GENETIC MANIPULATION OF YEASTS FOR ETHANOL PRODUCTION FROM XYLOSE

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 Yeasts are commercially valuable to a variety of industries. These microorganisms, mainly known in the baking and brewing industries, play an important role in animal nutrition (single-cell protein) and in production of alcohol fuels, chemical feedstocks, and, more recently, enzymes. Although species of *Saccharomyces* are the most widely studied yeasts, both genetically and biochemically, they do have certain drawbacks, such as the limited secretion of products, low ethanol tolerance, and restricted carbohydrate utilization. Other yeasts, which respond to unique environmental stimuli, may actually be preferred. Previously, organisms with desirable traits have been selected by screening culture collections or by mutagenesis and subsequent isolation of the desired strains. However, mutations are difficult to achieve with commercial polyplloid yeast strains. The polyplloid nature hinders mutagenesis and selection of dominant characteristics, yet it is important for maintaining the stability of the strain.

With the development of molecular biological techniques, improvement of polyplloid or imperfect yeast strains may be obtained by circumventing the problems associated with conventional genetic methods. Fusion of protoplasts within a single species creates recombinant products with properties unique to the parent. Unfortunately, fusion products are often unstable and may express undesirable parental traits. The best opportunity for successful, stable gene expression may lie with the use of recombinant DNA technology that inserts desirable genes into vectors (plasmids) and then transfers the plasmids to host organisms. Expression of the desired trait can be optimized through the selection of suitable vectors that will increase either intracellular or extracellular levels of products.

Ethanol Production

Modification of yeasts to increase ethanol production for liquid fuels and chemical feedstock industries is an open area for research. Improvement of strains could overcome low tolerance to ethanol and high substrate concentrations, characteristics of most commercial yeast strains. If the two characteristics are related, then an osmotolerant strain could possibly be altered to achieve high ethanol and substrate tolerance more easily than a strain that is not tolerant to high osmotic conditions. Characteristics such as flocculence and relative stability to fluctuations in temperature and pH may also be transferable.

Another area for strain improvement, one that could substantially reduce ethanol production costs, is that of increasing the variety of carbohydrates that a yeast will metabolize. Starch, cellulose, and xylose are abundant carbohydrates found in plant materials. Other fungi, as well as bacteria, have been identified as being capable of breaking down these complex carbohydrates. Combining the genetic material of these various organisms could result in a unique individual capable of coding for the enzymes necessary to attack a number of carbohydrates simultaneously.

In the immediate future, the primary goals for increased alcohol production are the development of organisms that have high osmotolerance; degrade cellulose, starch, and xylose; and have high tolerance to ethanol. Such organisms could make ethanol production much more competitive.

Protoplast Fusion

The development of the protoplast fusion technique allows genetic manipulation of strains that normally do not mate. In this procedure, the cells are treated with an enzyme, often zymolyase (Miles Laboratories, Elkhart, IN) or glucluase (Endo Laboratories, Garden City, NY), to remove most of the cell wall (Fig. 1). The resulting spheroplast is very fragile and must be maintained in an osmotically suitable buffer, usually 0.8-1.2 M sorbitol, pH 7. After a gentle washing with this buffer, the spheroplasts are then subjected to a calcium chloride or lithium acetate treatment. The actual fusion of the spheroplasts occurs when polyethylene glycol is added or when the cells are subjected to an electrical field (electrofusion). The fused cells are placed in a suitable medium to undergo cell wall regeneration, then placed on selective media to eliminate the parental cells.

A major problem with protoplast fusion is the selecting out of the fused cells from the parents. To aid in selecting fused cells, the two parents must have some unique, easily identifiable trait. This may take the form of an amino acid or vitamin requirement. However, since single gene traits are often difficult to obtain in a polyplloid strain, other identifiable characteristics, such as ability to grow on a unique carbohydrate, may be substituted. The choice must be such that the parentals have a low reversion rate of the marker gene, for the successful rate of fusion is often quite low (1/10,000).

In spite of the difficulties of working with industrial yeast strains, success with protoplast fusion has been achieved by using nutritional requirers (Perez et al., 1984; Legmann and Margalith, 1983; Johansson et al., 1984) and differential carbohydrates (Spencer et al., 1985, Figueroa et al., 1984; Taya et al., 1984; Groves and Oliver, 1984). The hybrids are often unstable, but once a stable isolate is selected, analysis of the DNA content may be performed. In general, the nuclear contents of a hybrid are often a unique parent type with a few genes or an extra chromosome of the other parental; therefore, there is no significant increase in the amount of DNA present in the fused product (Taya et al., 1984). Occasionally, a doubling of the nuclear contents in the fused cell occurs (Groves and Oliver, 1984).

