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STUDY OF INCREASING THE PRODUCTION OF VOLATILE FLAVOR COMPOUNDS BY THE YEAST *Kluyveromyces marxianus* THROUGH OPTIMIZATION OF CARBON AND NITROGEN SOURCES

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ABSTRACT

The regulation of growth and the production of flavor compounds by *Kluyveromyces marxianus* were accomplished according to the nutritional requirements (yeast extract, ammonium sulphate and glucose concentrations) by using Response Surface Methodology experiments. Results proved that increasing both initial yeast extract (YE) and glucose concentrations in the fermentation medium favored both the growth and production of fusel alcohols. The major fusel alcohol (isoamyl alcohol) and the acetate ester compound (ethyl acetate) produced in all flasks were determined in the concentration range 1299-3996 µg L^{-1} and 1558 to 3122 µg L^{-1}, respectively. In a scale-up attempt, productions were accomplished in a 5 L stirred tank bioreactor. The highest productivity values for major volatile flavor compounds, i.e., ethyl acetate (fruity), isoamyl alcohol (banana), 2-phenylethyl acetate (floral) were obtained during the exponential growth of the yeast in a 5 L stirred tank bioreactor. Additionally, the descriptive sensory terms "sourdough", "flower" and "sweet aromatic" were characteristics for volatile compounds produced by *K. marxianus*. This work also demonstrated high product losses due to the stripping effect when the production scaled up from flasks to bioreactor.

Keywords: *Kluyveromyces marxianus*, Natural flavor compounds, Optimization, Bioreactor, Sensory evaluation

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Introduction

Due to an increasing preference of the consumers for natural food additives and other compounds of biological origin, there has been increasing trend towards the production of flavor compounds by biotechnological process (Janssens *et al*. 1992; Medeiros *et al*. 2000; Mantzouridou *et al.* 2013; Berger, 2015). Although several bacteria, yeasts and fungi have been reported for the production of flavor compounds, a few species of yeasts and fungi have generally been preferred, and only a few of these find industrial application due to their GRAS (generally recognized as safe) status. Among the different producers, food grade yeast *Kluyveromyces marxianus* has been pointed out as a promising organism for the production of natural flavor compounds such as fruit esters, carboxylic acids, ketones, furans, alcohols, monoterpene alcohols, and isoamyl acetate in liquid fermentation (Medeiros *et al.* 2000; Gethins *et al.* 2015; Morrissey *et al.* 2015). Because of its ability to grow on a broad variety of substrates, at higher temperatures and rapid growth, many efforts have been made with this yeast like production of enzymes (Panesar, 2008), single cell protein (Aggelopoulos *et al*. 2014), reduction of lactose content in food products (Manera *et al.*, 2008). With regard to the production of flavor compounds using *K. marxianus*, most progress has been made specifically with the production of 2-phenylethanol (2-PE) (Etschmann *et al.* 2003; Etschmann *et al.* 2004), ethyl acetate (Urit *et al.* 2003a; Urit *et al.* 2003b) and the production of total volatile compounds by using inexpensive waste-stream medium as the growth medium (Medeiros *et al.* 2000; Wilkowska *et al*, 2014; Guneser *et al.* 2015). However, industrial production of sufficient quantities of yeast as starter cultures implies characterization of yeast physiology and nutritional requirements. Therefore, it is important to assess what kind of flavor compounds are synthesized by *K. marxianus* when growing on defined and/or semidefined culture medium rather than growing on waste stream (Gethins *et al.* 2015). Medium composition especially carbon and nitrogen sources, is one of the critical factor for both growth and production flavor compounds by yeasts (Fabre *et al*. 1998; Gethins *et al.* 2015; Löser *et al.* 2015). Hence, to obtain high biomass and metabolite productivities, it is important to find appropriate types of nutrients and their optimum concentrations in growth medium of the yeast (Manera *et al.* 2008; Fonseca *et al.* 2013; Yilmaztekin *et al.* 2013). Additionally, reducing the cost of culture medium by optimizing its composition is the basic research for industrial applications (Etschmann *et al*. 2004; Manera *et al.* 2008). Etschmann *et al.* (2004) optimized the growth medium of *K. marxianus* CBS 600 for production of 2-PE. In a recent study, it was also revealed that nitrogen and carbon source had pronounced effects on production of volatile metabolites by four strains of *K. marxianus* (Gethins *et al.* 2015). It was concluded that, nitrogen source and carbon source had pronounced effects on production of volatile metabolites. However, there was no information about the specific nutritional requirements and their optimum concentrations in the medium for maximizing the growth and production of each volatile flavor compounds by *K. marxianus*.

