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Application of Nuclear Magnetic Resonance Spectroscopy to Analysis of Ethanol Fermentation Kinetics in Strains of *Zymomonas mobilis*

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Nuclear Magnetic Resonance (NMR) spectroscopy has been used for following the progress and for determining the physiological status of microbial fermentation. This noninvasive NMR methods that allow us not only to identify the presence of particular metabolites within a biological sample, but also to monitor reaction rates, enzyme activities, and membrane transport *in vivo* have been developed. Despite the low levels of the carbon-13 isotope (1.1%), natural-abundance ^{13}C-NMR studies have proven useful for monitoring the progress of fermentation processes. ^{31}P-NMR can provide noninvasive information pertaining to cellular metabolism, because the technique provides information on the energy status of the cells, by virtue of its ability to identify the various nucleotide phosphates and other energy-rich compounds therein, as well as to characterize the intracellular pH from the chemical shifts of internal phosphate and other phosphorylated metabolites. In this paper, we will briefly summarize the use of NMR as an analytical tool for ethanol fermentation technology and also discuss some examples that illustrate how NMR can be used to obtain information about the progress of fermentations in *Zymomonas mobilis*.

Advantages of the NMR as an Analytical Tool

In recent years, NMR has become a popular method for metabolic research of intact biological systems. We now have a fairly detailed understanding of the mechanisms that control the activity of some individual enzymes. Biotechnologists as well as biochemists are therefore becoming increasingly interested in studying the organization and control of integrated metabolic pathways, and in relating their observations both to the activities of the constituent enzymes of the pathways and also to the physiological state of the system under investigation.

A major advantage of the NMR is that not only can one follow the rates of substrate breakdown and product formation, but it is also possible to study the appearance and disappearance of intracellular metabolic intermediates in a noninvasive and quantitative manner (1, 2, 3). In addition, following the introduction of aqueous shift reagents, it has become possible to differentiate concentrations of cations, sugars, or metabolites between cells and extracellular medium. In classical metabolic research, metabolite levels in biological systems are commonly measured using the technique known as 'freeze clamping'. This methods involves rapid freezing

of cells or tissues followed by extraction of metabolites, and subsequent analysis for the required components.. In this process, information about the intracellular organization or compartmentalization of metabolites is lost. Furthermore, great care has to be taken to ensure that an insignificant amount of metabolism take place during the interval between freezing the cells and analysis. In addition, the time course of metabolic processes can not be followed within a single preparation because of the destructive nature of the technique. Since NMR can be performed on intact cells, we can get more accurate information about cellular metabolism as well as compartmentalization of metabolites.

Analysis of Xylose Metabolism by Recombinant *Zymomonas mobilis* ZM4 (pZB5)

Z. mobilis has attracted widespread interest for fuel ethanol production because of its higher specific rates of sugar uptake and ethanol production, higher ethanol tolerance, and higher ethanol conversion efficiencies when compared to the traditionally used yeasts (4). However, wild type strains of *Z. mobilis* can only utilize glucose, fructose and sucrose, and lack a pentose metabolism pathway necessary to ferment xylose or arabinose. There have been many effort to broaden substrate specificity of *Z. mobilis* to ferment pentose which are abundant in lignocellulosic materials. Recently metabolic engineering of a pentose metabolism pathway in *Z. mobilis* was successful (5). The genetically engineered strains can convert the pentose sugar xylose to ethanol by the combined use of Entner-Doudoroff pathway and pentose uptake and assimilation via the cloned enzymes xylose isomerase, xylulokinase, transketolase, and transaldolase. The specific rates of growth, substrate utilization and ethanol production as well as yields of biomass and ethanol production on xylose of the recombinant *Z. mobilis* ZM4 (pZB5) were much less than those on glucose or glucose/xylose mixtures (6, 7, 8). Furthermore typical fermentations with ZM4 (pZB5) growing on glucose/xylose mixtures followed two phase growth kinetics with the initial uptakes of glucose and xylose being followed by slower growth on xylose after glucose depletion. Following glucose depletion, the cell growth rate decreased gradually to zero, while the slower uptake of the residual xylose and ethanol production continued in increasingly uncoupled metabolism. The reductions in rates and yields from xylose metabolism were studied using NMR (9). The major reasons were the production of by-products identified as xylitol, acetate, lactate, acetoin and dihydroxyacetone by ^{13}C-NMR and HPLC; growth inhibition due to xylitol with xylose being converted to xylitol by an aldose reductase and then xylitol being converted to the inhibitory compound xylitol phosphate by a side reaction of the cloned xylulokinase in the recombinant strain; and the slower rates of xylose assimilation (resulting from the lower selectivity of the Glf transporter) and metabolism and thus less energized state of ZM4 (pZB5) cells during xylose fermentation. *In vivo* ^{31}P-NMR studies showed that the levels of nucleoside triphosphates (NTP) and UDP sugars of ZM (pZB5) using xylose were less than those of ZM4 (pZB5) using glucose and this energy limitation is likely therefore to restrict the growth of this recombinant strain on xylose media.