Efforts to improve ethanol production by fusion have been successful between *S. cerevisiae* and *S. diastaticus*, the latter being a yeast which produces enzymes that degrade starch. However, most reports thus far describe the success of the fusion, leading to the conclusion that the achievement of better fermentations is apparently limited. However, one *S. cerevisiae/S. diastaticus* fusion product did produce more ethanol than either parent, presumably by utilizing both the starch and the glucose (Figueroa et al., 1985). It is unknown what actually caused the greater increase in ethanol.

Increases in ethanol production rates for fused products over parentals have been noted in other strains. A fusion of an osmotolerant strain (*S. mellis*) with *S. cerevisiae* produced a better fermentor (as measured by CO₂ evolution) than either parent (Legmann and Margalith, 1983). Brewing strains have also been used to obtain better ethanol producers (Johansson and Sjostrom, 1984; Seki et al., 1985; Panchal et al., 1982).

In many studies involving protoplast fusion, respiratory-deficient (RD) strains are used. Respiratory deficiency is characterized by a loss of mitochondrial function so that oxidative processes no longer occur and the cell must, therefore, function fermentatively. RD strains can easily be detected by their lack of growth on glycerol, a carbohydrate

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Reprinted from Food Technology 40(10) 99-103
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OCTOBER 1986—FOOD TECHNOLOGY 99
most yeasts use oxidatively. The genetic lesion for respiratory deficiency can be located within the mitochondrial genome (forming rho− or mit− mutants) or in the nuclear genome (forming pet− mutants). Unless the lesion for respiratory deficiency is clearly defined (which is often not the case), fusion of an RD cell with a respiratory-competent cell may produce a recombinant that is due to either nuclear or mitochondrial complementation, a fact that may be important in the cell’s oxidative processes. However, respiratory deficiency has proven to be a suitable marker for interspecific crosses involving S. cerevisiae and Candida utilis (Perez et al., 1984; Richard and van Broock, 1984) and S. cerevisiae and S. diastaticus (Figueroa et al., 1984; 1985; Spencer et al., 1981). If the end result is an increase in ethanol production, the source of the lesion, at least initially, may not be important.

**Transformation by Recombinant DNA Techniques**

Although a good deal of work has been done using protoplast fusion methods, it is obvious that successful production of improved yeast strains has been limited. Transformation of yeast by recombinant DNA techniques is more likely to produce stable isolates carrying a desired characteristic. However, to increase the chance of successful transformation, one needs to know the background of both the donor and recipient cells. Increasing the knowledge of the basic genetic background by knowing the biochemical pathways, cofactor requirements, genetic controls, and genes involved will allow for more successful genetic manipulation.

The basic techniques for yeast transformation are presented in Figure 2. Nuclear DNA of the donor cell is isolated, restricted with an endonuclease (such as by partial digestion with Sau3A to give a range in the sizes of pieces), and mixed with a plasmid that has been cut once by a comparable recognition-site enzyme. The mixture of DNA fragments undergoes ligation (to fuse the cut ends) and the plasmids are then inserted into an Escherichia coli host. The cells are placed onto restrictive media (in the example in Fig. 2, ampicillin and tetracycline), and the cells containing recombinant plasmids (ampicillin-resistant, tetracycline-sensitive) are isolated. After amplification of the recombinant plasmids (see Maniatis et al., 1982), yeast cells are transformed and the desired yeasts are selected.

A suitable plasmid that can transform the host cell is essential to developing a cloning system. A transforming plasmid must contain at least three elements: an origin of replication recognized by the host DNA polymerase; a promoter region recognized by the host RNA polymerase; and a functional gene that serves as a genetic marker. This latter factor can be a nutritional marker, such as the leu2 gene in the plasmid YEp13 (Broach et al., 1979), resistance to copper (Cantwell et al., 1985), or resistance to antibiotics such as G418 (Yocum et al., 1985). Even genes coding for an enzyme can be used as a plasmid marker, such as using the lactose permease gene from Kluyveromyces lactis to mark transformation in S. cerevisiae (Sreekrishna and Dickson, 1985). The origin of plasmid replication may also come from different types of DNA, depending on the host employed. Autonomous replicating sequences (ARS) from Candida (Ho et al., 1984), Kluyveromyces (Das and Hollenberg, 1982) and Schizosaccharomyces (Recht and Nurse, 1981) as well as the 2μ origin from Saccharomyces, have all been used successfully.

While the majority of research has been done on circular plasmids, linear plasmids are also useful. To increase their meiotic stability, telomeric sequences may be added (Szostak and Blackburn, 1982; Guerrini et al., 1985) or centromeric regions (CEN sequences) added (Clarke and Carbon, 1980). In this way, the plasmid acts like a minichromosome and is stable during mitosis and meiosis. Circular plasmids, although they may be present in high numbers, may be lost during cell division.

