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Screening of Native Yeast from *Agave duranguensis* Fermentation for Isoamyl Acetate Production

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ABSTRACT

In this work, fifty yeast strains, isolated from the spontaneous alcoholic fermentation of Agave duranguensis *to produce mezcal, were tested using the double coupling system. These yeasts were from the genera* Pichia, Torulaspora, Saccharomyces, Kluyveromyces, Deckera, Hanseniaspora, *and* Candida. P. fermentans *ITD00165 was the best isoamyl acetate producer, yielding 0.38 g/L of ester after incubation for 24 h, while* K. marxianus *ITD00211 produced 0.32 g/L of ester. Thus* P. fermentans *ITD00165 could be considered as an excellent choice for use in optimization studies of the culture medium and bioreactor operating conditions to develop a process for biotechnological production of isoamyl acetate.*

Key words: Banana aroma, Food additive, Native yeast strains

INTRODUCTION

A vast array of compounds, such as alcohols, esters, fatty acids, and sulphur compounds may be responsible for the flavor of foods (Gratfield. 1988; Dubal et al. 2008; Krings 1998). Food processing can cause a weak aroma in the final product, hence, it is necessary to use the additives (Lemos et al. 2010). Most of these compounds are produced by the chemical synthesis, but a rapid shift to biosynthesis is taking place (Janssens et al. 1992) because consumers have developed a tendency to prefer the food with a "natural" label (Janssens et al. 1992; Lemos et al. 2010). Isoamyl acetate is an ester with great interest in the food industry. It has a consumption of 74,000 kg per year due to its characteristic banana smell (Torres et al. 2009).

Yeasts produce esters by esterification of alcohols with acetyl co-enzyme A (Verstrepen et al. 2003). Two genes coding for the enzyme alcohol acetyltransferase have been identified in *Saccharomyces cerevisiae* (Mason and Dufour. 2000). This enzyme catalyzes the reaction between acetyl co-enzyme A and alcohols. Yeasts also produce enzymes with ester hydrolase activity and the balance between these two antagonistic enzyme activities determines the final concentration of isoamyl acetate in the fermentation system (Inoue et al. 1997; Fukuda et al. 1998; Yoshimoto et al. 1999; Rojas et al. 2001). Oda (1996) developed a system for the production of esters, called a doubled coupled system (DCS),

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which consisted in an immobilized microorganism between two the phases, one solid and one liquid. The hydrophilic solid surface contains the nutrients necessary for the microorganism, while the liquid phase is composed of an organic solvent in which the alcohol to be esterified is dissolved. After alcohol esterification with acetyl co-enzyme A, the resulting ester accumulates in the hydrophobic phase of the system, where it can be quantified and recovered.

Like several studies on alcoholic beverage production (Valero et al., 2002; Abbas. 2006), the objective of this work was to select yeast strains with high capacity of isoamyl acetate production, using the doubled coupled system in order to take advantage of the microbial biodiversity in the region as an alternative to produce a natural banana aroma.

MATERIALS AND METHODS

Chemicals

Isoamyl acetate (GC grade), isoamyl alcohol, decane, anhydrous sodium carbonate, potassium sodium tartrate, sodium sulphate, copper sulphate pentahydrate, sulphuric acid, ammonium molybdate, sodium arsenate heptahydrate, and glucose were supplied by Sigma-Aldrich (USA). Peptone, yeast extract, malt extract, and agar were supplied by BD Bioxon (Mexico).

Yeast Strains

Fifty non-*Saccharomyces* strains belonging to the genera *Candida, Hanseniaspora, Kluyveromyces, Pichia, Torulaspora,* and *Dekkera,* isolated from the spontaneous alcoholic fermentation of *Agave duranguensis,* were obtained from the Microbial Biotechnology Lab's Culture Collection at the Durango Institute of Technology.

Screening procedure

The synthesis of isoamyl acetate was carried out following the protocol described by Oda (1996). Two hundred microliters from 24 h cultures in GPYM medium were spread onto Petri dishes with solid GYMP medium and incubated at 30 °C for 24 h. After incubation, 8.0 mL of a 1% (v/v) isoamyl alcohol solution in decane was placed onto the agar plate and incubated at 30 °C for 24 h with shaking (100 rpm). A negative control was subjected to the same process by adding an isoamyl aceteate in decane solution (0.3 g/L) onto Petri dishes without microorganism. Aliquots of the decane solution were taken after incubation for 6, 12, and 24 h for analysis by gas chromatography (GC). The GYMP medium contained (g/L): 40 glucose, 5 peptone, 3 yeast extract, 3 malt extract, and 1 magnesium sulfate heptahydrate. The pH of the medium was adjusted to 6.0.