Response surface methodology (RSM) is a collection of mathematical and statistical techniques useful for designing experiments, establishing models, and analyzing the effects of several independent factors. The main advantage of RSM is the reduced number of experimental trials needed to evaluate multiple factors. Also, study of the individual and interactive effects of these factors will be helpful in efforts to find the target value. Hence, RSM provides an effective tool for investigating the aspects affecting desired response if there are many factors and interactions in the experiment (Garrido-Vidal *et al*. 2003; Elibol, 2004). The aims of this study were two-fold: first, on the basis of optimization of the growth medium, the present author intended to better understand the effect of carbon (glucose) and different nitrogen substances (yeast extract and ammonium sulphate) and to find their optimum medium composition for increasing the growth and the production volatile flavor compounds by *Kluyveromyces marxianus* NRRL YB-6373; the second aim was to determine the changes in the production of flavor compounds during the yeast growth in 5 L bioreactor in the optimized culture medium.

Materials and Methods

Microorganism and Inoculum Preparation

The *Kluyveromyces marxianus* NRRL YB-6373 was obtained from ARS Culture Collection (NRRL collection, Peoria, Illinois, USA). The inoculant culture was grown in a medium containing 10 g of glucose, 6 g of yeast extract and basal salts comprising 1 g of KH_2PO_4, 1 g of $MgSO_4.7H_2O$ per liter of deionized water. For flask cultivations belonging to experimental design, fermentations were carried out in 250-mL Erlenmeyer flasks containing 100 mL fermentation medium. Erlenmeyer flasks were closed with caps. After sterilization, each flask was inolucated at a concentration of 10^6 cfu mL^{-1} into 100 mL medium and incubated at 120 rpm in an orbital shaker at 30 °C. Fermentation was ceased after 24 h (Guneser *et al.* 2015).

Determination of Cell Dry Weights (g/L)

Cell dry weights (g L^{-1}) were obtained by separating the yeasts from a defined volume of suspension via centrifugation (at 7000 rpm for 10 min), washing the pellet twice with

distilled water, drying at 103 °C until constant weight and cooled to room temperature in a desiccator before weighing (Löser *et al.* 2015).

Extraction, Identification and Quantification of F&F Compounds

Volatile compounds in fermented liquid were extracted by solid-phase microextraction (SPME) for gas chromatography-mass spectrometry (GC–MS) analysis (Pawliszyn, 2012; Gethins *et al.* 2015; Guneser *et al.* 2015). Two mLs of the sample was added in 40 mL amber colored screw top vial with hole cap PTFE/silicon septa (Supelco, Bellafonte, USA), and 1 g of NaCl was added to the vial. The vial was kept at 40 °C in a water bath for 15 min to equilibrate the volatiles in headspace. Then, SPME (2 cm to 50/30 µm DVB/Carboxen/PDMS, Supelco, Bellafonte) needle was inserted into the vial and was exposed in the headspace for 15 min at 40 °C in a water bath. The sample was then immediately injected into GC-MS for identification and quantification of volatile compounds.

Volatile compounds were identified by GC–MS. Nonpolar HP5 MS column (30-m 9 0.25-mm i.d. 9 0.25-lm film thickness, J&W Scientific, Folsom, CA) was used for separation of flavour compounds. GC–MS system consisted of an HP 6890 GC and 7895C mass selective detector (Agilent Technologies, Wilmington, DE, USA). GC oven temperature was programmed from 40 to 230°C at a rate of 10°C min^{-1} with initial and final hold times of 3 and 15 min, respectively. Helium was used as a carrier gas at a flow of 1.5 mL min^{-1}. The MSD conditions were as follows: capillary direct interface temperature, 280°C; ionization energy, 70 eV; mass range 35–350 amu; scan rate, 4.45 scans/s. Flavor compounds were identified based on comparison of the mass spectra of unknown compounds with those in the National Institute of Standards and Technology (NIST), Wiley Registry of Mass Spectral Data. Compound identification was performed on chromatograms containing a peak at identical retention times with greater than 85% similarity to NIST and Wiley mass spectrums. Flavor compounds were quantified based on relative abundances of the compounds by Eq. 1 (Guneser *et al.* 2015). 2-methyl-3-heptanone was used as internal standard (IS) for neutral-basic compounds at a concentration of 0.82 µg.

