The Production of Isoamyl Acetate from Amyl Alcohol by *Saccharomyces cerevisiae*

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ABSTRACT

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Isoamyl acetate is a natural flavour ester, widely used as a source of banana flavour by the food industry. Fusel alcohols such as amyl alcohol are produced in significant quantities as a waste product, sometimes referred to as "lees oil" or "fusel oil", of the alcohol distilling industry. By manipulation of brewing yeast fermentation conditions, a significant portion of added amyl alcohol was shown to be converted to isoamyl acetate. This was achieved by the addition of L-leucine and amyl alcohol in fermentations carried out by a high ester-producing brewing yeast strain of *Saccharomyces cerevisiae* and by the use of alkaline fermentation conditions coupled with high gravity media. Mutant strains selected on 5,5,5 trifluoro-DL-leucine produced substantially high levels of isoamyl acetate. The adjustment of fermentation conditions outlined in this paper may act as a stepping stone for the potential use of *Saccharomyces cerevisiae* and other yeasts to produce high levels of natural flavour esters.

Key words: Fermentation, headspace-gas chromatography, isoamyl acetate, lees oil, fusel oil, molasses, *Saccharomyces cerevisiae.*

INTRODUCTION

Esters are amongst the most important flavour compounds in alcoholic beverages[10,17]. They are formed as by-products of alcoholic fermentation at comparatively low levels[13]. One of the principal esters produced during alcohol fermentation is isoamyl acetate (banana flavour), which also has widespread use in the food industry[4].The level of isoamyl acetate in alcoholic beverages varies from 0.8 to 6.6 ppm, depending on the fermentation conditions, type of strain and the beverage in question[15].

Yeasts produce esters by esterification of alcohols with acetyl coenzyme A[12]. Isoamyl acetate is produced from a reaction between amyl alcohol and acetyl coenzyme A catalyzed by the enzyme isoamyl alcohol acetyl transferase[11].

There is little evidence that brewing and wine making yeast produce isoamyl acetate from amyl alcohol and acetic acid by esterase activity[14]. In fact, the evidence would suggest that esterase activity is more likely to reduce ester concentration than enhance it[9].

The conversion of amyl alcohol, the main component of "lees oil" a waste product of the distilling industry into isoamyl acetate, a valuable flavour compound, is theoretically possible using yeast. This would have potential for the production of natural banana flavour if the levels produced were high enough to make it commercially viable. So-called natural flavours command a higher value in the marketplace than do "nature identical" or chemically produced flavour compounds. This paper focuses on the factors that influence isoamyl acetate production by *Saccharomyces cerevisiae,* with the aim of increasing the levels substantially over those that would be produced under normal alcoholic fermentation conditions.

MATERIALS AND METHODS

Chemicals

Ethyl acetate (GC grade), isoamyl acetate (GC grade), L-leucine, 2-pentanone (GC grade), amyl alcohol (GC grade), ethyl methyl sulfonate (EMS), YPD agar and YNB without amino acids were supplied by Sigma-Aldrich Co. Ltd., Dorset, UK. Ale wort was supplied by Carlow Brewing Company, Station Rd., Carlow, Ireland. Lees oil was supplied by United Distillers Group, Dublin, Ireland. Molasses was supplied by Carlow Sugar Beet Company, Athy Road, Carlow, Ireland. The 5,5,5 trifluoro-DL-leucine (TFL) was supplied by PCR Incorporated, PO Box 1466, Gainesville, Florida 32602, U.S.A.

Yeasts

Saccharomyces cerevisiae strain 5610 of the Guinness Research Centre, St. James's Gate, Dublin 8, Ireland, was used in all experiments except where indicated. Yeast strains were maintained on YPD agar slants and re-cultured monthly.

