Review: Ethanol production at elevated temperatures and alcohol concentrations: Part I – Yeasts in general

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There are a number of process advantages which could be exploited through the use of thermophilic microorganisms for ethanol production. Energy savings through reduced cooling costs, higher saccharification and fermentation rates, continuous ethanol removal and reduced contamination have stimulated a search for routes to thermophilic or thermotolerant yeasts. These routes have included screening existing culture collections, temperature adaptation, mutagenesis and molecular techniques and finally isolating new strains. Varying success has been achieved, however, the most thermotolerant yeasts have come from fresh isolations from environments which experience high temperatures. Thermotolerant yeasts have been investigated for the following potential applications: simultaneous saccharification and fermentation of cellulose, where the high fermentation temperature allows more rapid and efficient enzymatic cellulose hydrolysis; whey fermentation, where high salt and low fermentable substrate concentrations make conditions difficult; and fermentation of D-xylose and cellobiose, which is essential for efficient conversion of woody biomass to ethanol. Ethanol and temperature tolerance are important characteristics for commercial yeast strains. Both characteristics are interactive and generally decrease with increasing temperature and ethanol concentration. Considerable research has been directed towards investigation of fatty acid composition changes in response to these stresses and the role of heat shock proteins in tolerance mechanisms. If thermotolerant yeasts are to be used in commercial processes, bioreactor configuration will play an important part in the design of production processes. Batch and fed-batch systems have been shown to be useful in some circumstances as have continuous flow systems, however, some of the newly isolated thermotolerant yeasts such as Kluyveromyces marxianus do not show the high growth rate under anaerobic conditions that is characteristic of Saccharomyces cerevisiae. Various immobilization techniques appear to offer a means of presenting and maintaining high biomass in anaerobic continuous flow reactors.

Key words: Alcohol, ethanol, thermophilic, thermotolerant, yeast.

In the past few years there has been an upsurge of interest in thermophilic microorganisms mainly due to the increase in most reaction rates, product yield and final product resistance to degeneration at higher temperatures. The potential applications of thermophilic microorganisms, or their derivatives, in industrial applications have been widely appreciated and therefore an exponential increase in research related to this topic is still in progress. Industrial ethanol production is dependent on microbial activity, particularly that of yeasts. In this process high temperature advantages include energy savings achieved through a reduction in cooling costs or in avoiding frequent cessations in production due to overheating problems usually encountered in areas/seasons of high ambient temperature where cooling is unavailable. Three other advantages of processing at higher temperatures include the possible use of continuous ethanol stripping as a method of harvesting ethanol, significant restriction of contamination chances and reduction in the volume of distillery cooling-wastewater effluent.

Extreme thermophiles are restricted to prokaryotes, however, thermophilic filamentous fungi, capable of growth at up to 60 °C have been known for a long time (Cooney & Emerson 1964; Tansey & Brock 1972 & 1978). Cooney and Emerson (1964) have restricted the term “thermophile”, as applied to fungi, to organisms that have a maximum growth temperature of 50 °C or above and a minimum growth temperature at or above 20 °C. With regard to yeasts, Stokes (1971) stated that the maximum temperature for yeast growth is between

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46–48 °C and accordingly Watson (1987) concluding that the term ‘thermophile’, as specifically applied to yeasts, must be taken in the context of an upper temperature limit of about 48 °C.

The vast literature on yeasts mainly consists of studies on those growing in the range of 30–35 °C. The biochemical and physiological deviations in these yeasts have been studied only occasionally and the use of the terms ‘thermophilic’ or ‘thermotolerant’, in describing yeasts, has not been clear-cut (Arthur & Watson 1976; Watson et al. 1978; Arthur et al. 1978). Until recently thermophilic yeasts were largely unknown and those generally capable of growth at above 40 °C have been mostly referred to as thermotolerant (Krouweel & Braber 1979; Hacking et al. 1984; Hughes et al. 1984; Anderson et al. 1986a & b; Szczodrak & Targonski 1988; D’Amore et al. 1988, 1989; Lee et al. 1993) although mutants of Saccharomyces cerevisiae capable of growth at a maximum of 33–35 °C have been reported as thermotolerant yeasts (Kida et al. 1992; Morimura et al. 1997). Other thermotolerant yeasts belonging to the genus Kluyveromycetes were reported to produce alcohol at above 40 °C and to have a maximum growth temperature of 49 °C (Hughes et al. 1984) or even up to 52 °C (Banat et al. 1992) which, according to the criteria of Watson (1987) (with maximum growth temperature ≥48 °C), would categorize them as thermophilic yeasts.

