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Screening of Thermotolerant Yeast for Use as Microbial Feed Additive

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Abstract With the objective of identifying the commercial potential of new direct-fed microbials, several temperature-tolerant strains were isolated from cane molasses at 39°C and tested for their tolerance to pH, bile salts, and a mixture of volatile fatty acids (acetic acid:propionic acid:butyric acid=6.5:2.0:1.5). It was found that the isolated strain DY 252 grew very well up to pH 2.0 and was resistant to relatively high concentrations of bile salts. Among the strains tested, DY 252 was least inhibited by the addition of volatile fatty acids to the growth medium at 39°C. Accordingly, it would appear that strain DY 252, identified as yeast *Issatchenkia orientalis*, may be a potential candidate for use as a microbial feed additive.

Key words: Bile salts, direct-fed microbials, thermotolerant yeast, volatile fatty acids

Direct-fed microbial feed additives (previously called probiotics) are currently used as feed additives to foster good animal health and optimal production. The term direct-fed microbial (DFM) has been employed to describe microbial-based products [13, 14]. Many microorganisms have been used in DFM formulations. The most common organisms found in DFM products include *Saccharomyces*, *Bacillus*, and *Aspergillus* [15]. Many earlier studies have documented the positive effects of feeding DFMs to animals, although further studies on the mechanism of how DFMs improve animal production are still required. A number of studies document significant increases in milk or fat-corrected milk production from yeast- and fungal-supplemented diets [9, 12, 17], while others have described the role of *Saccharomyces cerevisiae* and fungal fermentation extracts in animal feeds [3, 18].

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Yeasts, in particular *S. cerevisiae*, have been widely used in brewing, wine-making, and baking processes for several thousand years. They are widely distributed in nature and commonly associated with fruits and vegetables. Several criteria have been used for screening microorganisms for DFMs [2, 10], including acid tolerance, bile resistance, and nonpathogenicity. In addition, the tolerance of the microorganisms to high temperature is also important, since body temperatures of most animals are within the range of 37–43°C. The aim of the current study was to isolate a thermotolerant yeast from cane molasses to improve over the currently marketed feed additives.

Isolation of Temperature-Tolerant Yeasts from Molasses
Temperature-tolerant yeasts were isolated from cane molasses using the same method as reported previously [16]. Five grams of fresh cane molasses was added to 250-ml flasks containing 100 ml of fermentation medium (glucose 100 g/l, yeast extract 5 g/l, urea 6.4 g/l, KH_2PO_4 1.2 g/l, and Na_2HPO_4 0.18 g/l). The incubation was carried out at 39°C for 24 h, and 0.1 ml of the fermentation broths diluted up to 10^2–10^4 times was plated onto YM agar plates. Several colonies on the plates were randomly selected and tested for ethanol production. Single colonies were taken from the culture plates and transferred to sterile Erlenmeyer flasks containing 100 ml of the fermentation medium. The fermentations were carried out with shaking (100 rpm) for 24 h at 30°C. The ethanol concentrations were measured by a gas chromatograph (Flame Ionization Detector, N_2 gas flow rate=30 ml/min, detector temperature=200°C). Figure 1 shows the characteristics of the ethanol fermentation with the isolated strains DY 133 and DY 252, along with eight typical ethanol producers, such as *S. cerevisiae* KCTC 7106, KCTC 7112, KCTC 7904, KCTC 7905, KCTC 7906, KCTC 7910, and KCTC 7911, and *S. uvarum* KCTC 7918. As seen in Fig. 1, the ethanol concentrations with the isolated strains were within a range of 42–47 g/l.

