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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 as 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 (Krouwel & 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 *Kluyveromyces* 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 (Krouwel & 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

to 43 °C) however, their fermentative capability at high temperature was severely impaired and the fusants' thermostability was lower than that for both parent cells.

4. Mutagenesis techniques. Treatment with chemical mutagens or exposure to UV radiation to kill >99% of the cells has been used to induce favourable mutations; this is followed by a selection programme at elevated temperatures (Zeng *et al*. 1992; Wati *et al*. 1996). Moderate improvement in relation to increasing thermal inactivation temperature in addition to enhancing the duration of tolerance upon exposure to elevated temperatures up to 44 °C was achieved in *S. cerevisiae* mutants (Wati *et al*. 1996).

5. Molecular biology techniques. More elaborate construction programs using different molecular biology techniques to construct or modify desired strains have also been reported (Takagi *et al*. 1983; Seki *et al*. 1983). Limited successes were reported as one needs to have the desired characteristics in a suitable strain and knowledge of the genes encoding for these characteristics to be able to harness or transfer them into other strains. Most of previously mentioned investigations involved using traditional strains of *S. cerevisiae*. The obvious shortcomings is that such strains have a maximum operational temperature of around 37 °C.

6. Isolation from nature. The most successful technique reported to date involves isolating and selecting strains from nature that exhibit the capability of growth at higher temperatures while producing ethanol. There are several thermophilic filamentous fungi in existence and since yeast are considered to be an evolutionary reduction of filamentous fungi (Moore 1987) there is no *a priori* reason why thermophilic yeasts should not exist. Several investigators followed this approach including Rose (1976), Chotickai (1983), Ryu *et al*. (1988), Anderson *et al*. (1988b), lbanez and Goyre (1992), Hsie (1993) and Lee *et al*. (1993). All of the above investigators enriched and isolated at temperatures between 40 °C and 42 °C and had success in obtaining some strains growing at 42 °C. Finally, Banat *et al*. (1992) enriching at 45–50 °C succeeded in isolating several strains of thermophilic yeasts (IMB1, IMB2, IMB3, IMB4 & IMB5) all capable of growth at temperatures up to 52 °C (Banat *et al*. 1992) and all effectively able to produce ethanol from a wide range of substrates at 45 °C (Banat & Marchant 1995). Two of these strains have been identified as *Kluyveromyces marxianus* var. *marxianus*. The characteristics and capabilities of these and other similar isolates have been extensively studied and are presently exploited for a number of different potential industrial situations as discussed below.

Potential Applications of Thermotolerant Ethanol Fermenting Yeasts

Simultaneous Saccharification and Fermentation of Cellulose

Cellulose is one of the most abundant low cost carbon source substrates in the world, however, hydrolysis of this polymer to yield a fermentable feedstock has always been problematic. Chemical hydrolysis is possible but expensive while enzymatic hydrolysis can only be rapidly achieved at temperatures above 45 °C. Enzymatic hydrolysis and subsequent fermentation therefore was best carried out in a two stage process. The cellulase enzymes are most active at up to 50 °C (a temperature at which most yeasts fail to grow let alone produce alcohol). Simultaneous saccharification and fermentation (SSF) processes in which enzymatic hydrolysis is coupled to fermentation, in one reaction vessel, by a thermotolerant yeast has therefore been actively pursued.

Several yeasts and bacteria have been tested for suitability for SSF of cellulose. Blotkamp *et al*. (1978) looked at some *Saccharomyces* and *Candida* species for SSF at 40 °C and although this was followed by copious reports on improvements to the SSF process, it became obvious that higher temperatures are essential to increase the rate of the cellulose saccharification step. Both Szozodrak & Targonski (1988) and Spindler *et al*. (1988b) screened for thermotolerant yeasts suitable for SSF. Among 58 yeasts strains tested, Szozodrak & Targonski (1988) selected a strain *Fabospora fragilis* CCY51-1-1 as the most suitable for ethanol production (56.0 g ethanol l^{-1} from 140 g glucose l^{-1}) at 43 °C. This strain's performance however, dramatically decreased (≈40%) at 46 °C. Spindler *et al.* (1988a & b) screened ten promising yeasts strains for thermotolerant simultaneous saccharification and fermentation of cellulose, concluding that the use of a mixed culture of such yeasts is advantageous to achieve higher product yields at temperatures up to 41 °C.