Isoamyl acetate and isoamyl alcohol concentrations were determined using standard solutions. Measurements reported are the average of three independent plate cultures. Statistical comparisons were made using Analysis of Variance (ANOVA) and the Fisher Least Significant Difference (LSD) post-hoc test with the program Statistica version 7.0 (StatSoft, USA).

The concentration of isoamyl acetate was quantified on an Agilent Technologies Network System Gas Chromatograph 6890N (Agilent Technologies, USA) equipped with a flame ionization detector (FID) and an HP-Innowax column (Hewlett-Packard, USA) (length, 30 m; inside diameter, 0.25 mm; film thickness, 0.25 mm). The temperatures of the injector block and detector were 220 and 300 °C, respectively. The oven temperature was programmed as follows: equilibrated at 80°C for 10 min, 80 to 150 °C at 1.5 °C/min, heated to 170 °C at 4°C/min, heated to 250 °C at 20 °C/min, and maintained at 250 °C for 2 min.

Liquid culture for acetate ester formation

To measure the ester formation, liquid cultures were grown in 125 mL flasks containing 50 mL of sterilized GPYM medium at pH 6.0. Flasks were incubated at 28 °C with shaking (120 rpm). Aroma compounds in the culture were measured by the headspace gas chromatography. Isoamyl alcohol (1%), sterilized with a 0.2 µm filter, was added to flasks at 0 and 12 h of fermentation to look at its effect on ester production. Yeast cell counts were determined by a Neubauer chamber. Experiments were carried out in triplicate.

Reducing sugar concentrations were measured as described previously (Nelson 1944; Somogyi 1945). Aroma compounds were measured the by headspace analysis of the culture in a gas chromatograph Shimadzu model 17A (Shimadzu corp., Japan), equipped with a flame ionization detector at 230 °C. The operation conditions were as follows: HP-5 capillary column (length, 30 m; inside diameter, 0.32 mm), column temperature of 40-150 °C at a rate of 20° C/min, and an injector temperature of 230 °C. Volatile compounds were

expressed as ppm/L of the headspace. External standard compounds were used to identify and quantify the microbial aroma compounds.

RESULTS AND DISCUSSION

The interest in naturally derived products has grown constantly in the society, but many bioprocesses for bioflavor production are not yet commercially available due to low yields (rarely above 0.1 g/L). Thus, these processes are economically unattractive (Medeiros. 2000; Vandamme. 2002). On the other hand, the advantages of yeast, such as their ease of production, have favored industry to adopt biotechnology as a way to produce bioflavors. Nevertheless, a better understanding of yeast biochemistry and the enzymes involved, fermentation optimization, metabolic regulation, and genetic improvement are primordial to enhance the theyields (Abbas. 2006; Etschmann. 2004; Vandamme. 2002). Studies on ester production by yeast have found that modifying parameters, such as components of the culture media and aeration conditions, have increased aroma production by three times (Etschmann. 2004; Lilly. 2006; Medeiros. 2000; Rojas. 2002)

Except for 13 strains of *Torulaspora delbrueckii*, all the tested strains were able to produce isoamyl acetate after incubation for 24 h. The tested strains of *T. delbrueckii* could be considered as intermediate producers of isoamyl acetate. However, not all the authors have reported isoamyl acetate as an ester produced by *T. delbrueckii* (Plata et al. 2003; Renault et al. 2009). In this study, some strains had no production of this aroma. Table 1 shows the yeasts in each genus with the highest isoamyl acetate production. According to the statistical analysis, *P. fermentans* ITD00165 was the best isoamyl acetate producer, followed by *K. marxianus* ITD00211 and *T. delbrueckii* ITD00233.

Table 1. Yeast strains with high production of isoamyl acetate after incubation for 24 h.

Yeast	Isoamyl Acetate* (g/L)
Candida diversa ITD0073	0.1348 ± 0.01^{hi}
Candida pseudointermedia ITD00101	0.1458 ± 0.02^{hi}
Deckera anomala ITD00015	0.1219 ± 0.02^{hij}
Hanseniaspora uvarum ITD00108	0.0822 ± 0.003^{k}
Kluyveromyces marxianus ITD00211	0.3280 ± 0.006^{b}
Pichia fermentans ITD00165	0.3829 ± 0.05^{a}
Saccharomyces cerevisiae ITD00215	0.0903 ± 0.01^{jk}
Torulaspora delbruekii ITD00233	0.2399 ± 0.05^{c}

*Means sharing the same letter are not significantly different at confidence level of 95%.