Fermentation

Yeast inocula were prepared in 500 mL conical flasks containing 100 mL of wort (O.G. 1045) pH 4.90, shaking at 100 rpm at 21°C for 2 days. The yeast was harvested by centrifugation at 4000 rpm for 15 min and used to pitch 3 L at 3.0 g/L in tall stainless steel cylinders, one meter high, diameter 70 mm, containing wort (O.G. 1045, pH 4.90) and molasses (O.G. 1045, pH 6.3). The tubes were fitted with a rubber bung, which had a hollow glass tube

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(2.5 mm diameter) inserted ¾ length inside the stainless steel tube to allow for aeration. The wort and molasses were supplemented with various additions as specified in each experiment. Upon pitching of the yeast the wort and molasses was aerated briefly (four hours) to stimulate growth by connecting an air pump to the hollow glass tubing. Fermentations took 6 days at 21°C under static conditions and the stainless steel tubes were held at a slant of 30°. The final gravity and pH were checked at the end of each fermentation. At the end of fermentation, samples were taken directly from the tall stainless steel cylinders for analysis by Headspace-Gas Chromatography (HS-GC).

Analysis of volatile esters

A 50 mL syringe was attached to the glass rod and samples were withdrawn under suction. Working in a refrigerated room (4°C) 10 mL of the sample from the syringe was placed into the headspace vials (22.5 mL) (in triplicate). The internal standard (2-pentanone) was then added to each vial to give a final concentration of 20 ppm. Aluminum caps with PTFE seals were used to seal the vials using a handcrimper. At the time of thermostatting the vials were removed from the cold room and placed in a shaking (100 rpm) water bath at 30°C for 30 min. Using a pre-heated (50°C) 2.5 mL Luer-lock gas tight syringe, 1.0 mL of the headspace was withdrawn from the appropriate vial and injected manually into the GC. A capillary column (ZB WAX (60 meter × 0.25 mm I.D. × 0.25 μm film coating) using nitrogen as the carrier gas (60 kPa) was used to separate the esters and alcohols. Stationary gas: air (50 kPa). GC settings were as follows: oven temperature 70°C, split ratio: 1:50 (checked using a digital flow meter), injection temperature: 200°C, flame ionization detector (FID): 225°C, make-up gas: nitrogen (8 kPa). Analysis time was 30 min. All samples were analyzed in triplicate. The percentage standard deviation (% S.D.) from the mean in all experiments was less than 8%.

Selection and isolation of 5,5,5 trifluoro-DL-leucine resistant mutants

A modification of the method used by Ashida *et al.*[2] was used to select for mutants. *Saccharomyces cerevisiae* 5610 was grown up for 16 hours in YPD broth at 21°C shaking (100 rpm). A reduced level of EMS was then added – 100 μL per 200 mL of culture. The culture was then spread-plated (50 μL) per plate onto YNB plates containing 1.0 mM 5,5,5 trifluoro-DL-leucine (TFL). Plates were incubated at 21°C for 3–5 days. Resistant colonies were re-streaked onto YNB plates again containing 5,5,5 TFL and were incubated at 21°C for 3–5 days.

TABLE I. Volatile alcohol/ester levels in beer from a commercial microbrewery.

Alcohol/ester conc. (ppm)	Strain				
	A	B	C	D	E
Ethyl acetate	17	29	21	25	16
Isoamyl acetate	1.9	2.3	3.5	4.9	3.8
Ethyl hexoate	0.19	0.14	0.24	0.08	0.16
Isobutyl acetate	0.03	0.08	n.d.	0.08	n.d.
Ethyl butyrate	0.11	0.09	0.09	0.09	n.d.
Propanol	20	24	24	28	34
Isobutanol	14	18	22	33	26
Amyl alcohol*	74	88	95	149	107

D = Ale yeast strain 5610; A, B, C, E = other ale strains. n.d. = not detected

*The compound "amyl alcohol" referred to in this study is a mixture of 2- and 3-methyl butanol, which co-elute in the GC method used in this study and are therefore indistinguishable from each otherr

RESULTS

Table I shows the volatile levels in beer of typical ale yeast brewing strains from a commercial microbrewery (original gravity 1045–1050). As can be seen, the levels of isoamyl acetate produced are in the region of 1.9 to 4.9 ppm. With a taste threshold of 1.6 ppm, this is just enough to impart a slight fruity flavour to beers and is considered desirable. On average, the amount of isoamyl acetate produced in beer fermentation is about 50–100 times lower than that of amyl alcohol.

