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Enhanced production of isoamyl acetate from beet molasses with addition of fusel oil by *Williopsis saturnus* var. *saturnus*

Murat Yilmaztekin, Huseyin Erten, Turgut Cabaroglu *

Cukurova University, Faculty of Agriculture, Department of Food Engineering, 01330 Adana, Turkey

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ABSTRACT

Fusel oil which contains high level of amyl alcohols (approximately 45–55%) is a by-product obtained from the distillation of alcohol made by fermentation of molasses. *Williopsis saturnus* is a yeast which is able to convert isoamyl alcohol into isoamyl acetate. The aim of this study was to increase the formation of isoamyl acetate by the addition of fusel oil at the ratios of 1%, 2% and 3% (v/v) to molasses based fermentation medium using *W. saturnus*. It was found out that bioconversion of added fusel oil into isoamyl acetate was possible and an addition of 1% fusel oil led to an increase in isoamyl acetate concentration from 118 to 354 mg/L.

1. Introduction

Flavours and fragrances find wide application in the food, feed, cosmetic, chemical and pharmaceutical industries. Traditionally, flavours and fragrances are obtained by isolation from natural sources (plants, animals), chemical synthesis, or fermentation (Franssen, Alessandrini, & Terraneo, 2005). However, a rapid switch towards the production and use of flavour compounds from microbiological origin is observed. The reasons are, among others, the facts that chemical synthesis results often in an environmentally unfriendly production process and in undesirable racemic mixtures. Furthermore, consumers have developed a fearful attitude towards chemical or synthetic (even nature-identical) compounds, especially when related to food and products used at home (Vandamme & Soetaert, 2002). Recent US and European legislations have meant that 'natural' flavour substances can only be prepared either by physical processes (extraction from natural sources) or by enzymatic or microbial processes, which involve precursors isolated from nature. The 'natural' routes for flavour production are the bioconversions of natural precursors using biocatalysis, de novo synthesis (fermentation) and isolation from plants and animals (Serra, Fuganti, & Brenna, 2005).

Esters of short-chain fatty acids are important flavour and fragrance compounds widely used in the food and beverage industries. Isoamyl acetate, the character impact compound of banana flavour and pear drops, is widely used by food industries with the production of 74 tonnes per annum. The amount of natural flavour esters isolated from plant materials is often in short supply. Enzymatic synthesis can be attractive, however, they are very selective and are carried out at moderate temperatures (Krishna, Divakar, Prapulla, & Karanth, 2001). A wide range of microorganisms are known to produce flavour compounds from simple nutrients via de novo synthesis (e.g. sugars and alcohols) (Janssens, de Pooter, Schamp, & Vandamme, 1992). Several yeasts are capable of producing large amounts of fruity ester flavours. The genus *Williopsis* synthesizes important levels of volatile esters in a YPD medium (yeast extract, peptone and glucose), e.g. isoamyl acetate, at a concentration of 58–73 mg/L (Iwase, Morikawa, Fukuda, Sasaki, & Yoshitake, 1995).

In countries where ethanol is produced in large scale by-product utilization has become an important issue in making ethanol production less polluting and more profitable. Among these by-products, fusel oil obtained from distillation of fermented agricultural products is currently used as raw material for the production of the amyl and butyl alcohols. However, a great portion of fusel oil has generally been discarded (Kucuk & Ceylan, 1998). Main components of fusel oil are ethanol (13%), butanol (15%), and amyl alcohols (51%). Remaining constituents are small proportions of other secondary alcohols and water (15%). The yeast *Williopsis saturnus* can convert higher alcohols into the corresponding acetates (Janssens, 1991), for example the concentration of isoamyl acetate can be increased by addition of fusel oil as a cheap source of higher alcohols to fermentation medium (Vandamme & Soetaert, 2002). If levels produced biotechnologically are high enough to make it

* Corresponding author. Tel.: +90 322 338 69 97; fax: +90 322 338 66 14.
E-mail address: tcabar@cu.edu.tr (T. Cabaroglu).

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commercially available, it would be an alternative way to obtain natural isoamyl acetate from cheap agricultural residues.

The aim in this work was to increase the production of isoamyl acetate by the addition of fusel oil to molasses based fermentation medium using *W. saturnus* var. *saturnus* HUT 7087. To the best of our knowledge, there is no report on improved production of isoamyl acetate in a molasses based medium with the addition of fusel oil by *W. saturnus* var. *saturnus*.