Mean relative abundance (µg L^{-1}) = concentration of IS x peak area of compound/peak area of the IS

(Eq. 1)

Residual Sugar

The substrate glucose was recognized and quantified by UHPLC (Thermo, USA) with a Phenomenex Rezex RHM Monosaccharide (H+) 300 mm × 7.8 mm ion exchange column, using a Shodex Refractive Index Detector. The column and detector temperatures were 65 °C and 45 °C, respectively, and the injection volume was 15 µL. A solution of H_2SO_4 (5 mM) was used as the mobile phase at a flow rate of 0.8 mL/min (Isleten-Hosoglu *et al.* 2012).

Batch Cultivation in Stirred Tank Bioreactor

Batch cultivation was also performed in a 5 L stirred tank bioreactor (STR) (Biostat A-plus, Sartorius, Melsungen, Germany) with 4 L working volume. The STR was equipped with two six blade disk impellers (diameter 53 mm). A dissolved oxygen probe (Hamilton Oxyferm FDA 225, Bonaduz, Switzerland) and a pH sensor (Hamilton Easyferm K8 200, Bonaduz, Switzerland) were installed on the top plate of the bioreactor. The initial pH was 5.6 and was not controlled throughout the experiments. Aeration rate and temperature were set as 0.5 vvm, 30 ±1°C, respectively with 120 rpm agitation rate. There was no intervention on process parameters during the production.

The exponential growth phase (EGP) was determined as the linear region on an ln (X) versus time plot, where X is the cell concentration in terms of cell dry weight (g L^{-1}). The maximum specific growth rate (μ_{max}) was determined as the slope of this linear region. The biomass yield on substrate ($Y_{x/s}$) was detrmined as the slope of the line on an X versus S plot, exclusively including points belonging to the EGP, where S is the substrat (glucose) concentration (Doran, 1999).

Sensorial Analysis

A roundtable discussion was conducted as defined in Guneser *et al.* (2015) to determine descriptive sensory properties and changes in aroma profiles of during the production in a bioreactor (at 9 h, 12 h, 24 h, 48 h, 72 h) versus control samples. Seven trained panelists were involved in sensory evaluation with no restriction on descriptive terms. Samples (10 mL) were put in small containers with cap and containers were kept in water bath at 40 °C for 15 min for ensuring the accumulation of volatiles in the headspace. Panelist's quantified the attributes using 15-point product-specific scale anchored to the left with 'not' and on the right with 'very' (Meilgaard *et al.* 1999).

Experimental Design and Data Analysis

Response surface methodology (RSM) designs such as Box–Behnken and Central Composite Design (CCD) model

probable curvature of the response function (Mandenius *et al*. 2008). Central composite design (CCD), a method of response surface design in Design Expert software (version 7.0.0, Stat-Ease Inc., Minneapolis, MN), was used to perform the experimental design for the optimization of carbon (glucose), nitrogen concentrations (yeast extract and ammonium sulphate) in the medium. A total of 20 runs were used to optimize the range and levels of the chosen variables. This cubic design is characterized by a set of points located at the midpoint of each edge of a multi dimensional cube and center point replicates (n = 6) whereas the 'missing corners' help the experimenter to avoid the combined factor extremes. This property prevents a potential loss of data in those cases (Box & Behnken, 1960). For predicting the optimum point, a second order polynomial function is fitted to correlate relationship between independent variables and response. For three factors, the corresponding equation is:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

Where Y represents the response variable, β_0 is model constant, β_1, β_2 and β_3 are linear coefficients, β_{12}, β_{13} and β_{23} are interaction effect coefficients, β_{11}, β_{22} and β_{33} are quadratic coefficients, and X_1, X_2 and X_3 are the coded levels of independent variables. The terms $X_1 X_2$ *and* X_i^2 (i=1,2 or 3) represent the interaction and quadratic terms, respectively.

One-way analysis of variance (ANOVA) was used to compare the differences in intensities of flavor compounds obtained by GC–MS analysis during the production in bioreactor. The ANOVA model used in this study is given in the equation $Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$, where Y_{ij} is the j^{th} observation value in i^{th} sample; μ is the general population mean; α_i is the effect of i^{th} sample; and ε_{ij} represents random error terms (Winer *et al*. 1991). Nonmetric Multidimensional Scaling (MDS) method was also applied in this study to indicate relationship between intensities of flavor compounds obtained by GC–MS analysis and descriptive sensory analysis (Mackay & O'Mahony, 2002). For all statistical analysis, SPSS (version 18.0; SPSS Institute Inc., Chicago, IL) was used.