In vivo Kinetic Analysis of Lignocellulosic Toxic Compound Inhibition

The ethanol fermentation efficiency of the recombinant strains with the hemicellulose hydrolysate can be substantially hindered by the toxic substances produced during the hydrolysis of hemicelluloses. Among the identified inhibitory compounds, acetic acid has been proved to be most significant for fermentation (10). The fermentation characteristics and effects of lignocellulosic toxic compounds on a recombinant *Z. mobilis* ZM4 (pZB5) and its parental strain ZM4 have been characterised using ^{13}C- and ^{31}P-NMR *in vivo* (11). Kinetic studies of sugar uptake and ethanol production in *Z. mobilis* strains were conducted using carbon-13 natural-abundance NMR. Acetic acid was shown to be highly inhibitory to ZM4 (pZB5) on xylose medium, with xylose utilisation and ethanol production being completely inhibited at pH 5 or below in the presence of 10.9 g/L sodium acetate. However ZM4 (pZB5) or ZM4 on glucose medium could metabolise glucose to ethanol at the lower pHs, 4.5 or 4.0, in the presence of 10.9 g/L sodium acetate although decreasing pH caused a greater inhibitory effect. These results suggest that acetic acid causes more inhibitory effects on the cloned enzymes for xylose uptake and assimilation rather than on those of the Entner-Doudoroff pathway. From the ^{31}P NMR results, the addition of sodium acetate caused decreased NTP production and sugar phosphates formation, together with acidification of the cytoplasm. Intracellular de-energization and acidification appear to be the major mechanisms by which acetic acid exerts its toxic effects. The level of NTP of ZM (pZB5) using xylose was less than that of ZM4 (pZB5) or ZM4 using glucose. This is evidence of the intrinsically slower xylose uptake and assimilation and a less energized state of ZM4 (pZB5) cells during xylose fermentation.

In vivo Kinetic Analysis of Acetate Inhibition and Mechanism of Resistance

In order to develop effective ethanol fermentation processes using lignocellulose hydrolysates, it will be necessary to utilize acetic acid tolerant recombinant strains. For this purpose, an acetic acid tolerant mutant of *Z. mobilis* ZM4 has been isolated as a possible host strain, and its ethanol production kinetics have been characterised (12). Also *in vivo* ^{31}P-NMR and metabolic studies were carried out on an acetic acid tolerant mutant, *Zymomonas mobilis* ZM4/Ac^R, and compared to those of the parent strain, *Z. mobilis* ZM4, to evaluate possible mechanisms of acetic acid resistance (13). ZM4/Ac^R showed multiple resistance to other lignocellulosic toxic compounds such as syringaldehyde, furfural, hydroxymethyl furfural, vanillin and vanillic acid. The mutant strain was resistant to higher concentrations of ethanol or lower pH in the presence of sodium acetate, compared to ZM4 which showed more additive inhibition. *In vivo* ^{31}P-NMR studies revealed that the internal pH and the energy status of ZM4/Ac^R were less affected by sodium acetate compared to the parent strain. This resistance to pH change and de-energization caused by acetic acid is a possible explanation for the development of resistance by this strain.

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