In a more recent selection program, Ballesteros *et al*. (1991) achieved best conversion of 10% Solka-floc cellulose substrate to ethanol (with yields of ≈3.8% w/v) using both *K. marxianus* L.G. and *K. fragilis* L.G., separately, at temperatures up to 42 °C; higher temperatures dramatically reduced ethanol yields. A strain development program for *K. marxianus* was subsequently carried out (Ballesteros *et al*. 1993) using a mutagenic treatment program. This approach resulted in many mutants capable of conversion yields of ethanol of ≈3.3% (w/v) at 45 °C using the same substrate concentration. Media supplementation with lipids and nutrients in an attempt to enhance ethanol production in a simultaneous saccharification and fermentation process using a thermotolerant strain of *K. marxianus* had the opposite effect (Ballesteros *et al*. 1994). Nutrient additions did not affect the process while the addition of unsaturated fatty acids

and sterols decreased cellulose hydrolysis yields by the cellulase enzymes. It was concluded that the medium used had sufficient nutrient content including nitrogen and vitamins and that lipid supplementation seems to lead to denaturation of the cellulase complex rather than acting as a surfactant that interferes with the enzyme's surface-active properties (Ballesteros *et al*. 1994).

Whey Fermentation to Ethanol

In most European countries dairy industries produce large quantities of cheese. The whey by-product (large quantities, running into million of cubic metres per year) has low value as animal feed and poses a disposal problem. In recent years these industries have developed methods for the removal of milk protein from the whey which leaves an effluent (whey permeate) of equal volume but with even less market value as an animal feed additive. Lactose (≈40 g l^{-1}) is the principal carbon source in whey permeate. However, the concentration of salt in whey is quite high (≈6% w/v) which makes lactose concentration prior to fermentation impractical.

Extensive attention has been paid during the last 15 years to the evaluation of whey permeate as a potential alternative fermentable substrate for alcohol production, mainly at mesophilic temperatures. EI-Samragy & Zall (1988) tested nine yeast strains and reported only two capable of utilizing the available lactose in 6% salt whey permeates. Five other strains of *Kluyveromyces* adapted to high lactose concentration (225 g l^{-1}) were grown in concentrated whey permeate solutions and were found capable of producing alcohol, although only at low concentrations due to salt inhibition (Mahmoud & Kosikowski 1982). Among the lactose-fermenting yeasts, only strains of the genera *Kluyveromyces* and *Candida* appear to have high alcohol production capability (Duvnjak *et al*. 1987; Ruggeri *et al*. 1987; Taylor & Mawson 1989; Zayed & Hunter 1991).

Other recent experiments involving *Kluyveromyces* and *Candida* species have been reported by Grubb & Mawson (1993), Jones *et al*. (1993) and Ferrari *et al*. (1994). They reported varying degrees of ethanol production using different production techniques to avoid either substrate or product inhibition. These techniques included: membrane recycle bioreactors (Vienne & Stockar 1985; Tin & Mawson 1993), immobilization with β-galactosidase (Hahn-Hägerdal 1985), calcium alginate-immobilized cells (Marwaha *et al*. 1988), batch cultures at elevated solute concentrations (Grubb & Mawson 1993), fed batch production (Jones *et al*. 1993; Ferrari *et al*. 1994) and extractive fed batch cultures (Jones *et al*. 1993).

To our knowledge the only thermophilic yeasts with the capability to grow well on lactose are the *K. marxianus* IMB cultures (Table 1). They all produced appreciable amounts of alcohol on whey permeate medium; IMB2 in particular produced 1.7% (w/v) ethanol from 4.0% (w/v) lactose which constitutes ≈79% of the theoretical maximum (Banat & Marchant 1995). Further improvements in ethanol production using this substrate may be achievable by applying conventional mutagenic techniques to that microorganism.