The genus *Pichia* has been reported previously (Oda and Ohta. 1997; Rojas et al. 2001) as a good ester producer. Oda (1996) reported *P. heedii* and *P. quercuum* as the strains with very good activities in the doubled coupled system for the esterification of aliphatic alcohols and terpenes. Rojas and co-workers (2001) assayed a selection of strains for their ability to esterify the alcohols using the double coupling system. They found that the strain *P. annomala* was able to esterify isoamyl alcohol better than the rest of the strains, providing 3 g/L isoamyl acetate. Among the factors responsible for these variations are the genus and species of the strain, the media composition, and the culture conditions (Gatfield. 1988; Verstrepen et al. 2003; Saerens et al. 2008). The production of isoamyl acetate by all the strains of *K. marxianus* tested here agreed with the results reported by Plata and co-workers (2003), who found that the presence of esters was detected only when the fermented media reached an isoamyl alcohol concentration of 0.01 g/L. They also found that, at this point, alcohol acetyltransferase was active. These may be signs that the production of isoamyl acetate is determined, at least in part, by the availability of isoamyl alcohol (Fabre et al. 1995; Plata et al. 2003). The strains of *Torulaspora* have ester hydrolase activity, which is more noticeable in the stationary phase (Renault et al. 2009). The strain ITD00233 showed a slight decrease in the concentration of isoamyl acetate from 0.27 to 0.23 g/L during the period from 6 to 12 h, which was minimal compared with a reduction of 50% reported previously (Plata et al. 2003).

All the strains from the two genera with the highest isoamyl acetate production are compared

in Figure 1. This figure showed that *P. fermentans* ITD00165, *P. fermentans* ITD00154, and *K. marxianus* ITD00211 were the best producers. Thus, these strains were characterized in terms of their production kinetics.

Figure 2 compares the kinetics of isoamyl acetate production by the two best producing strains (*P, fermentans* ITD00165 and *K. marxianus* ITD00211), as well as one strain with low production (*P. fermentans* ITD00154). *P. fermentans* ITD00165 had the largest production (0.39 g/L at 24 h) and it produced 75% of the total isoamyl acetate in 6 h (Fig. 2A). *K. marxianus* ITD00211 produced 0.33 g/L at 24 h, but produced only 33% of the total isoamyl acetate in 6 h (Fig. 2B). *P. fermentans* ITD00154 had the lowest production (0.14 g/L), which was reached at 6 h (Fig. 2C). In the control experiment without microorganism, the isoamyl acetate concentration remained constant throughout the incubation; nevertheless, the isoamyl alcohol concentration decreased from 0.8 to 0.4 g/L after incubation for 6 h (Fig. 2D). This behavior was very similar to that observed for isoamyl alcohol in the other graphs of this figure, independent of the kinetics of isoamyl acetate production in each graph. These results were strong evidence that isoamyl acetate was the product of yeast metabolism and not due to the esterification of added isoamyl alcohol. Yilmaztekin and co-workers (2009) reported a different behavior for the yeast *Williopsis saturnus.* They found that isoamyl acetate production by *W. saturnus* was enhanced 3-fold by the addition of 1% isoamyl alcohol (as a fusel oil). These results suggested that *W. saturnus* had a different metabolism than that of *P. fermentans* reported here, since *W. saturnus* esterified isoamyl alcohol to produce isoamyl acetate.

Isoamyl acetate production by *P. fermentans* ITD00165 was also studied in liquid culture. The maximum ester concentration into the headspace of the culture system was obtained after incubation for 12 h (Fig. 3). The aroma production reached 33 ppm/L with 93.75% reducing sugar consumption. Figure 1 showed that the isoamyl acetate production observed almost coupled to growth. Once sugar consumption and growth stopped, isoamyl acetate production and its concentration decreased, probably due to loss by evaporation. Rossi and co-workers (2009), also measuring aroma concentration in the headspace of Erlenmeyer flasks, reported similar kinetics of isoamyl acetate production by the fungus *Ceratocystis fimbriata* in solid substrate fermentation using citric pulp. Another possible contribution to the decrease in product concentration was the action of the enzyme ester hydrolase, since the quantity of ester produced depended on the balance of the activities of alcohol acetyltransferase and ester hydrolase, which differed in each yeast (Lilly. 2006; Bisson. 2010; Garde-Cerdán 2008). For example, the ester hydrolase activity in *P. heedii* CECT 11452 was 28 times higher than in *P. anomala* CECT 10590, while the alcohol acetyltransferase activity was 17 times higher in *P. heedii* CECT 11452 than in *P. anomala* CECT 10590 (Lilly 2006; Bisson 2010).