Results and Discussion

Optimization of Carbon and Nitrogen Sources in Growth Medium of K. marxianus for the Growth

Experiments were carried out in order to study the effect of two different nitrogen sources (yeast extract and ammonium sulphate) and glucose on the growth and the production of flavor compounds by the yeast *K. marxianus*. Central-composite design for three nutrient sources along with their low, medium and high levels was applied. According to the implemented design, twenty combinations were performed for 24 h. In the experimental design model, yeast extract (6-20 g L^{-1}), ammonium sulphate (6- 20 g L^{-1}) and glucose (12-40 g L^{-1}) were taken as input variables (Table 1). Cell dry weight (g L^{-1}) and the amount of each flavor compounds (µg L^{-1}) were taken as responses of the system. All responses in different experimental conditions based on the experimental design matrix were estimated and results of each have also been included in Table 2. Model equations can be obtained for whole responses. For example, a second order polynomial model for the response cell dry weight (g L^{-1}) wherein the interaction terms have been fitted to the experimental data obtained from the CCD experiment can be stated in the form of the following equation:

$$Y = 2.39 + 0.46 * X_1 - 0.22 * X_2 + 0.84 * X_3 + 0.044 * X_1 * X_2 + 0.13 * X_1 * X_3 + 0.031 * X_2 * X_3 - 0.14 * X_1 * X_1 + 0.21 X_2 * X_2 - 0.15 X_3 * X_3 \quad \text{(Eq.2)}$$

where Y is the predicted response, i.e. the cell dry weight (g L^{-1}), and X_1, X_2, and X_3 are the coded values of the test variables, yeast extract, ammonium sulphate and glucose concentrations (g L^{-1}), respectively.

Table 1. Experimental range and levels of the independent variables (yeast extract, ammonium sulphate and glucose) central composite design plan in actual value and observed responses

Variables (g L^{-1})	Symbol	Actual levels of coded factors				
	Coded	-1.682	-1	0	1	+1.682
Yeast extract	X_1	1.23	6	13	20	24.77
Ammonium sulphate	X_2	1.23	6	13	20	24.77
Glucose	X_3	2.45	12	26	40	49.55

The statistical analysis of the model was accomplished in the form of analysis of variance (ANOVA). The model *F*-value of 48.46 indicated that the model was significant for the cell dry weight (p<0.01). According to the ANOVA test, there was not a significant interaction between nutrient sources on cell dry weight of *K. marxianus* (p<0.01). However, the effect of each nutrient source was significant, indivually. Increasing the initial yeast extract and glucose concentrations in the growth medium enhanced the growth of yeast. On the other hand, the cell dry weight significantly decreased by increasing concentrations of ammonium sulphate. This may due to the rapid consumption of ammonium which leads to an ionic imbalance and acidification in the

medium. The growth of yeast can be effected by such acidification in the medium (Löser *et al.* 2015). In the present study, availability of both organic and inorganic nitrogen sources in the growth medium showed the preference of yeast for the organic nitrogen source. This may due to the rich amino acid content, vitamins, salts, growth promoting substances of yeast extract than ammonium sulphate which could possibly lead to improved nitrogen utilization for anabolic processes (Tanyol *et al.* 2015). In a similar manner, supplementing the whey medium with yeast extract or yeast extract plus ammonium sulphate increased the biomass concentration of *K. marxianus* in comparison to the control and supplementing with only ammonium (Parrondo *et al.* 2009). A production of yeast biomass and the right balance between yeast growth and synthesis of total volatile flavor compounds are important factors for the economy of the total volatile compounds (Löser *et al.* 2015). This balance can be optimized by using such kind of statistical approaches applied in this study. In the present study, biomass yield for flasks varied from 0.4 up to 3.85 g L^{-1} for 24 h cultivation (Table 2) which was higher than the previous study performed with other type of yeast strain in medium supplemented with different inorganic nitrogen sources (Löser *et al.* 2015).