Fermentation of D-xylose and Cellobiose to Ethanol

D-xylose is the most common pentose produced by acid or enzymatic hydrolysis of hemicellulose. The pentose content of some cellulosic biomass types reaches up to 35% of the total carbohydrate content (Rosenberg 1980). Most industrially-used *Saccharomyces* species cannot ferment xylose to ethanol. This

Table 1. Growth characteristics of five thermotolerant yeasts strains of *K. Marxianus* grown for 48 h on medium containing lactose at 45 °C (adapted from Banat & Marchant 1995).

Strain	DT (h)	Cell mass (g l^{-1})	Cell mass yield (g . g^{-1})	Ethanol (g l^{-1}) (final value)
Lactose (1.0% w/v)				
IMB 1	1.60	1.9	0.19	4.3
IMB 2	1.35	2.0	0.20	3.7
IMB 3	1.60	2.1	0.21	3.8
IMB 4	1.40	2.1	0.21	4.0
IMB 5	1.50	2.1	0.21	3.7
Whey permeate (≈4% w/v lactose)				
IMB 1	1.80	1.8	0.045	16.0
IMB 2	1.60	1.9	0.048	17.0
IMB 3	1.80	2.0	0.050	16.8
IMB 4	1.70	1.9	0.048	15.2
IMB 5	2.00	1.9	0.049	13.6

DT = doubling time (calculated during exponential growth phase); Cell Mass = Dry weight (final value); Cell Mass Yields = g cell mass/g total sugar consumed.

Table 2. Ethanol production from D-xylose by various yeasts.

Microorganisms	Temp. (°C)	Xylose (g l^{-1})	EOH (g l^{-1})	Ethanol Yield (g g^{-1})	Reference
Brettanomyces					
B. clausenii	MR	20.0*	21.4	0.43**	Parekh *et al.* (1988)
Candida					
C. tropicalis ATCC 1369	MR	100.0	5.4	0.11	Jeffries (1981)
C. shehatae NCL-3501	MR	10–80	NA	0.4–0.43	Abbi *et al.* (1996)
C. shehatae FPL-702	MR	NA	35.0	NA	Sreenath & Jefferies (1996)
Candida sp. XF-217	MR	100.0	30.0	0.42	Gong *et al.* (1981)
Clavispora					
Clavispora sp. 83-877-1	25.0	60.0	10.9	0.29	Nigam *et al.* (1985)
Kluyveromyces					
K. cellobiovorus KY 5199	28.0	100.0	30.0	0.31	Morikawa *et al.* (1985)
K. marxianus SUB-80-S	30.0	20.0	5.6	0.28	Margaritis & Bajpai (1982)
K. marxianus 83-SM16-10	25.0	20.0	5.2	0.26	Margaritis & Bajpai (1982)
K. marxianus IMB1,2,3,4&5	45.0	10.0	0.8–1.2	0.08–0.12	Banat & Marchant (1995)
Pachysolen					
P. tannophilus NRRL Y-2460	MR	20.0	5.3	0.27	Schneider *et al.* (1981)
P. tannophilus NRRL Y-2460	MR	115.0	23.0	0.30	Slininger *et al.* (1982)
P. tannophilus IFGB 0101	MR	30.0	3.8	0.13	Debus *et al.* (1983)
Pichia					
P. stipitis NRRL Y-5773	MR	30.0	12.0	0.36	Dellweg *et al.* (1984)
Schizosaccharomyces					
S. pombe	30.0	100.0	37.0	NA	Err-Cheng *et al.* (1986)

MR = mesophilic range; Ethanol yields = g ethanol produced/g substrate consumed; NA = none available.
* 20 g xylose in 70 g total wood sugar; ** ethanol yield is calculated in reference to total sugar.

prompted a search for yeasts capable of this conversion and several reports appeared in the literature in the early eighties describing a few yeasts capable of xylose conversion to ethanol (Table 2). Margaritis & Bajpai (1982) were the first to report several strains of *K. marxianus* capable of direct fermentation of D-xylose to ethanol. Morikawa and coworkers (1985) screened 213 strains of yeasts for xylose and cellobiose fermentation and reported a strain *K. cellobiovorus* to be the most effective. Cellobiose on the other hand, is a product of enzymatic hydrolysis of cellulose by cellulase. Cellobiose accumulation inhibits the action of cellulase and decreases the rate of cellulose hydrolysis if not removed by the action of the β-glucosidase enzyme.