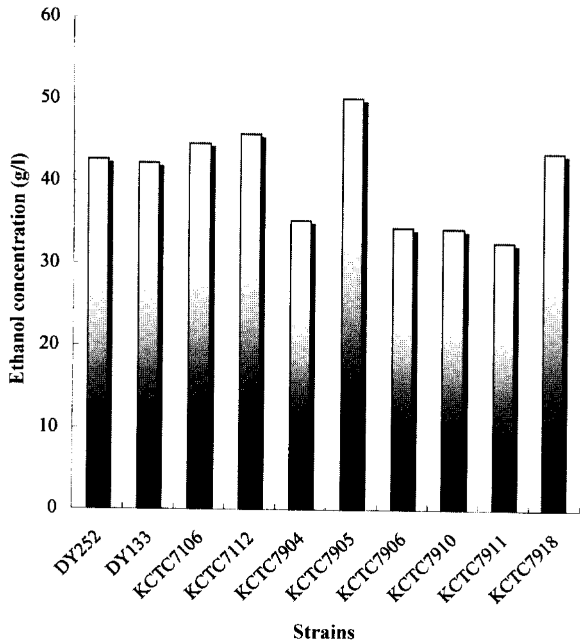

Fig. 1. Characteristics of ethanol fermentation by isolated strains together with eight typical ethanol producers.

However, the ethanol concentrations with strains KCTC 7904, KCTC 7906, KCTC 7910, and KCTC 7911 were much lower, probably due to formation of byproducts. It has been previously reported that a theoretical yield of 0.51 g ethanol can be obtained from the fermentation of 1 g glucose. Ethanol yields of 80–95% of theoretical values have been reported with yeasts [1, 4, 5, 7, 11].

Studies on Tolerance to pH, Bile Salts, and Volatile Fatty Acids

The microorganisms should have acid-tolerant properties, because they need to survive and remain metabolically active during the passage from stomach to the small intestine. In Fig. 2, the effect of pH on the growth of the yeasts at 30°C is shown. The composition of the growth medium per liter for this study was as follows: 20 g glucose, 10 g yeast extract (Difco), and 2 g KH_2PO_4. Throughout this experiment, 5% (v/v) inoculum was employed. As seen in Fig. 2, DY 252 grew very well at pH 2.0, while most of the *S. cerevisiae* strains did not. However, above pH 3.0 the growth of all the yeasts were no longer significantly affected by pH.

The success of a DFM is dependent on ensuring that the selected strains have bile-resistant qualities. The importance of bile tolerance was established by Gilliland *et al.* [8]. The effect of bile salts (Difco) within a range of 0.3–0.9% (w/v) on the growth of the various yeasts is shown in Fig. 3. The relative growth was calculated by measuring the absorbances at 640 nm (Spectronic 20, Bausch & Lomb) using 20-fold diluted samples. As shown in Fig. 3, most of the yeasts, except for *S. cerevisiae* KCTC 7106, exhibited bile salt-tolerant characteristics with up to 0.9% bile salts, which is much higher than that found in the intestines of most animals [2].

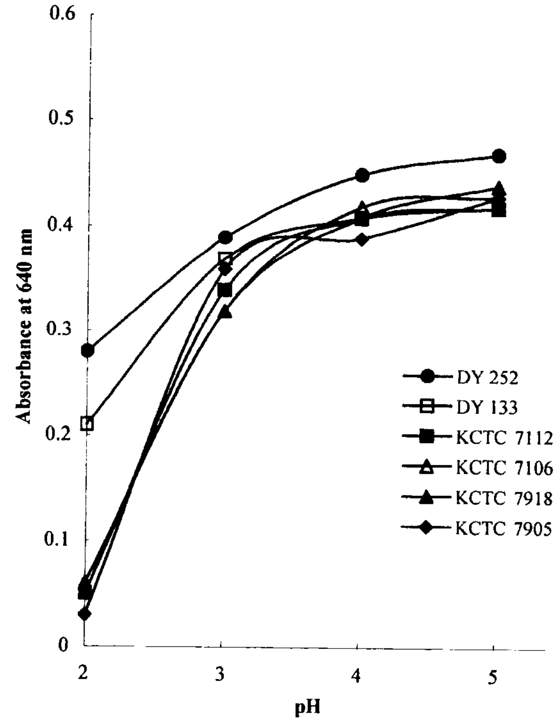

Fig. 2. Effect of pH on growth of various strains at 30°C.