The effect of EDTA on ester and amyl alcohol production in wort and molasses

EDTA was employed in an attempt to "clean up" molasses, as the presence of trace metals such as Mg, Fe, Mn and Cu in high concentrations in the fermentation medium can be toxic to the yeast. In addition, these trace metals influence the pH and ionic strength of the medium. Ergun *et al.*[3] investigated the potential of employing EDTA as a conversion enhancing agent for the improved production of ethanol from molasses using *S. cerevisiae.* In this method the metal complex acts as a "metal buffer" which reversibly dissociates to release ions as they are utilized by the yeast, or combines with excess metal ions added to the system. Analysis of the volatile components by HS-GC is shown in Table II.

In wort, the addition of EDTA had little effect on isoamyl acetate production (up to 0.5 ppm increase over the control). Likewise with molasses, where there was only 0.7 ppm of an increase in isoamyl acetate compared to the control. However, there was an increase of 8.4 ppm in the production of ethyl acetate where EDTA was added to molasses when compared to the control.

TABLE II. HS-GC analysis of esters and amyl alcohol in wort and molasses – the effect of EDTA.

	Wort			Molasses		
	Ethyl acetate (ppm)	Isoamyl acetate (ppm)	Amyl alcohol (ppm)	Ethyl acetate (ppm)	Isoamyl acetate (ppm)	Amyl alcohol (ppm)
Control	23.4	4.9	119.5	22.7	4.4	124.0
0.03 g/L	24.8	4.9	116.2	24.8	4.7	122.0
0.06 g/L	24.2	5.0	118.9	25.3	4.7	128.0
0.12 g/L	24.6	5.3	125.2	29.4	5.1	121.9
0.24 g/L	24.9	5.4	121.6	31.1	5.1	122.3

TABLE III. HS-GC analysis of esters and amyl alcohol in wort and molasses – the effect of zinc.

	Wort			Molasses		
Zinc sulphate	Ethyl acetate (ppm)	Isoamyl acetate (ppm)	Amyl alcohol (ppm)	Ethyl acetate (ppm)	Isoamyl acetate (ppm)	Amyl alcohol (ppm)
0	26.5	4.9	121.8	28.2	4.7	126.1
0.015 g/L	27.1	5.3	146.3	29.6	5.3	153.6
0.030 g/L	30.6	6.2	150.4	29.2	5.7	155.2
0.060 g/L	27.5	7.3	155.1	29.0	6.8	157.0
0.120 g/L	30.5	7.5	160.7	31.4	7.0	157.7

The effect of zinc and pitching rate on isoamyl acetate and amyl alcohol synthesis

Zinc is an important mineral nutrient for yeast and has both structural and catalytic functions and it has been shown that the addition of zinc can improve sluggish fermentations[17]. Seaton *et al.*[17] proposed that zinc addition to lager fermentations resulted in an increase in the synthesis of higher alcohols by stimulating the breakdown of α-keto acids to their corresponding higher alcohol.

Increasing concentrations of zinc sulfate (from 0.015 to 0.12 g/L) were added to the fermentation media of both wort and molasses to investigate its effect on ester and amyl alcohol synthesis. As the addition of zinc also led to faster fermentation rates (data not shown), this suggests that zinc may be a limiting factor for yeast growth both in wort and molasses. Ester and amyl alcohol analyses are shown in Table III. The addition of 0.12 g/L zinc sulphate resulted in a 1.5 fold increase in the amount of isoamyl acetate for both wort and molasses and approximately a 25% increase in amyl alcohol production.