It is commercially attractive to search for yeasts capable of fermenting this disaccharide to ethanol. Such yeasts are few. Morikawa *et al*. (1985) reported ethanol production from cellobiose by the same strain of *K. cellobiovorus* which was capable of D-xylose fermentation. All of the previously mentioned xylose and cellobiose fermentations however, were carried out at mesophilic temperatures of 25–30 °C. All five thermophilic yeasts strains isolated at our laboratory showed an ability to ferment xylose (albeit at relatively low rates) (Table 2) and cellobiose (Table 3) at 45 °C.

Table 3. Growth characteristics of five thermotolerant yeast strains of *K. Marxianus* grown for 48 h on medium containing 10 g cellobiose l^{-1} at 45 °C, (adapted from Banat & Marchant 1995).

Strain	DT (h)	Cell mass (g l^{-1})	Ethanol (g l^{-1})	% of Theoretical yield
IMB 1	3.60	2.0	4.4	80.5
IMB 2	3.40	1.2	5.2	95.2
IMB 3	3.70	1.4	3.1	56.8
IMB 4	3.90	1.2	3.5	64.1
IMB 5	2.30	2.2	3.1	56.8

DT = doubling time (calculated during exponential growth phase); Cell Mass = cell biomass (final values); and % theoretical yield = % of maximum possible ethanol theoretical yield.

Ethanol Tolerance in Yeasts

The process of ethanol production, excretion, regulation and most importantly tolerance is not entirely understood and considerable attention has been directed towards better understanding of this process in yeasts (Oliver 1984; D'Amore *et al*. 1990) and bacteria (Ingram 1990).

Plasma membrane phospholipids have been shown to play an important role in the tolerance mechanism

(Ingram & Buttke 1984). Ethanol appears to alter the degree of polarity of the cell membrane and the cytoplasm, causing disruption of growth at higher concentration (Lynd *et al*. 1991). This disruption may be in the form of increased membrane fluidity at higher ethanol concentrations (Lloyd *et al*. 1993). Higher concentrations of membrane unsaturated fatty acids, vitamins and proteins appear to enhance ethanol tolerance (Ingram 1984; D'Amore & Stewart 1987). Other physiological factors such as medium composition and mode of substrate feeding (Yamamura *et al*. 1988; Dombek & Ingram 1986a&b), intracellular ethanol accumulation (D'Amore *et al*. 1988), temperature and osmotic pressure can all contribute to ethanol tolerance (Jones *et al*. 1981; Ohta & Hayashida 1983; Vienne & Stockar 1985; D'Amore and Stewart 1987). Changes in the cell wall components in response to higher ethanol concentrations were innovatively utilized by Umesh-Kumar an co-workers (1990) to obtain ethanol-tolerant strains. They identified some thermostable antigenic components in *S. cerevisiae* which are produced in larger quantities by cells growing at higher ethanol concentrations. Antibodies were then produced against these antigenic components and used to specifically isolate *S. cerevisiae* cells with ability to produce and tolerate high ethanol concentrations (Umesh-Kumar *et al*. 1990).

Bajpai & Margaritis (1982) investigated ethanol inhibition kinetics in a strain of *K. marxianus* and concluded that high initial ethanol concentration inhibited the maximum specific growth (μ_{max}) rate but had no effect on final ethanol concentration and cell yields or sugar utilization. They also reported an ethanol tolerance level in this strain similar to those reported for *S. cerevisiae*. On the other hand, both Rosa & Sa-Correia (1992) and Fernanda & Sa-Correia (1992) found lower ethanol tolerance in *K. marxianus* compared to *S. cerevisiae* and correlated this with the activity of the plasma membrane ATPase. Lower ethanol tolerance in another close strain of *K. fragilis* was however reported to be due to the inhibition of the carbon source transport system (lactose in this case). This inhibition increased exponentially with increasing ethanol concentrations (Sa-Correia & Van Uden 1983b). Several yeast strains were tested for ethanol tolerance in continuous cultures and found to suffer total inhibition at concentrations ranging between 6.3–9.4% (w/v) (Lynd *et al*. 1991), and to have a threshold level of ≈4.0% (w/v) (Piper *et al*. 1994). Ethanol production rate patterns for the five thermotolerant *K. marxianus* IMB strains (Figure 1) had an uninhibited rate for three of the strains up to ≈8.0% (w/v) ethanol concentration which seems to be both their threshold and maximum tolerance level. Reduced threshold and tolerance levels of ≈5.5% (w/v) were noticed for the other two strains (Figure 1) (Banat & Marchant 1995).