Optimization of Carbon and Nitrogen Sources in Growth Medium of K. marxianus for Production of Volatile Flavor Compounds

Under the different experimental conditions tested due to the experimental design matrix (Table 2), seven volatile flavor compounds were detected at levels above control conditions (noninoculated medium). The major compounds were belonging to the esters and alcohols. The volatile flavor compounds were ethanol, ethyl acetate (fruity), isoamyl alcohol (banana), isoamyl acetate (fruity), 2-phenylethyl alcohol (rose), 2-phenylethyl acetate (floral) and 2-phenylethyl propanate (floral) (Table 2). There were significant differences between flasks in respect to flavor compounds. When a general evaluation made, isoamyl alcohol was the major compound produced in all flasks, followed by ethyl acetate and then 2-phenylethyl acetate (2-PEA) (Table 2). Aggelopoulos *et al.* (2014) observed that isoamyl acetate, phenyl ethyl alcohol (2-PE) and ethyl alcohol could be produced from the mixture of food waste including orange pulp, molasses, potato pulp, whey, brewer's spent grains using *K. marxianus* IMB3 with solid state fermentation. Major aroma compounds produced by *K. marxianus* changed depending on the fermentation medium composition also stated by Medeiros *et al.* (2000), previously. When yeast extract and glucose were used as major nutrient compounds by different strains of *K. marxianus*, strain–to-strain variations were also observed for the overall profile of major aroma compounds (Gethins *et al.* 2015). When striving to produce particular flavor compounds, those characteristics can be considered for proper strain selection.

When we consider the effect of nutrient sources on production of flavor compounds individually, ethanol production was affected by both yeast extract and glucose concentrations in the medium. Increasing the initial YE concentration decreased the production level of ethanol by *K. marxianus*. On the contrary, increasing concentration of glucose enhanced the ethanol production in the fermentation medium (p<0.01). Nitrogen-limited growth can regulate yeast metabolism and provoke ethanol formation at aerobic conditions stated by Löser *et al.* (2015), previously. Similarly, YE and glucose had a significant effect on the production of fusel alcohols (2-PE and isoamyl alcohol) by *K. marxianus*. Unlike the ethanol production, increasing both the initial YE and glucose concentrations in the fermentation medium favored the production of fusel alcohols. Similarly, significant effect of glucose on aroma production was previously reported for *K. marxianus* strain in solid-state fermentation (Medeiros *et al.* 2000). The aroma production was strongly inhibited in the absence of glucose suggesting that glucose was the main factor controlling the production reported by Medeiros *et al.* (2000). On the other hand, it was concluded that the carbon source (glucose, fructose and lactose) did not have a major effect on the production of fusel alcohols (Gethins *et al.* 2015; Fabre *et al.* 1998). However, the stimulating effect of yeast extract was also observed by Gethins *et al.* (2015) on production of fusel alcohols; the levels of both alcohols were the highest when yeast extract as opposed to ammonium sulphate or peptone was the nitrogen source. 2-PE has been subject to detailed study in *K. marxianus* (Etschmann *et al.* 2003; 2004). It is widely used in the flavor industry because of its pleasant rose-like aroma and sweet taste and important fragrance in the perfume industry. In this study, concentration of 2-PE varied between 440 and 1645 µg L^{-1} in flasks (Table 2). The maximum concentrations of 2-PE in tomato and pepper pomace fermented by *K. marxianus* in shake flask conditions were reported as 201 µg kg^{-1} and 79 µg kg^{-1}, respectively (Guneser *et al.* 2015). Concentrations of 2-PE produced by different strains of *K. marxianus* in medium containing glucose and yeast extract changed also between 450 and 740 µg L^{-1} (Gethins *et al.* 2015). Additionally, the concentration of isoamyl alcohol which was the major volatile compound produced in all flasks changed between 1299 µg L^{-1} and 3996 µg L^{-1} in the present study. It is clear that the most important factor influ-

encing both fusel alcohol productions is the strain used, nature and concentration of substrates utilized, C/N ratio and the combination of factors (Fabre *et al.* 1998; Etschmann *et al.* 2004; Yilmaztekin *et al.* 2013). Since the fusel alcohols are the product of amino acid and sugar metabolism, conditions which promote yeast growth such as high level of nutrients especially glucose may increase the production of fusel alcohols (Vidal *et al.* 2013; Yilmaztekin *et al.* 2013). This may explain high production levels of fusel alcohols by *K. marxianus* in this study of which medium consisted of yeast extract, ammonium sulphate and glucose as nutrients.