Varying the pitching rate from 3.0 to 15.0 g/L had no appreciable effect on isoamyl acetate or amyl alcohol concentration. Also, stirring the fermentation under anaerobic conditions had no effect on amyl alcohol production and gave rise to a slight decrease in isoamyl acetate production (data not shown).

The effect of yeast re-pitching on ester and amyl alcohol synthesis in wort and molasses

It is generally accepted that aged cultures of yeast are better for producing higher quantities of esters than young cultures[19]. This phenomenon was investigated by re-pitching with the same yeast seven times in succession. The effect of yeast re-pitchings on ester synthesis is given in Fig. 1.

Repeated yeast re-pitching lead to substantial increases in isoamyl acetate production (up to 2.25 fold increase using wort as the fermentation medium) while ethyl acetate production increased from 25 ppm to 32 ppm. With each re-pitching the level of isoamyl acetate increased steadily up until the fifth re-pitching, after which it began to fall. A parallel trend for amyl alcohol was also found (Fig. 2).

The effect of pH on ester and amyl alcohol formation in wort and molasses

An investigation was carried out to evaluate the profile of ester production under different pH conditions. Yoshioka and Hashimoto[20] studied the effect of pH on the en-

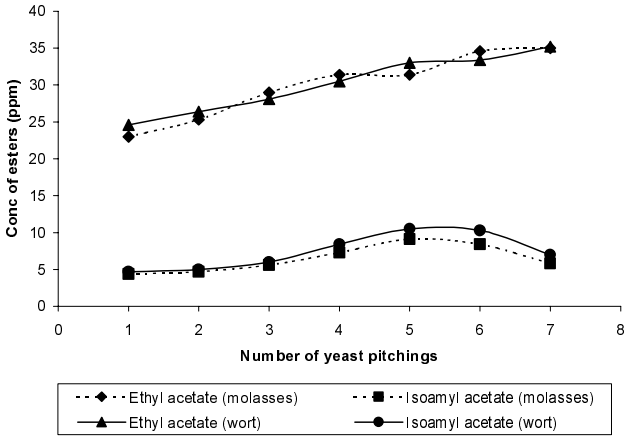

Fig. 1. The effect of yeast re-pitchings on ester synthesis.

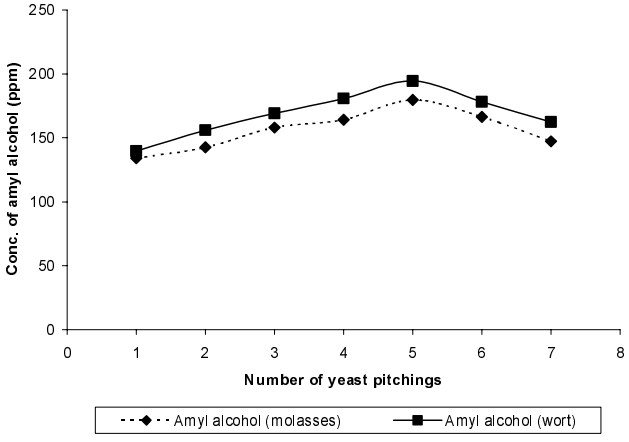

Fig. 2. The effect of yeast re-pitchings on amyl alcohol synthesis.