2. Materials and methods

2.1. Materials

Sugar beet molasses was obtained from Ozmaya Co. (Adana, Turkey) and fusel oil from Malatya Sugar Factory (Malatya, Turkey). Ethyl acetate, isoamyl acetate, 2-methyl-1-butanol (active amyl alcohol), 3-methyl-1-butanol (isoamyl alcohol), ethanol, H_2SO_4, NaOH and Malt Extract Agar were purchased from Merck (Darmstadt, Germany).

2.2. Microorganism and medium

Williopsis saturnus var. *saturnus* HUT 7087 was obtained from HUT Culture Collection (Higashi-Hiroshima, Japan). Yeast was maintained on Malt Extract Agar slants and re-cultured monthly. Molasses was diluted with deionized water to 10° Brix and was used as preculture and fermentation medium.

2.3. Pretreatment of molasses

Molasses solution was adjusted to pH 3.0 with 1 N H_2SO_4 to separate heavy metal ions that could affect yeast growth. The liquid was allowed to stand for 24 h and then centrifuged at 2800*g* for 15 min (Roukas, 1998). The pH of the supernatant was adjusted to 5.0 with NaOH and the solution sterilized at 121 °C for 15 min before fermentation experiments.

2.4. Fermentations

A preculture was carried out in 250 mL shake flasks containing 50 mL sterilized molasses solution at 25 °C at 100 rpm for 48 h. The yeast was harvested by centrifugation at 2000*g* for 15 min and used to inoculate 500 mL shake flasks containing 100 mL sterilized treated molasses at a rate of 1×10^7 cells/mL. Cultures were continuously shaken at 25 ± 1 °C at 100 rpm on a rotary shaker. Fusel oil sterilized with 0.2 μm filter was added to flasks at ratios 1%, 2% and 3% (v/v) at the end of exponential growth phase which fell to 72 h of fermentation. Experiments were carried out in duplicate.

Table 1 shows the composition of fusel oil used. The fermentation was monitored as the fall in density which is measured by a Mettler Toledo digital density meter. Yeast count and percentage viability were determined by a modified methylene blue staining method on a Thoma chamber (Erten, 1997). Samples for esters and higher alcohols analysis were centrifuged and supernatants were kept at −20 °C until analyzed.

Table 1
Composition of fusel oil

Component	% (v/v)
Ethanol	28.7
1-Propanol	1.7
2-Methyl-1-propanol	5.3
1-Butanol	0.1
2-Methyl-1-butanol	22.9
3-Methyl-1-butanol	23.7
Water	17.6

2.5. Analysis of esters and higher alcohols

The determination of products (isoamyl acetate, ethyl acetate, 2-methyl-1-butanol (active amyl alcohol) and 3-methyl-1-butanol (isoamyl alcohol)) was carried out by direct injection of 1 μL samples into a gas chromatograph, Shimadzu GC-14B model (Kyoto, Japan) equipped with a split injector and a flame ionization detector as described by Erten and Campbell (2001). Esters and higher alcohols were separated using a Chrompack CP-WAX-57CB capillary column (0.25 mm i.d. ×60 m × 0.4 μm film thickness) (Middelburg, The Netherlands). GC settings were as follows: injection temperature: 160 °C; oven temperature: 4 min at 40 °C, then increased by 1.8 °C per minute up to 94 °C and 40 °C per minute up to 180 °C and finally 4 min at 180 °C; detector temperature: 180 °C; carrier gas: He (1.3 mL/min); split rate: 1:50. The quantification was performed by using internal standard (3-pentanol) method. Standard solutions containing all compounds were prepared and analyzed in duplicate. Relative response factors (RRF) were calculated from peak areas for each compound using the following equation

$$\mathrm{RRF}: [(A_{is})/(A_c)] \times [(C_{is})/(C_c)] \quad (1)$$

where A_{is} is the area of internal standard; A_c, area of compound; C_{is}, concentration of internal standard and C_c is the concentration of compound. A linear plot was obtained with a correlation coefficient of at least 0.999 for all compounds. The results given represent the means for two determinations each, with their standard deviations.

2.6. Statistical analysis

The mean values and standard deviation were calculated from data obtained from two independent cultivations. One-way analysis of variance was used to test the effect of fusel oil concentration on isoamyl acetate production and these data were then compared by Duncan's multiple range method (SPSS, 1999).