The third element for a successful plasmid, that of having a suitable promoter region, can be obtained from the host genome or from a related organism that carries an easily identifiable gene. An example of this is to use the β-galactosidase promoter and gene from E. coli. If there is successful transcription and translation of this genetic material in the host, the colonies turn blue on a suitable substrate. A desired gene could then be inserted into a β-galactosidase gene, and expression would result from the β-galactosidase promoter region.

Despite the problems involved in transforming yeasts with plasmids, limited goals have been reached. Successful transformation resulting in increased alcohol production has not been attained yet, in part because not all the processes involved are understood. Limited areas and specific problems must be attacked first. The utilization of complex carbohydrates by yeasts is one area. The three major complex carbohydrates constituting biomass are cellulose, starch, and xyllose. These are potential surplus carbon sources and are renewable resources. The literature on the research for conversion of these complex carbohydrates into useful compounds is extensive, and the reader is referred to reviews on cellulose fermentations (Montenecourt and Eveleigh, 1985; Wood, 1975) and starch fermentations (French, 1981; Stewart, 1981; Stewart et al., 1983); research on xyllose fermentation will be discussed below.

**Xylose Fermentation**

The application of protoplast fusion and recombinant DNA techniques to xylose fermentation has been rapidly expanding. Since xyllose represents a large fraction of biomass that could be used for production of chemicals, it is one area that is amenable to improvement by these techniques. Until 1980, no yeasts had been identified that could ferment xyllose to ethanol. However, since then, screening programs have identified several yeasts that produce alcohol from xyllose (Maleszka and Schneider, 1982; Suihko and Drazic, 1983; Toivola...
et al., 1984; du Preez and Prior, 1985; Nigam et al., 1985). Three of these, *Pachysolen tannophilus*, *Pichia stipitis*, and *Candida shehatae*, have been studied in detail. *P. tannophilus* has been studied the most (for review, see Slininger et al., 1986) and is perhaps the strain most amenable to genetic analyses, even though it is a homothallic strain. Limited genetic analyses have been done with *P. tannophilus* (James and Bolen, 1982) by adjusting the culture conditions to encourage spore formation and tetrad analysis. *Candida* and *Pichia* are imperfect yeasts and are, as such, more amenable to the newer methods of genetic recombination.

In the first step of the xylose-degradation pathway (Fig. 3), xylose is converted to xylulose. Bacteria generally accomplish this in one step, using xylose isomerase; however, yeasts commonly use a two-step process producing the intermediate xylitol (see Jeffries, 1988). In yeast, xylose reductase, requiring the cofactor NADPH, reduces xylose to xylitol, which is then converted to xylulose by the NAD<sup>+</sup>-requiring xylitol dehydrogenase (Smiley and Bolen, 1982). This two-step process is very demanding on the supply of NADPH and NAD<sup>+</sup> and can easily upset the cellular NADPH/NAD<sup>+</sup> pool (Bruinenberg et al., 1983). This cofactor specificity for the xylose-degrading enzymes in yeasts is a serious limitation to the use of these yeasts for the commercial production of ethanol.

Increasing ethanol yields from xylose may be accomplished by inserting the gene for xylose isomerase into these yeasts. Since, at least in *P. tannophilus* (Alexander, 1985), no significant amount of xylose isomerase activity has been detected, it appears that this would be an ideal area for strain improvement. To date, however, success has been limited. Maleszka et al. (1982) used the Clarke-Carbon *E. coli* gene bank to isolate recombinant plasmids that would complement xylose-negative mutants of *E. coli* and *Salmonella typhimurium*. A number of clones were identified that contained the region of DNA coding for xylose isomerase activity. One such plasmid contained a region including the genes for a regulatory element as well as the structural genes for xylose isomerase and D-xylulose kinase. The xylose isomerase gene has also been isolated in separate work (Úeng et al., 1985) from the same *E. coli* clone bank and inserted into a plasmid that is capable of transforming *Schizosaccharomyces pombe*. The transformed yeast cells are capable of expressing the xylose isomerase gene. Ho et al. (1983) also isolated the xylose isomerase region from the *E. coli* clone bank and have subcloned the gene into *YEp13*, a plasmid which can transform both *E. coli* and *S. cerevisiae* with high efficiency. Because of the apparent inability of the bacterial promoter to direct transcription in yeast cells, subsequent work has dealt with inserting a eukaryotic promoter in front of the bacterial xylose isomerase gene (Ho, 1985.) Transposons have also been used to develop clone banks containing the xylose isomerase gene from *E. coli* (Lawlis et al., 1984) and *Bacillus subtilis* (Wilhelm and Hollenberg, 1984). Plans include moving the xylose isomerase gene to a region under the control of a yeast promoter (Wilhelm and Hollenberg, 1984). Successful transformation of a *Saccharomyces* capable of converting xylose to ethanol may be possible in the near future.