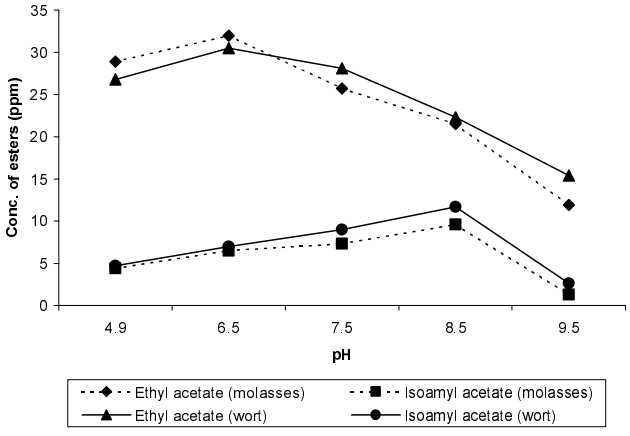

Fig. 3. The effect of pH on ester synthesis.

zyme alcohol acetyltransferase (AATase) in brewer's yeast. They found that the enzyme was most active in the pH range of 7 to 8. In this experiment a broad series of pH values was investigated – ranging from pH 4.9 to pH 9.5. The effect of pH on ester synthesis is given in Fig. 3.

The results suggest that while ethyl acetate levels decrease with increasing the pH above 6.5, that isoamyl acetate reaches a peak at pH 8.5 and then decreases. This may be a reflection of the decrease in amyl alcohol, which occurs at the same time (Fig. 4). This may also reflect an increase in AATase activity at alkaline pH, a finding also confirmed by Hammond and Pye[7], suggesting that there are two opposing trends between pH 6.5 and pH 8.5. This suggests that the two esters may be the result of two different enzyme systems in the cell.

The effects of L-leucine addition on isoamyl acetate and amyl alcohol synthesis

As L-leucine is a precursor for amyl alcohol[18] and by extension isoamyl acetate, the level of exogenous L-leucine would be expected to have an influence on the level of amyl alcohol and isoamyl acetate. Various quantities of L-leucine were added to both fermentation media to look at its effect on amyl alcohol and ester production.

As expected, the addition of L-leucine gave rise to nearly a 2.3 fold increase in isoamyl acetate production (4.8 to 11.0 ppm – see Fig. 5) and a 27% increase in amyl alcohol production (see Fig. 6). Ethyl acetate levels increased marginally. Beyond 4.0 g/L addition of L-leucine there was no further increase in isoamyl acetate or amyl alcohol production, suggesting that L-leucine catabolism may be saturated. These findings agree with those of Sablayrolles and Ball[16] who claimed that the addition of L-leucine strongly increases the production of the corresponding fusel alcohol (amyl alcohol), which subsequently leads to an increase in the corresponding ester (isoamyl acetate).

The effect of lees oil addition on ester and amyl alcohol synthesis in wort and molasses

Fusel oil was added to the fermentation media in the form of lees oil (54.5% amyl alcohol, 17.8% 2-methylbutanol, 11.2% isobutanol and 9.6% propanol). The catabolic or Ehrlich pathway of fusel alcohol formation starts by transamination of an amino acid and α-ketoglutarate. This results in a corresponding α-keto acid, which is decarboxylated and reduced to fusel alcohol.

The addition of lees oil to the fermentation media (Fig.

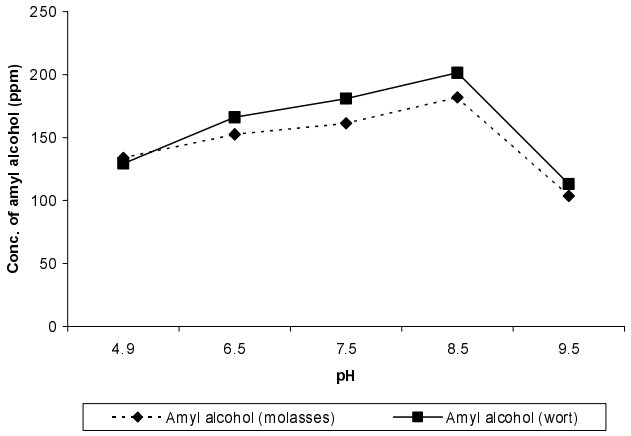

Fig. 4. The effect of pH on amyl alcohol synthesis.