According to the implemented design, the effects of yeast extract and glucose on acetate esters production were significant ($p<0.05$). Two different types of nitrogen sources affected differently on the production of esters by *K. marxianus*. Increasing concentration of yeast extract in the growth medium decreased the production of esters, whereas ammonium didn't have any effect on synthesis of those compounds. On the other hand, glucose was favoring the ester production. For acetate esters, a carbon or nitrogen content higher than that in standard fermentation medium is correlated with greater acetate ester production reported by Saerens *et al.* (2008). The rate of ester formation during fermentation depends on the availability of the two substrates (acetyl-CoA and amyl alcohols) and the activity of enzymes (alcohol acetyl transferase (AATase)) (Yilmaztekin *et al.* 2013). Loughlin *et al.* (2015) mentioned that repressing effect of ammonium on the production of 2-PEA and interpreted as ammonium may act to repress the activity of AATase. On the other hand, no significant effect of nitrogen source on production of isoamyl acetate or ethyl acetate has been described by same researchers. The major acetate ester compound produced in all flasks was ethyl acetate with the concentration range 1558 to 3122 µg L^{-1} in this study (Table 2). Those values are comparable or even higher than those previous reported in literature with *K. marxianus* strains, e.g. 432 µg kg^{-1} with immobilized cell on appleberry/chokeberry pomace (Wilkowska *et al.* 2014), and with four strains of *K. marxianus* grown in synthetic media, using variety of carbon and nitrogen sources (Gethins *et al.* 2015). The maximum concentrations of 2-PEA and isoamyl acetate (3276 µg L^{-1} and 761 µg L^{-1}, respectively) after 24 h cultivation were also higher than those reported in defined media enriched with different carbon and nitrogen sources (Gethins *et al.*, 2015) and on tomato and pepper pomace medium (Guneser *et al.* 2015) and also on appleberry/chokeberry pomace and appleberry/cranberry pomaces (Wilkowska *et al.* 2014). The ester profile is highly strain dependent and strongly affects the perception of the flavor of the food product produced by yeast, especially in breweries (Rojas *et al.* 2001; Saerens *et al.* 2008). Hence, the regulation of the ester levels by nutritional variables for different yeast strains is important from this point of view. In this study, the total volatile formation was considerably higher than for *K. marxianus* cells grown on agricultural waste mediums (Wilkowska *et al.* 2014; Guneser *et al.* 2015).

Batch Cultivation in 5 L Stirred Tank Bioreactor

The optimized conditions (yeast extract 6 g L^{-1}, ammonium sulphate 6 g L^{-1}, glucose 40 g L^{-1}) were applied to aerobic bench-top bioreactor (5 L) for maximizing the production of flavor compounds by *K. marxianus*. Main objective of this part was to follow the process of volatile flavor compounds accumulating during the growth of yeast in the bioreactor and to compare the production levels occurred in flasks and the bioreactor condition.

K. marxianus cells at first exhibited exponential growth (Figure 1), but the duration of exponential growth was short (the first 9 h of cultivation). Meanwhile, cells grew with a specific growth rate (µ) of 0.39 h^{-1} and a growth yield ($Y_{x/s}$) of 0.33. After this period, the biomass concentration increased linearly with time which was most probably as a result of an undefined nutritional limitation. The growth behavior of the yeast on semi-defined medium was compatible with the findings of several studies conducted with *K. marxianus* and other yeast strains (Duarte *et al.* 2008; Guneser *et al.* 2015; Löser *et al.* 2015). The glucose was entirely depleted and cells also entered the stationary phase after the fermentation time of 24 h. The biomass productivity was 0.24 g L^{-1} h^{-1} which was 2 fold higher than those obtained with shake flasks (0.12 g L^{-1} h^{-1}) with the same medium composition. The growth on a glucose ($Y_{x/s}$) seemed to be slower than other *K. marxianus* strain grown on glucose, whereas this value was almost similar for growth on the remaining sugars (Fonseca *et al.* 2013). This is probably a consequence of the difference between the initial glucose levels of studies. This might decrease the rate with which the substrate is metabolized. Variations of medium pH with time were also depicted in Figure 1. The medium pH decreased with time within the first 12 h and reached a steady level around pH 3.8. Generally, the initial pH of around 5.0-6.0 was considered as the most suitable one for the growth and the production of volatile metabolite by the yeast (Kargi & Ozmihci, 2006).

