* NRRL B593. Abstr. 97th Gen. Meet. Am. Soc. Microbiol. H-8, p. 286.

**D. Thesis**

Kasap, M. 1997. Hydrogenase of *Clostridium acetobutylicum* ATCC 824. M.S. thesis. Virginia Polytechnic Institute and State University, Blacksburg.

Colby, G. D. 1993. CoA-transferase and 3-hydroxybutyryl-CoA dehydrogenase: acetoacetyl-CoA-reacting enzymes from *Clostridium beijerinckii* NRRL B593. Ph.D. thesis. Virginia Polytechnic Institute and State University, Blacksburg.

Yan, R.-T. 1991. Enzymology of butanol formation in *Clostridium beijerinckii* NRRL B592. Ph.D. thesis. Virginia Polytechnic Institute and State University, Blacksburg.

Thompson, D. K. 1989. Acetoacetyl coenzyme A-reacting enzymes in solvent-producing *Clostridium beijerinckii* NRRL B593. M.S. thesis. Virginia Polytechnic Institute and State University, Blacksburg.