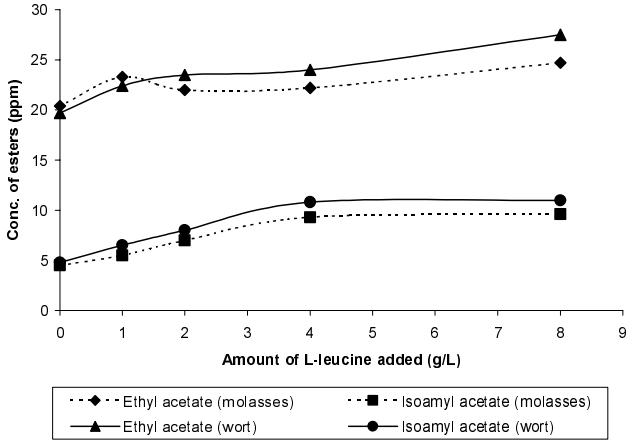

Fig. 5. The effect of L-leucine on ester synthesis.

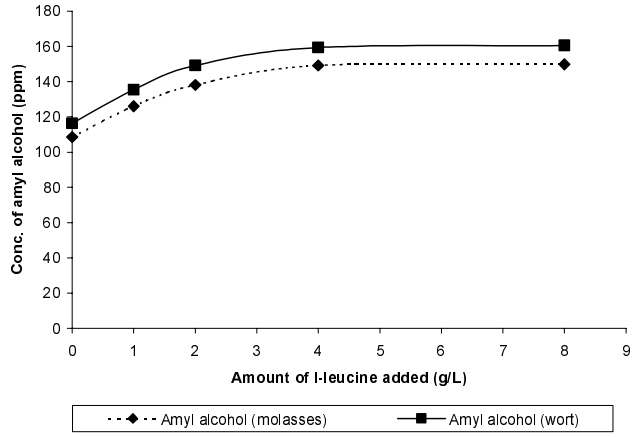

Fig. 6. The effect of L-leucine on amyl alcohol synthesis.

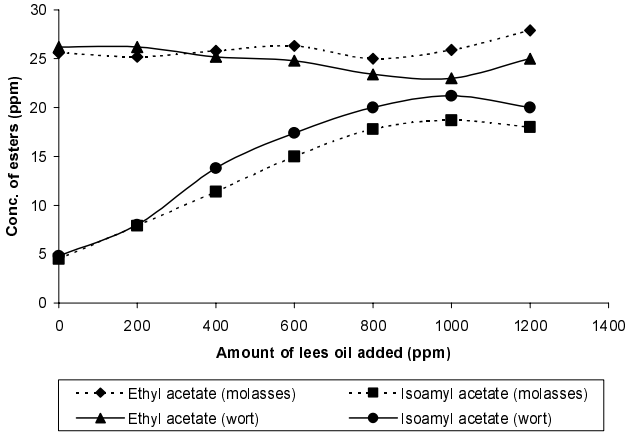

Fig. 7. The effect of lees oil on ester synthesis.

7) greatly stimulates isoamyl acetate production in wort and molasses (up to 4.4 fold in wort). This would indicate that there is a direct correlation between amyl alcohol levels and isoamyl acetate synthesis. At very high levels (above 1000 ppm lees oil) the AATase enzyme becomes saturated and no further increases in isoamyl acetate were found. The addition of lees oil to molasses and wort had a negligible over-all effect on ethyl acetate synthesis.

The effect of high gravity fermentation media on ester and amyl alcohol production in wort and molasses

It is well known that disproportionately high ester synthesis occurs in high gravity fermentations (1085) when compared with normal (1045–1050) brewery fermentations[1]. In this experiment a series of high gravity fermentation media (wort and molasses) in ascending order were fermented to analyse their effect on ester synthesis.