Ethanol production attained the peak value of 741.5 µg L^{-1} during the fermentation time between 6 and 9 h, i.e., the exponential phase of the process. The increase in glucose consumption by the cells during this period may have been responsible for the increase in production of ethanol which was also the same for growth on lactose (Zafar & Owais,

2006). Fermentation time had significant effect on the concentration of volatile compounds which were produced in bioreactor ($p \leq 0.01$). The exponential growth of cells was accompanied by more rapid accumulation of ethyl acetate, fusel alcohols (isoamylalcohol and 2-PE) and 2-PEA than other volatile compounds in the fermentation broth (Table 3). Concentrations of all volatile compounds reached their maximum levels at 9 h fermentation time and remained almost in the same range with slight increases and decreases for some compounds at 12 h cultivation in bioreactor. After 12 h fermentation, concentrations of all volatiles except 2-PE and isoamyl acetate decreased rapidly (Table 3). There was no significant difference in the level of isoamyl acetate and a slight decrease in the concentration of 2-PEA until the end of the fermentation. Significant decreases in the concentrations of all volatiles were also observed before through the fermentation (Medeiros *et al.* 2000; Guneser *et al.* 2015). Decreases in the concentrations of volatile compounds during the production in a bioreactor can be ascribed to the loosing them from liquid culture medium via exhaust gas in aerated cultivation system which is known as stripping effect (Urit *et al.* 2013a; 2013b; Guneser *et al.* 2015).

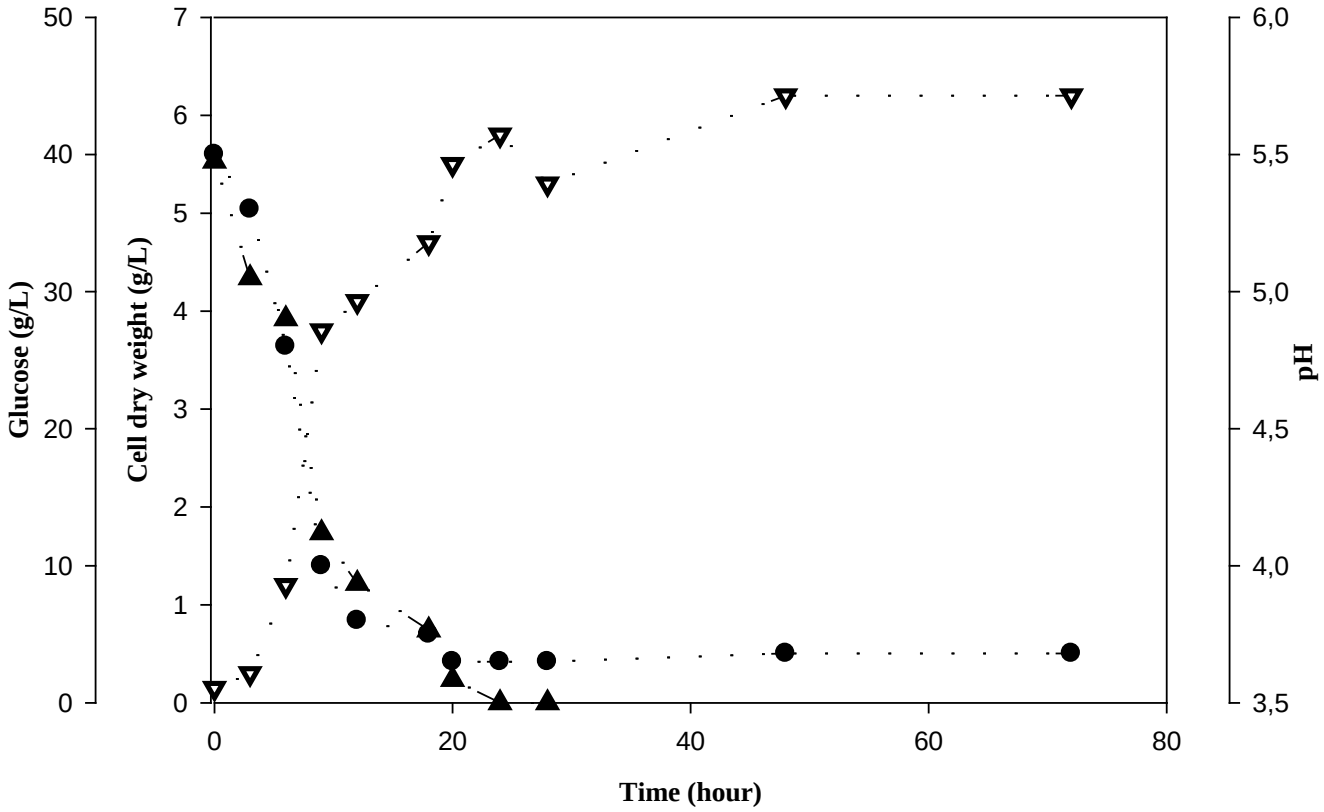

Figure 1. Growth, glucose consumption and pH changes of *K. marxianus* grown in a 5 L stirred tank bioreactor. Cell dry weight (g L^{-1}, Δ), glucose consumption (g L^{-1}, ▲), pH (●).