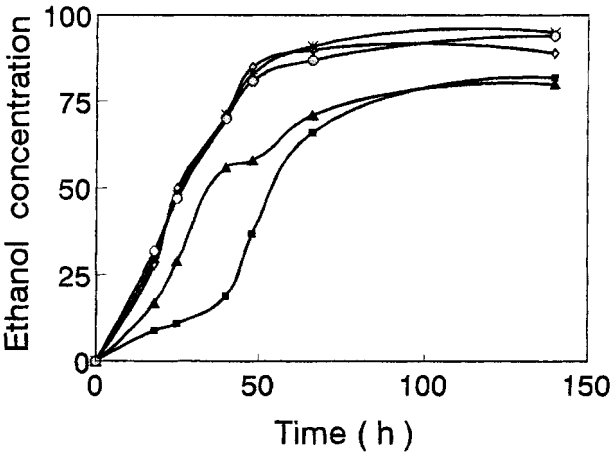

Figure 1. Ethanol production rate and maximum ethanol tolerance in five yeast strains of *K. Maxinas* 1MB1 (○), IMB2 (▲), IMB3 (∗), IMB4 (■) and IMB5(◇) grown at 40 °C in batch flasks on mineral medium containing 250 g glucose l^{-1} supplied gradually at 150 + 50 + 50 g l^{-1} batches added at 0.0 h, 18 h and 40 h, respectively (Banat & Marchant, 1995).

Trehalose was found to act as both a membrane stabilizer and a protectant for yeast cells under stressful conditions, and its intracellular concentration has been suggested to play an important role in the ability to tolerate higher ethanol concentrations (Kida *et al*. 1993; Majara *et al*. 1996).

In recent review Piper (1995) concluded that heat and ethanol stresses exhibit extensive similarity and functional overlap and are the result of stress protein synthesis, membrane lipid composition, plasma membrane H^+-ATPase changes and reduction in membrane-protective trehalose induction (Piper *et al*. 1994; Piper 1995). Also recently Xu *et al*. (1996) reported a three method approach to achieving high ethanol concentrations (up to 15.0% w/w) using a strain of *S. cerevisiae*. This approach combined: (1) extra nutrient supplementation (mainly as yeast extract) (2) immobilization on alginate beads and (3) using a high (30% w/v) glucose medium in a step-fed batch process.

Temperature Tolerance in Yeasts

High temperature is thought to cause increased fluidity in membranes generally and yeasts respond to this physical change by changing their fatty acid composition (Ohta *et al*. 1988). Increasing temperature leads to higher saturated esterified fatty acids such as palmitic and palmitoleic acids in yeast cell membrane at the expense of unsaturated acyl chains such as oleic, linoleic and linolenic acid (Van Uden 1984a; Suutari *et al*. 1990). This is usually associated with a decrease in the amount of membrane phospholipids to maintain optimal membrane fluidity for cellular activities which are possibly

part of an adaptive response (Rose 1993; Lloyd *et al*. 1993). Similar membrane changes have also been shown to occur in the ethanol-producing bacterium *Zymononas mobilis* (Benschoter & Ingram 1986).

In addition to membrane changes, increasing the growth temperature usually leads to the synthesis of heat shock proteins (Hsps) which are suggested to play an important role in conferring thermal and ethanol cross-tolerance in various microorganisms (Michel & Starka 1986). The trigger for their induction is probably the cytoplasmic accumulation of aberrant or partially-denatured protein (Piper *et al*. 1994).