However, the use of bacterial genes in eukaryotic systems poses several problems. One is that the promoter of a prokaryotic gene may not be recognized by a eukaryotic polymerase. Other factors involved in gene expression are inducer/repressor sequences, enzymatic cofactor requirements, processing enzymes (of RNA and proteins), and transport factors. All in all, obtaining expression of a foreign gene, and having it fit properly into a sequence of events of a pathway, is a complex process. It may be more advantageous to clone a eukaryotic isomerase gene instead of a prokaryotic one into *Saccharomyces*.

If xylose isomerase cloning attempts are unsuccessful in producing a *Saccharomyces* capable of converting xylose to ethanol, improvement of strains may be accomplished by cloning the eukaryotic genes for xylose reductase and xylitol dehydrogenase. However, instead of one gene, two or possibly more genes must be transferred. *Pachysolen* appears to have two genes coding for xylose reductase. One gene codes for an enzyme that requires NADPH as a cofactor (Ditzelmuller et al., 1984), and another gene codes for an enzyme that binds with NADH (Ditzelmuller et al., 1985) or both NADPH and NADH (Verduyn et al., 1985a; Bolen et al., 1985). *Pichia* however, appears to have a single enzyme which has a dual cofactor specificity for either NADPH or NADH (Bruinenberg et al., 1984; Verduyn et al., 1985b).

The type of xylose reductase (dual or single cofactor specificity) encoded by the yeast cells most likely determines the cells’ reactions to the presence of oxygen. *Pichia*, containing a dual cofactor-specific xylose reductase, can use xylose...
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aerobically or anaerobically. Presumably, xylose reductase supplies the \( \text{NAD}^+ \) necessary for the xylitol dehydrogenase. *Pachysolen*, which produces mainly \( \text{NADPH} \)-specific xylose reductase, ferments xylose better aerobically when the \( \text{NAD}^+ \) required by xylitol dehydrogenase is supplied by mitochondrial activity. The limitation of xylose fermentation by *Pachysolen* at the step requiring \( \text{NAD}^+ \) and xylitol dehydrogenase is suggested by the observation that even in cells with little detectable xylose reductase activity, xylitol accumulates in the medium (Alexander, 1985).

Further support for the theory that \( \text{NAD}^+ \) is limiting fermentation comes from our most recent work, (Alexander, 1986), which shows that upon addition of the hydrogen-accepting compound, acetone, the ethanol yield of *Pachysolen* increases by 50–70% over that of control cultures. This helps to explain why *Pachysolen* cultures do better under aerobic or semiaerobic conditions, where the oxygen acts as a hydrogen receptor with regeneration of \( \text{NAD}^+ \). Therefore, it might be more advantageous to clone the xylose reductase gene from *Pichia* into a host yeast, because the xylose reductase enzyme will provide the \( \text{NAD}^+ \) necessary for the second cloned gene (xylitol dehydrogenase) and will not be a drain on the cellular pool of \( \text{NAD}^+ \). However, the recombinant cells (*Pichia* genes and *S. cerevisiae* as host) might still require oxygen, for it has been reported that *S. cerevisiae* requires functioning mitochondria to metabolize xylulose to ethanol (Maleszka and Schneider, 1984). This may be due to the requirement for \( \text{ATP} \) in the phosphorylation step of xylulose to xylulose-5-\( \text{P}_4 \).

In spite of the complexity of xylose metabolism in yeast, protoplast fusion of *C. shehatae* has resulted in an increase in ploidy level and a slightly increased level of ethanol production from xylose (Johannsen et al., 1985). It may be that *C. shehatae* is not a suitable host for increasing xylose fermentations by the method of introducing high copy numbers of the xylose reductase and xylitol dehydrogenase genes because of high requirements for cellular \( \text{NAD}^+ \).

The lack of a suitable transformation system in *Pachysolen* also needs to be defined. We are presently using nutritional mutants that have low reversion rates (<1 \( \times 10^{-9} \)) and transforming them with a clone bank containing *Sau3A* fragments of *Pachysolen* nuclear DNA which have been inserted into the plasmid YEp13. These transformation conditions may then be suitable for transformation by other plasmids, perhaps by one carrying the xylose isomerase gene. *Pachysolen* may then yield ethanol at near theoretical rates.

References


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![Fig. 3 — Simplified Pathway of xylose degradation in *P. tannophilus*](image-url)


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Based on a paper presented during the IFT Food Microbiology Division/IFT Biotechnology Division program, “Application of Genetic Engineering to Fermentation,” at the 46th Annual Meeting of the Institute of Food Technologists, Dallas, Tex., June 15-18, 1986.

—Edited by Neil H. Merriman, Senior Associate Editor