3. Results and discussion

3.1. Yeast growth and density during fermentation

Fig. 1 shows the yeast growth over a period of 240 h. The exponential growth phase lasted over 72 h. The maximum number of cells reached after the addition of fusel oil was approximately 2×10^8 cells/mL with 1% fusel oil added experiment. Yeasts showed similar growth patterns in the experiments with 1% and 2% fusel oil added. The rate of growth was at the lowest level with 3% fusel addition. Increased amount of added fusel oil affected yeast growth and yeast viability (Fig. 2). Viability expressed as the percentage of viable cells in the total cell number of cultures used to determine the effect of fusel oil on yeast growth. Percentage viability of yeast cells remained above 97% in the control, and 95%, 90% and 73% with 1%, 2% and 3% fusel oil added experiments, respectively. Yeast viability decreased by increasing amounts of fusel oil added.

Fermentation was monitored with decrease in density as an indication of the utilization of fermentable sugars. There was no more decrease in density after 216 h of fermentation in all experiments. The density was around 1.015 g/cm^3 with all experiments at the end of fermentation. The effect of fusel oil supplementation on density was unclear throughout fermentation, because of dilutions by the addition of fusel oil.

3.2. The effect of fusel oil addition on isoamyl acetate production

Higher alcohols are formed by yeast via catabolic route (Erlich pathway) in the presence of amino acids and from the anabolic

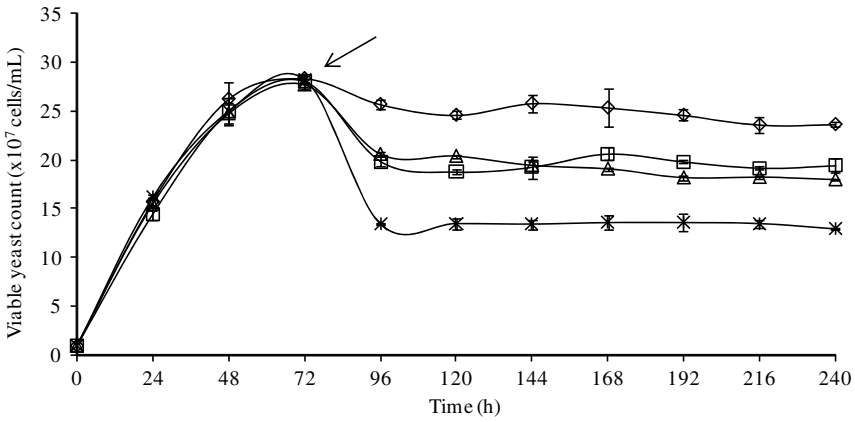

Fig. 1. Growth of yeast during fermentation of molasses based medium supplemented with fusel oil. Control medium without fusel oil (◇). Mediums supplemented with fusel oil; 1% (□), 2% (Δ) and 3% (∗). The arrow indicates the time of fusel oil addition. The bars indicate standard deviations for two independent cultivations.

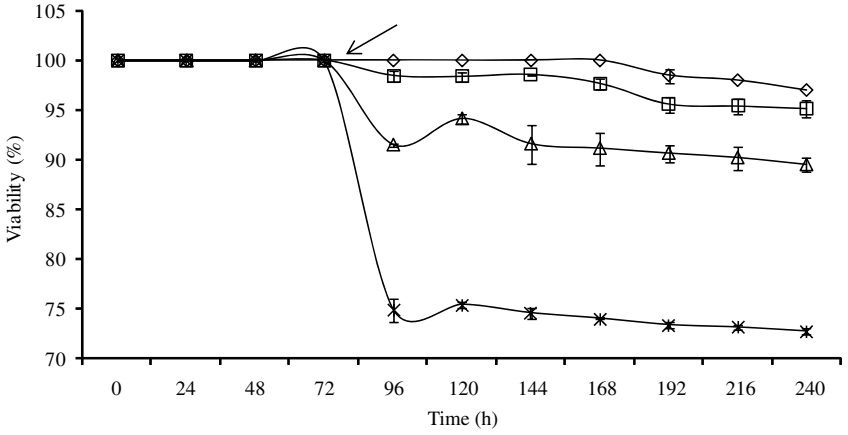

Fig. 2. Changes in the viability of yeast cells during fermentation of molasses based medium supplemented with fusel oil. Control medium without fusel oil (◇). Mediums supplemented with fusel oil; 1% (□), 2% (Δ) and 3% (∗). The arrow indicates the time of fusel oil addition. The bars indicate standard deviations for two independent cultivations.