Suutari *et al*. (1990) studied adaptation to temperature in several yeasts. Temperature-induced changes included either an increase in the mean fatty acid chain length or an increase in the degree of fatty acids unsaturation, or both. The use of oils such as linseed oil or mustard oil (at 0.5% w/v) or fatty acids from those oils were shown to lessen the effects of both ethanol and temperature damage on yeast cells (Saigal & Viswanathan 1983 & 1984). Trehalose accumulation has also been associated with heat stress, both in the ability to accumulate it and in its cellular concentrations contributing to the heat resistance of yeasts (Attfield *et al*. 1994; Ling *et al*. 1995; Majara *et al*. 1996; Arguelles 1997).

The effects of high temperature are often exacerbated by ethanol concentrations above ≈3% (w/v), both optimum and maximum temperatures of growth becoming appreciably depressed (Sa-Correia & Van Uden 1983a; Van Uden 1984b). Both heat and ethanol cause membrane disordering and protein denaturation (Piper 1993 & 1995), in addition to inhibiting glycolysis and enhancing mutations (Van Uden 1984a & b). Both stresses also increase the permeability of the plasma membrane, resulting in an increased passive proton influx that acts to dissipate the electrochemical potential gradient that the cell maintains across this membrane, which in turn adversely affects nutrient uptake, maintenance of the potassium balance and the regulation of intracellular pH (Serrano 1991). Heat shock and high ethanol-induced protective responses in yeast show a high degree of similarity (Rosa & Sa-Correia 1992). Both dramatically stimulate the activity of plasma-membrane ATPase, the enzyme responsible for maintaining the proton gradient across the plasma membrane (Serrano 1991). These increases in ATPase activity cause an enhanced proton efflux that counteracts the dissipation of protonmotive force resulting from the stress-induced increase in membrane permeability. It is therefore not surprising that mutations that alter plasma-membrane ATPase activity also influence cellular tolerance of both ethanol and heat (Piper *et al*. 1994).

Several scientists have investigated the effect of temperature on the specific rates of thermal death and growth and also on growth yields with respect to carbon source intake (Sa-Correia & Van Uden 1983a; Van Uden 1984a; Fatichenti & Berardi 1987). According to Van Uden (1984a), when the logarithms of the specific growth rates (μ_g) and death rates (μ_d) are plotted in combination against reciprocal temperatures (Arrhenius plot) to obtain the temperature profile of the yeast strain in question, yeasts manifest two types of temperature profiles: a dissociative and an associative profile. In yeasts exhibiting a dissociative temperature profile, thermal death has no significant effect on exponential growth; the extrapolation of the thermal death plot does not meet the growth plot at a biologically significant rate. In contrast, in those exhibiting associative temperature profiles, thermal death is concomitant with exponential growth above the optimum temperature, and the plot of the thermal death rate meets that of the growth rate.

It has been concluded that yeasts with dissociative patterns are thermotolerant since growth yield coefficients do not vary significantly with temperature, while for yeasts with associative profiles, growth yield coefficients decline above the optimum temperature (Van Uden 1984a). Several yeasts belonging to genera *Saccharomyces*, *Hansenula*, *Candida*, *Cryptococcus* and *Kluyveromyces* have been reported exhibiting either of these temperature profiles (Van Uden 1984a; Fatichenti & Berardi 1987; Sampaio & Spencer-Martins 1989). Among members of the genus *Kluyveromyces*, one of the two main species capable of growth at temperatures above 40 °C, *K. fragilis SS-437* was found to be an associatively-profiled thermotolerant yeast (Fatichenti & Berardi 1987), while the other, *K. marxianus* var. *marxianus* was found to be a dissociatively-profiled yeast (Sampaio & Spencer-Martins 1989).

Production Techniques Suitable for Thermotolerant Yeasts

The two main approaches for culturing microorganisms in liquid medium are the batch and continuous culture conditions. A host of other production systems have also been reported and selected results with the thermotolerant yeast *K. marxianus* IMB3 obtained at the authors' laboratory are presented in Table 4.