Elevated temperatures usually encountered in several geographical regions may adversely affect alcohol and other industrial fermentation processes (Krouweel & Barber 1979; Sa-Correia & van Uden 1983a). In addition to the influence of external temperature increase, fermentation broths also get heated up due to exothermic metabolic reactions. In many warm countries, including India, summer temperatures frequently reach >40 °C (45–50 °C) and in the typical ethanol fermentation processes carried out at ambient temperatures with no cooling system an increase of up to 11 °C can be experienced due to exothermic metabolic reactions (Burrows 1970). Consequently, the fermentation vessel’s temperature rises to above 40 °C, leading to reduced ethanol productivities. In some of these alcohol production industries, this overheating problem is partially overcome by spraying cold water on the fermentation vessel walls which adds to production costs. The availability of thermotolerant yeast strains thus offers an advantage of enabling operation at elevated temperatures in industrial fermentations such as single cell protein production, baker’s yeast production, pharmaceutical yeasts and ethanol production; hence the need and search for more suitable heat tolerant strains continues. Thermophilic bacteria are abundantly available and have found application in the degradation of several organic compounds such as starch or cellulose, production of thermally stable enzymes and bioremediation processes. In contrast, thermotolerant yeast strains are few and their use in the production of fodder yeast, ethanol and baker’s yeast has yet to find widespread industrial applications.

**Enrichment, Isolation, Selection and Development of Thermotolerant Yeasts**

Thermotolerant yeasts capable of growth and ethanol production at temperatures above 40 °C have been actively sought, mainly through one of the following techniques:

1. **Screening of existing yeast strains.** Numerous strains are available at various culture collections or at the various research laboratories interested in yeasts. Several investigators tested as many yeast cultures as they could obtain, using elevated temperature(s) (>40 °C) and/or ethanol tolerance as selection pressures for the most suitable strain available (Hawke et al. 1983; Hughes et al. 1984; Hacking et al. 1984; Anderson et al. 1986b; Szczodrak & Targonski 1988; Spindler et al. 1988a&b; D’Amore et al. 1989; Ballesteros et al. 1991). Success using this route was usually limited, perhaps because most of the strains tested were originally obtained and thereafter maintained at mesophilic temperatures prior to testing.

2. **Temperature adaptation.** Modification of existing or newly isolated strains to growth and or ethanol tolerance at elevated temperatures. This technique involves incubation at gradually increasing temperatures and/or ethanol concentrations as an adaptive pressure and usually under continuous culture conditions (Arthur & Watson 1976; Brown et al. 1984; Suutari et al. 1990) or repeated-batch fermentation conditions (Morimura et al. 1997). Success using this technique has also been limited, particularly with regards to temperatures, due to the narrow range of adaptation possible for most microorganisms (around 2–4 °C).

3. **Protoplast fusion.** This involves the use of two different yeast strains with the desired capabilities including high temperature tolerance, high ethanol productivity and other relevant characteristics including flocculation or utilization of specific carbon sources have been also employed. Protoplasts from the desired strains were prepared and their fusion was carried out followed by a selection program for cells with the combined required capabilities (Seki et al. 1983; Groves & Oliver 1984; Kida et al. 1992; Sohn et al. 1994). Recently, Sakanaka et al. (1996) reported success in fusing yeast cells of a thermotolerant strain of K. marxianus and a high ethanol producing strain of S. cerevisiae. The fused cells obtained were capable of growth at high temperatures (up