route from sugars via biosynthesis. Esters are mainly produced via biochemical pathways which involve an intracellular enzymatic reaction between alcohols and acetyl-CoA and which catalyzed by alcohol acetyltransferase (Erten, 1997; Hammond & Pye, 1996; Peddie, 1990). For example, 2-methyl-1-butanol and 3-methyl-1-butanol are esterified to form 2-methyl-1-butylacetate and 3-methyl-1-butylacetate, respectively, and the sum of 2-methyl-1-butylacetate and 3-methyl-1-butylacetate corresponds to isoamyl acetate (Janssens, 1991). At the beginning of fermentation, ester synthesis is very slow due to the high metabolic demand for acetyl-CoA for yeast growth. After the active growth phase an equilibrium is established between acetyl-CoA consumption for growth and for ester production (Peddie, 1990). Thus, the fusel oil was added at the beginning of stationary phase, after 72 h of fermentation to establish a sufficient yeast population. The process applied in this study was a biomass formation phase, followed by bioconversion of added fusel oil during the stationary growth phase which was described previously (Janssens, 1991). Fig. 3 shows the utilization of 2-methyl-1-butanol and 3-methyl-1-butanol added in the form of fusel oil during fermentation. The amounts of 2-methyl-1-butanol and 3-methyl-1-butanol at time of addition were 948 and 989 mg/L for 1% addition, 1923 and 1918 mg/L for 2% addition and 2450 and 2650 mg/L for 3% addition, respectively. Amyl alcohols only in 1% fusel oil added experiment were much converted to isoamyl acetate. A certain selectivity was noticed in the ester formation, thus, 3-methyl-1-butanol was esterified into 3-methyl-1-butylacetate much faster and with higher yields (Janssens, 1991; Janssens, de Pooter, de Mey, Vandamme, & Schamp, 1989; Vandamme & Soetaert, 2002). According to Hammond and Pye (1996) the proportion of alcohol converted to ester varied markedly with the chain length and structure. They also stated that the K_m values of isoamyl alcohol isomers for alcohol acetyltransferase are very similar but the V_{max} for 2-methyl-1-butanol is only about 60% that for 3-methyl-1-butanol and this explains different degrees of isoamyl acetate formation from the two substrates (Hammond & Pye, 1996). The bioconversion yields of 2-methyl-1-butanol and 3-methyl-1-butanol were significantly influenced by fusel oil concentration. The higher bioconversion yield was obtained by 1% fusel oil added experiment and it was found 63.5% and 81% for 2-methyl-1-butanol and 3-methyl-1-butanol, respectively, in this study with duplicate experiments (Table 2). Janssens et al. (1989) reported bioconversion yields as 63% for 2-methyl-1-butanol and 90% for 3-methyl-1-butanol.

Fig. 4 shows the effect of fusel oil addition on the isoamyl acetate production. Addition of fusel oil lead to a 3-fold increase in isoamyl acetate production. On the other hand, there is a direct correlation between amyl alcohol levels and isoamyl acetate synthesis until a certain level of added fusel oil concentration. In present study, the maximum amount of isoamyl acetate was observed by 1% fusel oil added experiment at 144 h of fermentation at 354 mg/L (Fig. 4). There was a decrease in isoamyl acetate production with 2 and 3% fusel oil added experiments compared to con-

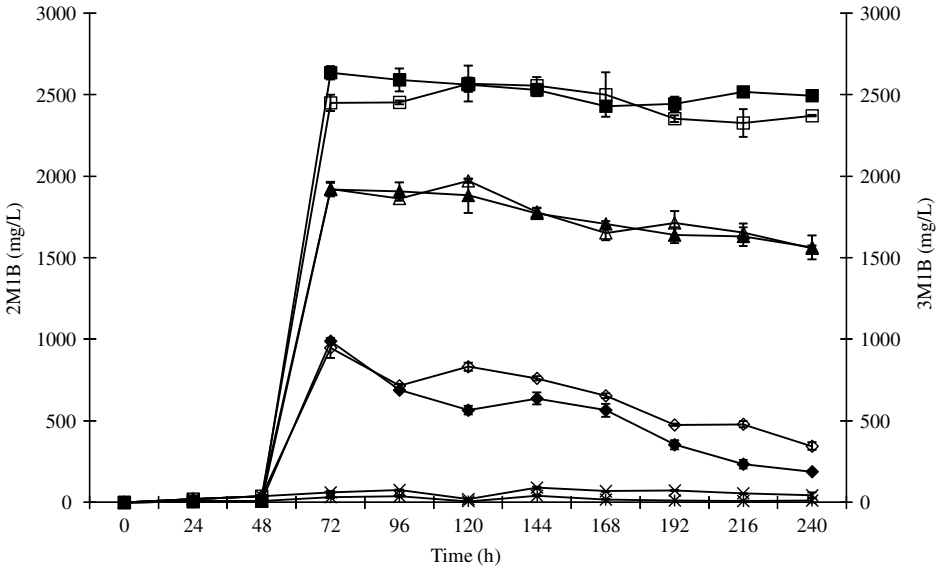

Fig. 3. Changes in 2- and 3-methyl-1-butanol concentration throughout fermentation. Open symbols: 2-methyl-1-butanol (2M1B) concentrations, Control (×), 1% (◇), 2% (Δ) and 3% (□). Filled symbols: 3-methyl-1-butanol (3M1B) concentrations, Control (∗), 1% (♦), 2% (▲) and 3% (■). The bars indicate standard deviations for two independent cultivations.