Batch and Fed-batch Culture Techniques

Several thermotolerant yeasts belonging mainly to the genera *Saccharomyces* and *Kluyveromyces* have been grown under batch conditions and have successfully produced appreciable concentrations of ethanol varying between 4–8% (w/v) (Hughes *et al*. 1984; Hacking *et al*. 1984; Anderson *et al*. 1986b; Szczodrak & Targonskim 1988; D'Amore *et al*. 1989; Ballesteros *et al*. 1991; Banat

Table 4. Performance of strain *K. Marxianus* IMB3 under different continuous fermentation conditions (Adapted from Banat *et al.* 1996).

Dilution rate (h^{-1})	Temp. (°C)	Cell Mass (g l^{-1})	Ethanol (g l^{-1})	Glucose (g l^{-1})		Ethanol Yield (g · g^{-1})
				in feed	in effluent	
Anaerobic chemostat condition						
0.05	45	0.92	14	75.0	48.0	0.51
0.10	45	0.85	11	75.0	44.0	0.35
0.15	45	0.83	6	75.0	56.0	0.32
Two fermenters in series (*aerobic-anaerobic*)						
0.20	40	10.0	34	150.0	49.0	0.34
0.20	45	8.99	30	150.0	30.0	0.25
Two stage anaerobic fermenter with cell recycle						
0.20	40	3.08	61	150.0	31.5	0.51
0.20	45	1.13	41	150.0	70.0	0.51

Ethanol yields = g ethanol produced/g substrate glucose consumed.

et al. 1992; Fleming *et al*. 1993; Barron *et al*. 1994; Brady *et al*. 1994; Banat & Marchant 1995; Banat *et al*. 1996).

Cultivation under batch conditions, however, has some limitation for several thermotolerant yeasts. Higher ethanol yields usually require high initial sugar concentration and this creates a high osmotic pressure, which when combined with elevated temperature of incubation, can inhibit growth (Grubb & Mawson 1993). The accumulation of ethanol in the fermentation vessel can also inhibit growth and temperature tolerance (D'Amore *et al*. 1990; Lynd *et al*. 1991).

To overcome these problems the fed-batch culture technique often has been employed. In this system part of the problem of the high initial sugar concentration can be overcome through gradual substrate addition after a fixed period from the culture's initiation time. This proved particularly useful when using high concentrations of the sugar substrate (Jones *et al*. 1993; Ferrari *et al*. 1994; Xu *et al*. 1996). This mode of growth however does not seem to reduce the inhibitory effects of ethanol accumulation on the culture.

Continuous Culture Technique

Under continuous culture conditions for ethanol production, a steady state is maintained in the reactor vessel for a theoretically unlimited period of time (Klapatch *et al*. 1994). Accordingly, the number of cells in the reactor should remain constant, since as many cells are removed from the vessel as are generated assuming ideal behaviour (no wall growth or flocculation). In this process the substrate (sugar) is constantly supplied to the reactor and the product (ethanol and cells) is continuously removed at the same rate. This should theoretically eliminate adverse effects due to both high sugar concentration and ethanol accumulation (Hack *et al*. 1994; Banat *et al*. 1996).

Several attempts have been carried out at the authors' laboratories to maintain thermotolerant yeasts using this technique. The results of some of this work are presented in Table 4. Complete substrate (glucose) utilization was not achieved under continuous culture conditions and in multistage fermentors even when cells were recycled. This is anticipated to be a problem for continuous culture productions due to the fact that growth and ethanol production in yeasts cell are best at opposing conditions. Growth is stimulated in the presence of oxygen which leads to biomass generation under mainly aerobic conditions while anaerobic conditions are required for fermenting existing carbohydrate sugars to ethanol (Banat *et al*. 1996). During ethanol production therefore the lower surplus energy means cell biomass is not produced and consequently cells cannot be generated to replace the harvested cells. This will eventually lead to cell washout and culture collapse.

The disadvantages encountered with both culture techniques and the continuous search for a more rewarding culture condition in terms of ethanol yield or productivity have compelled researchers to explore other production systems.