From Fig. 8 it can be clearly seen that with increasing specific gravity the levels of isoamyl acetate dramatically increases from 4.8 to 19.7 ppm in wort and from 4.2 to 17.4 ppm in molasses. A corresponding increase in amyl alcohol from 145.6 to 227.8 ppm in wort and 139.5 to 211.2 ppm in molasses was also observed (Fig. 9). Ethyl acetate levels increased gradually with increasing specific gravity.

The effect of high gravity wort + lees oil + alkali media + the addition of L-leucine + yeast re-pitching on ester and amyl alcohol synthesis

When the five variables which yielded the greatest increases in isoamyl acetate production were combined, the result was a large increase in isoamyl acetate production. These five variables were: high gravity wort (O.G. 1085), the addition of 1000 ppm of lees oil and 4.0 g/L L-leucine, maintaining the fermentation medium at pH 8.5 throughout fermentation by the addition of KOH, along with several re-pitchings of the yeast.

As can be seen (Fig. 10) when all five of the variants are combined, the increase in isoamyl acetate is significant. Up until and including the fifth re-pitching there is a steady increase in isoamyl acetate production (80.7 ppm) after which there is a decline. This suggests that alkali pH is a very important factor in producing high levels of iso-

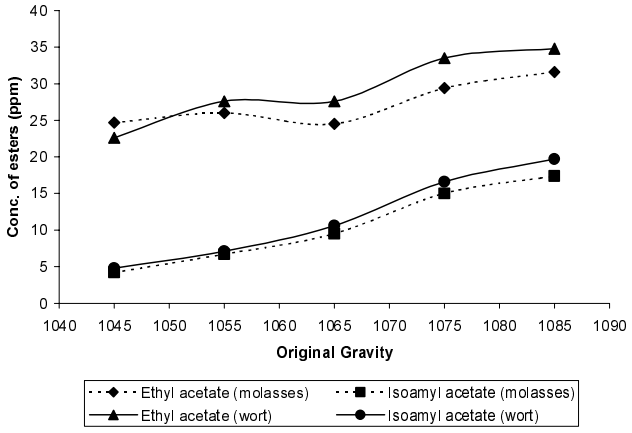

Fig. 8. The effect of high gravity fermentation media on ester synthesis.

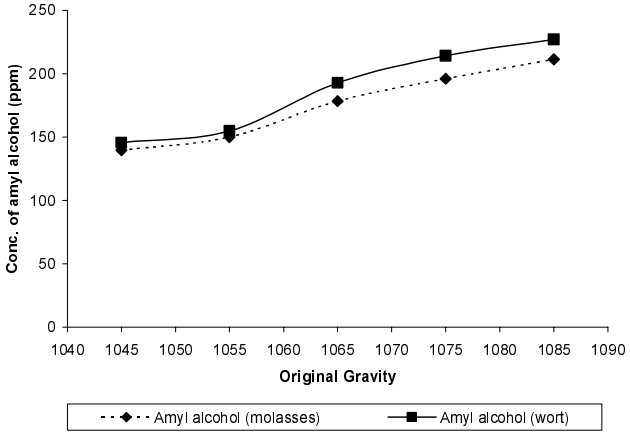

Fig. 9. The effect of high gravity fermentation media on amyl alcohol synthesis.

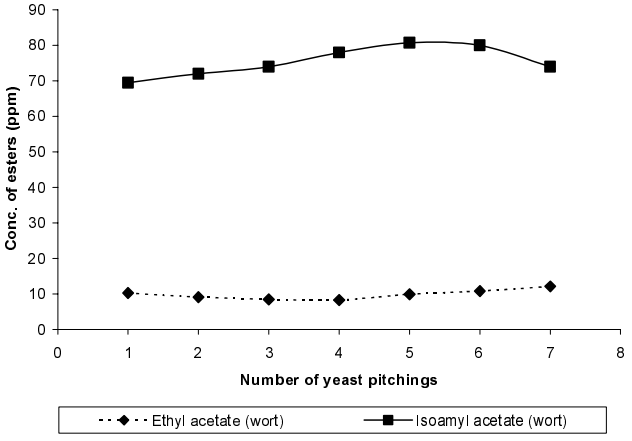

Fig. 10. The effect of high gravity wort + lees oil + alkali pH + the addition of L-leucine + yeast re-pitching on ester synthesis.