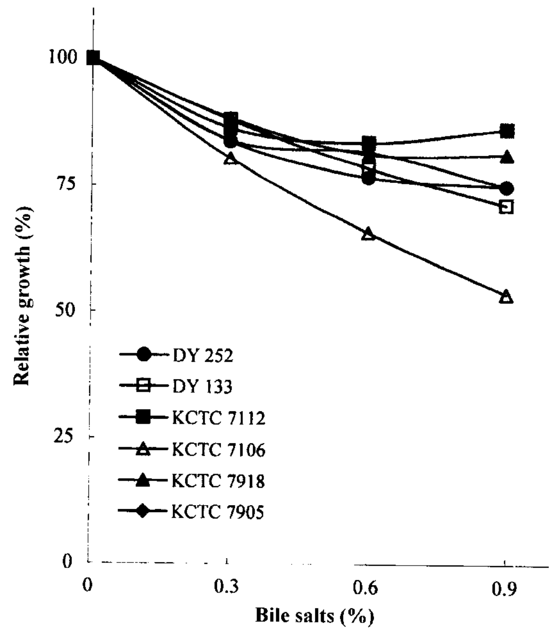

Fig. 3. Effect of bile salts on relative growth of various strains at 30°C.

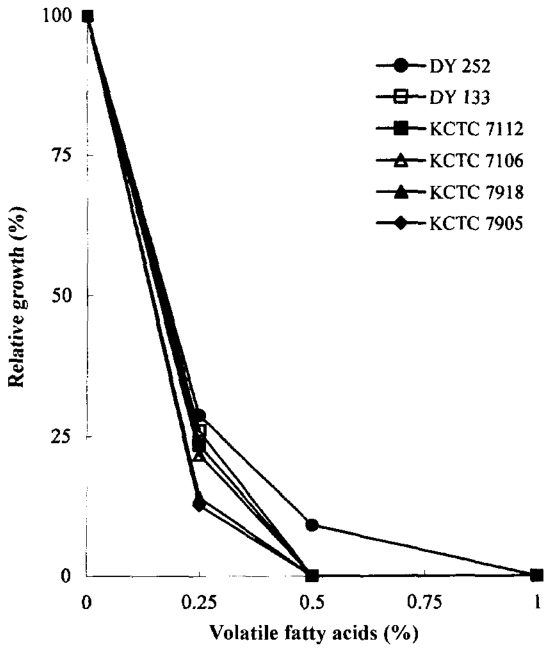

Fig. 4. Effect of volatile fatty acids on the relative growth of various strains at 39°C.

Resistance studies with volatile fatty acids (VFA) were also carried out for the six strains, as shown in Fig. 4. For this particular study, a mixture of VFA (acetic acid:propionic acid:butyric acid=6.5:2.0:1.5), a composition similar to that reported by Agarwal *et al.* [2], was added to flasks within a range of 0.25–1.0% (v/v). The incubation was carried out at 39°C, because this is the body temperature of ruminants. As can be seen in Fig. 4, significant growth inhibition by VFA was evident. Similar results were also reported previously [2]. One-quarter % of VFA exerted a significant inhibitory effect on all the strains. At 0.5% VFA concentration, equivalent to 80 mM of total volatile fatty acids, no growth was observed, except for DY 252. Therefore, DY 252 was identified as the strain with the highest tolerance to VFA. Since it has been reported that the concentrations of total VFA for ruminants are within a range of 70–130 mM [6], DY 252 appeared to have the best potential for the future development of a DFM.

Identification and Characterization of Strain DY 252

Based on the 26S rDNA sequencing of strain DY 252 together with a phylogenetic analysis (data not shown), DY 252 was identified as *Issatchenkia orientalis*. DY 252 had a 26S rDNA sequence that was identical to that of *I. orientalis* NRRL Y-5396, which belongs to the yeast family. Table 1 summarizes the fermentation characteristics of the isolated strain DY 252. When its sugar utilization was tested using an API ID32 C test kit (bioMerieux Co.), DY 252 was found to ferment both glucose and fructose, but not sucrose (see Table 1).

Table 1. Sugar utilization characteristics of isolated strain DY 252.

Sugar	Result
Cellobiose	–
D-Fructose	+
D-Galactose	–
D-Glucose	+
D-Ribose	–
D-Xylose	–
DL-Lactate	+
Erythritol	–
Glycerol	+
L-Arabinose	–
N-Acetyl-glucosamine	+
Raffinose	–
Rhamnose	–
Sorbitol	–
Sucrose	–

Although various microbial feed additives have already been commercially available, many improvements in strain selection and technology are still desired to reduce production costs. The current study provided evidence that strain DY 252 has the potential to be developed as a microbial feed additive.

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