Immobilized Cell Systems

Widespread use of inorganic compounds as immobilization materials to support ethanol-producing yeast cells has taken place recently. These inorganic supports have been found to improve the ethanol productivity of yeast and bacteria and are becoming widely used because they are simple and relatively cheap. Kanellaki *et al*. (1989) found that Y-alumina, a well-known carrier for biocatalysts, would promote ethanol production in *S. cerevisiae*. Kissiris, a volcanic material found in Greece was also reported to promote ethanol production of immobilized *S. cervisiae* (Kana *et al*. 1989) and *K. marxianus* (Nigam

et al. 1997). Immobilization on porous ceramics (Sueki *et al*. 1991; Demuyakor & Ohta 1992) and calcium alginate (Nolan *et al*. 1994) have also been reported for successful continuous and fed batch ethanol production respectively.

Dale *et al*. (1994) discussed osmotic inhibition of free and immobilized *K. marxianus* and reported that immobilized cells were more tolerant to high osmolalities when osmotic strength was increased gradually, indicating significant adaptation to exerted pressure. They also concluded that the immobilized cells' productivity was less inhibited by high osmolality than free cell productivity. Other advantages include increased yeast stability and simpler bioreactor design. Several other reports highlighted the advantages of immobilization of the thermotolerant strain *K. marxianus* IMB3 (Nolan *et al*. 1994; Barron *et al*. 1996; Brady *et al*. 1997). Among the advantages reported for an immobilized *K. marxianus* used for ethanol production from artichoke extract were: wider range of pH and temperature tolerance and effective ethanol production in comparison with the free cells systems (Bajpai & Margaritis 1987).

Specialized Production Systems

Several other production systems evolved to increase yeast tolerance and productivity at higher temperatures and ethanol concentrations. These systems included cell recycle through centrifugation, flocculation or precipitation (Kida *et al*. 1990; Hoshino *et al*. 1990; Banat *et al*. 1996) and membrane recycle bioreactor technique (Tin & Mawson 1993). In these techniques, part or complete cell recycle has been carried out to allow operation at continuous culture conditions in which cell biomass production at high ethanol production becomes limited. Other approaches included the use of a two stage fermentation; the first maintained under aerobic and the second under anaerobic conditions. This was carried out to maximize biomass production in the first and obtain high ethanol productivities in the second (Hack *et al*. 1994; Banat *et al*. 1996). Mehaia & Cheryan (1984) used a hollow fibre bioreactor running under continuous culture conditions for ethanol production from lactose (at 150 g l^{-1}) using a strain of *K. fragilis*. They reported higher ethanol productivity $\approx$100–150 g l^{-1} h^{-1} at dilution rates of 1–4 h^{-1}. However, care was needed to maintain the process for extended periods and for bleeding gas generation within the system.

Other approaches involving the use of a combination of several methods, as outlined above, has been reported to yield up to 15% (w/w) ethanol using a strain of *S. cerevisiae* (Xu *et al*. 1996). Finally, low temperature (20 °C) high gravity fermentations of wheat, oats or barley mashes (>300 g dissolved solids l^{-1}) with fortification with sugar adjuncts or yeast extract were carried out and ethanol yields in excess of 15% (w/w) were reported (Jones *et al*. 1995; Thomas & Ingledew 1995; Ingledew *et al*. 1995). These approaches however, were carried out at mesophilic temperature ranges. The fermentation efficiency of such combined methods in terms of total substrate (sugar) consumption, ethanol yields and minimum production of fermentation byproducts other than ethanol at thermophilic ranges remains to be investigated.

Conclusion

This article is part I of two reviews describing the process advantages in exploiting yeast cultures capable of retaining their metabolic activities at higher temperatures. Available literature shows the intensive and interesting work carried out by various researchers on different aspects related to properties and applications of thermotolerant yeast. Breakthroughs in obtaining such yeasts have mainly been achieved through fresh isolation programmes for new strains. Both temperature and ethanol tolerance appear to be heavily interrelated and progress in understanding this relationship has made significant advancement. Bioreactor configurations will play a major role in possible future commercial production strategies. Since this subject represents a topic of much industrial and biotechnological importance we concentrated our efforts on the isolation and characterization of a thermophilic/thermotolerant yeast strains from samples exposed to higher temperature in natural environment. One of these isolates *Kluyveromyces marxianus* IMB3 has been most promising and details of progress in its potentials are described in Part II of this review.

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