Table 2
Effect of fusel oil concentration on bioconversion yields of amyl alcohols

Fusel oil concentration (%)	2-Methyl-1-butanol (%)	3-Methyl-1-butanol (%)
1	$63.5^{a} \pm 4.9$	$80.1^{a} \pm 0.8$
2	$18.9^{b} \pm 1.0$	$18.5^{b} \pm 2.0$
3	$3.2^{c} \pm 1.8$	$5.4^{c} \pm 1.3$

The bioconversion yields were calculated by evaluation of amyl alcohol consumption at the end of the cultivations. The values were the average of duplicate cultivations with their standard deviations. Different letters in the same column indicate significant difference ($P \leqslant 0.01$).

Table 3
Effect of fusel oil concentration on isoamyl acetate production

Fusel oil concentration (%)	Isoamyl acetate (mg/L)
Control	$87.3^{a} \pm 4.8$
1	$354.1^{b} \pm 1.2$
2	$71.2^{c} \pm 4.3$
3	$10.5^{d} \pm 0.4$

Values were the average concentrations of 144 h of duplicate cultivations with their standard deviations. Different letters in the same column indicate significant difference ($P \leqslant 0.01$).

trol. The results showed that isoamyl acetate production was significantly influenced by fusel oil concentration (Table 3). The regulation of production of isoamyl acetate by the level of isoamyl alcohol was clearly demonstrated by the increased formation of this ester when wort supplemented with 3-methyl-1-butanol. Alcohol acetyltransferase in yeasts became saturated when more than 400 mg/L of 3-methyl-1-butanol (Calderbank & Hammond, 1994) and 1000 mg/L of fusel oil (Quilter, Hurley, Lynch, & Murphy, 2003) was added. No further increase in isoamyl acetate levels was observed with further additions.

4. Conclusions

Most of isoamyl acetate in industry is produced by chemical synthesis. However, there is a growing demand for naturally obtained flavour compounds. Employment of enzymes and whole cells as biocatalysts to produce natural flavour compounds is a production process that is frequently utilized. This experiment was the first attempt for the production of natural isoamyl acetate from beet molasses with addition of fusel oil by *W. saturnus* var. *saturnus*. With *W. saturnus* var. *saturnus* 354 mg/L isoamyl acetate was

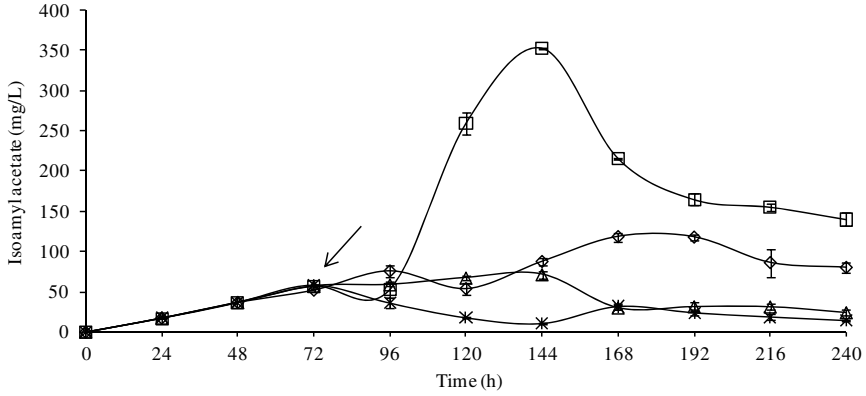

Fig. 4. Effect of fusel oil addition on isoamyl acetate production. Control medium without fusel oil (◇). Mediums supplemented with fusel oil; 1% (□), 2% (Δ) and 3% (∗). The arrow indicates the time of fusel oil addition. The bars indicate standard deviations for two independent cultivations.

obtained in this study. These encouraging result could be a candidate for large scale production. However, more research in strain development and process optimization needs to be performed to increase isoamyl acetate concentration.

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