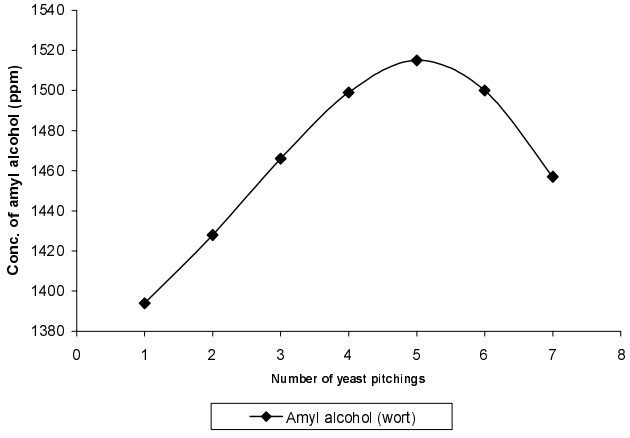

Fig. 11. The effect of high gravity wort + lees oil + alkali pH + the addition of L-leucine + yeast re-pitching on amyl alcohol synthesis.

TABLE IV. Screening of TFL resistant mutants for increased ester and amyl alcohol production.

Fermentation no.	Strain	Ethyl acetate (ppm)	Isoamyl acetate (ppm)	Amyl alcohol (ppm)
1	5610a (mutant)	14	162	1716
2	5610 (parent)	10	79	1489

amyl acetate. The same trend was observed for amyl alcohol (Fig. 11), suggesting that L-leucine catabolism may be more rapid at high pH.

Screening of trifluoro-DL-leucine resistant mutants for increased ester and amyl alcohol production

Fermentations, employing the conditions outlined in the previous experiment, were carried out on all mutant colonies that grew on YNB plates containing 5,5,5 trifluoro-DL-leucine.

The results show (see Table IV) that the mutant strain *S. cerevisiae* 5610a was able to produce up to twice as much isoamyl acetate as the parent strain. Also, 5610a produced more amyl alcohol (an increase of 15%) while ethyl acetate levels remained low.

DISCUSSION

The ester producing ability of brewing yeast to convert fusel alcohol sources such as "lees oil" to a potentially valuable product such as isoamyl acetate has not, to our knowledge, been reported previously. Fermentation conditions of ester-producing *S. cerevisiae* brewing yeast can be adjusted by the addition of L-leucine, amyl alcohol, using high gravity wort, by the use of alkaline pH conditions and several yeast re-pitchings to produce over 80 ppm isoamyl acetate. This amount of isoamyl acetate was doubled by using mutants isolated on 5,5,5, trifluoro-DL-leucine media. While the amount of isoamyl acetate produced is low in quantitative terms, it is one hundred times higher than the taste threshold for isoamyl acetate (1.6 ppm) and a concentrated product could be prepared by counter-current solvent extraction or other extraction techniques, such as freeze-trapping the off-gases of fermentation[4]. The ability of saké yeast strains to produce isoamyl acetate in increased quantities has been greatly enhanced by recombinant DNA technologies, such as gene dosing of the ATF1 gene[6] (encoding the AATase enzyme), also, by the disruption of the isoamyl acetate hydrolysing esterase gene[5] (EST2) and by various genetic manipulations of the leucine biosynthetic and catabolic pathways[8]. It is believed that a combination of these genetic manipulations, allied to the adjustments in fermentation conditions outlined in this paper, could ultimately lead to a situation where brewing strains of *S. cerevisiae* could be used to produce commercially viable levels of natural isoamyl acetate.

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