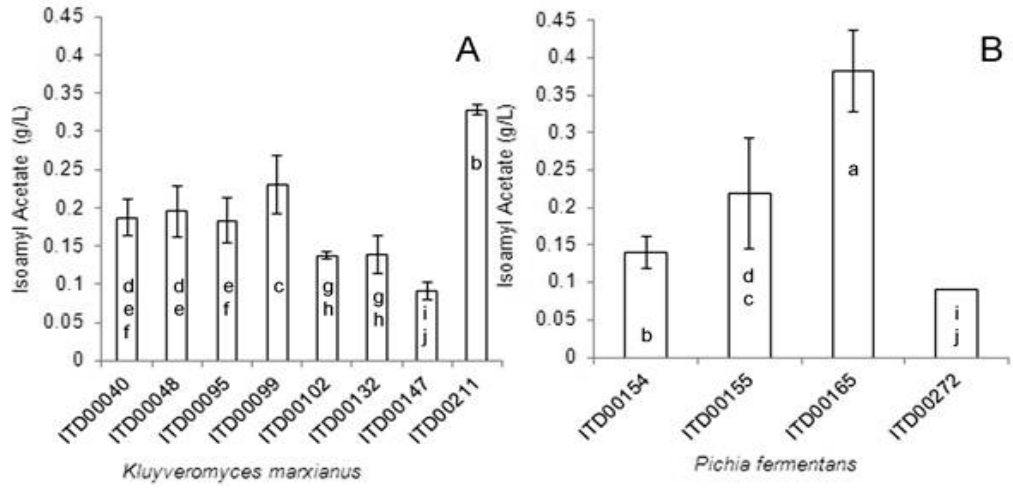

Figure 1 - Isoamyl acetate concentration in the organic phase of the doubled coupled system after incubation for 24 h with A) *Kluyveromyces marxianus* and B) *Pichia fermentans*.

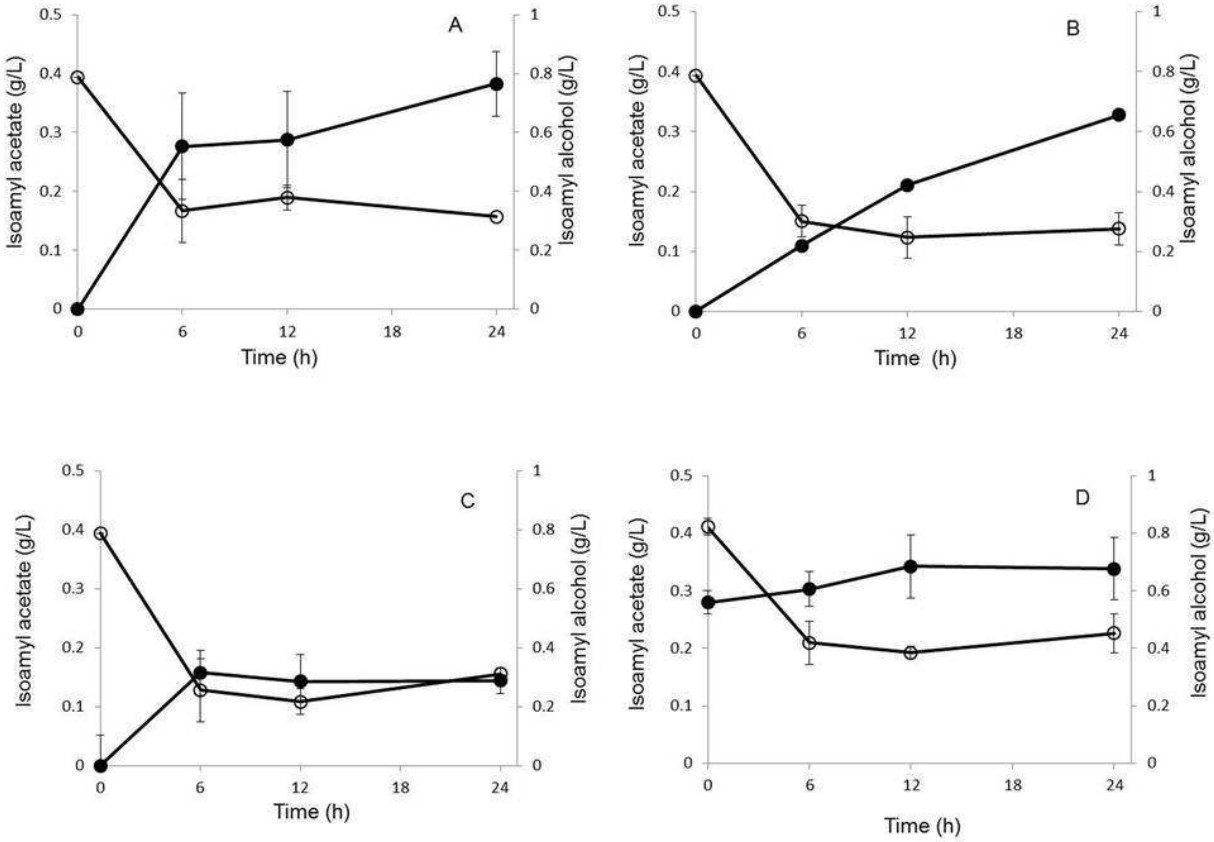

Figure 2 - Production of isoamyl acetate (●) and isoamyl alcohol (○) into decane throughout the incubation. A) *Pichia fermentans* ITD00165, B) *Kluyveromyces marxianus* ITD00211, C) *Pichia fermentans* ITD00154, D) Control without micoorganism. The bars indicate standard deviations for three independent incubations.

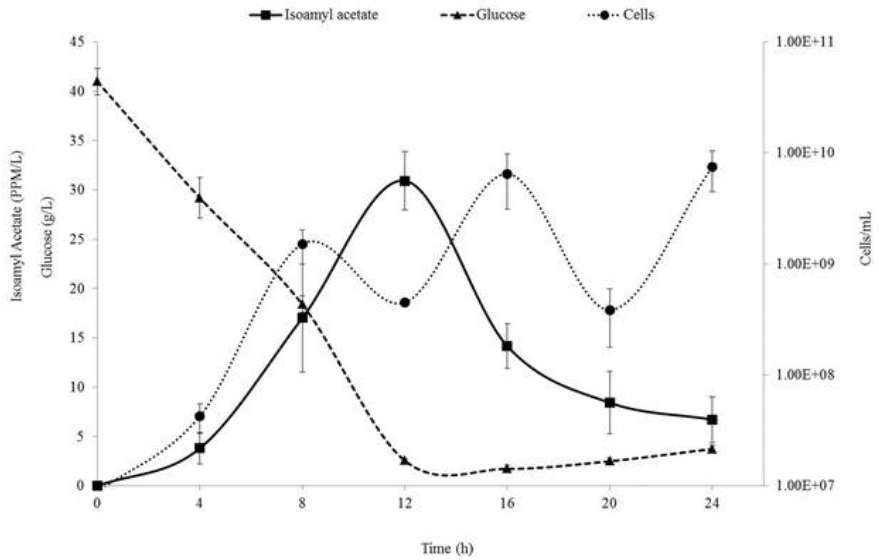

Figure 3 - Production of isoamyl acetate by *Pichia fermentans* ITD00165 in GYMP medium. Concentrations of biomass (●), isoamyl acetate (■), and reducing sugars (▲). The bars indicate standard deviations for three independent cultivations.

Peddie (1990) reported that ester synthesis was very slow at the beginning of fermentation in liquid cultures of *S. cerevisiae*. At this time, oxygen and acetyl CoA were rapidly consumed during the production of unsaturated fatty acids and sterols. Following this, equilibrium between acetyl CoA consumption for fatty acid and sterol synthesis and for ester production was established. Plata and co-workers (2004) found the highest concentrations of isoamyl acetate (0.15 ppm) with a high glucose concentration (250 g/L) under semi-anaerobic conditions (150 rpm) and the highest production occurred during the stationary growth phase after 240 h of the process. Rojas and co-workers (2001) reported that the highest isoamyl acetate concentration (10.21 ppm) was found in *P. anomala* cultures grown with glucose (40 g/L) under semi-anaerobic (120 rpm) conditions, which was approximately 19-fold higher than that produced by *S. cerevisiae* cultures after 48 h of the process. In contrast, in the present study, the synthesis of isoamyl acetate occurred during the exponential growth phase (after growth for 4-8 h), peaked at 12 h (32 ppm/L) when the stationary phase was reached, and then began to decline (Fig. 3). There was a 95% consumption of the initial reducing sugars during the first 12 h of incubation. Subsequently, there was an increase of about 5% during the following 12 h incubation. This could be due to the presence of invertase, which could be hydrolyzing the residual sucrose from the malt extract present in the culture medium. This increase was not considered significant, and in any case did not impact the production of isoamyl acetate.

CONCLUSION

The doubled coupled system permitted to identify the potential of non-*Saccharomyces* yeasts, isolated from the alcoholic fermentation of agave to produce acetate esters. In particular, the strain *P. fermentans* ITD00165 was an excellent choice for use in optimization studies of the culture medium and bioreactor operating conditions to develop a process for biotechnological production of isoamyl acetate.

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