Table 2. Central composite design plan in actual value and observed responses (cell dry weight, g L^{-1}; flavor compounds, µg L^{-1})

Runs	X_1	X_2	X_3	Cell dry weight (g L^{-1})	Etha-nol	Ethyl acetate	Isoamyl Alcohol	Isoamyl acetate	Phenylethyl alcohol	Phenylethyl acetate	Phenylethyl propanate
1	20	6	12	2.2	530	1900	3248	463	1080	903	121
2	13	13	49.5	3.3	1077	2173	3497	624	1272	1728	220
3	20	20	12	1.6	468	2185	2869	300	1008	1126	151
4	13	24.8	26	2.5	836	2277	3604	562	1270	1408	144
5	13	13	26	2.45	741	2157	2965	481	805	1080	137
6	13	13	26	2.65	844	2222	3410	615	1254	1539	204
7	24.8	13	26	2.70	638	2006	2813	425	1552	1489	167
8	6	20	12	1.05	1061	2233	2506	455	841	1346	119
9	13	13	26	2.3	823	2158	3458	570	1283	1383	164
10	6	6	40	2.95	1224	3122	3692	761	1645	3276	245
11	6	6	12	1.45	990	2298	2701	558	1143	1471	139
12	13	13	26	2.3	618	1784	2668	475	1225	1355	128
13	1.2	13	26	1.1	1527	2453	1617	733	771	2346	80
14	20	20	40	3.75	1009	2166	4088	601	1562	2151	166
15	13	13	26	2.2	750	2160	3000	570	1220	1300	140
16	20	6	40	3.85	989	2199	3996	640	1555	2211	202
17	6	20	40	2.3	1156	2001	2409	487	936	1879	92
18	13	13	2.5	0.4	105	1558	1299	311	440	632	45
19	13	1.2	26	3.25	654	2025	3200	504	1469	1483	168
20	13	13	26	2.5	750	2000	3000	570	1220	1103	140

Table 3. Flavor compounds produced by *K. marxianus* in optimized medium during bioreactor fermentation

Aroma compound	Concentration of aroma compound (µg L^{-1}) (Mean±SE)							
	Fermentation time (h)							
	6	9	12	18	24	48	72	Pre*
Ethanol	214.5±9.5^{D}	741.5±21.5^{A}	740.0±8.0^{A}	591.0±9.0^{B}	577.0±11.0^{B}	325.5±5.5^{C}	117.0±3.0^{E}	991.4
Ethyl acetate	1607.5±19.5^{A}	946.0±14.0^{B}	743.0±22.0^{C}	371.0±7.0^{D}	257.5±10.5^{E}	143.5±4.5^{F}	31.0±6.0^{G}	2864.5
Isoamyl alcohol	1164±15.0^{E}	2568±14.5^{B}	2726.0±14.5^{A}	1871.0±12.0^{C}	1701.0±31.0^{D}	893.5±6.5^{F}	421.5±13.5^{G}	3202.5
Isoamyl acetate	121.5±3.5^{B}	215.5±8.5^{A}	196.0±16.0^{A}	177.5±2.5.0^{A}	175.0±3.0^{A}	202.5±5.5^{A}	110.5±7.5^{B}	716.1
Phenylethyl acetate	639.0±2.0^{E}	1149.0±4.0^{A}	1089.0±7.5^{B}	862.0±3.0^{C}	731.5±3.5^{D}	400.5±4.5^{F}	383.5±1.5^{F}	2901.1
Phenylethyl alcohol	350.5±17.5^{E}	1847.0±22.5^{A}	1808.0±8.0^{A}	1463.5±17.5^{C}	1625.5±9.5^{B}	1433.0±6.0^{CD}	1379.0±3.0^{D}	1359.3
Phenylethyl propanate	12.5±2.5^{D}	109.0±2.0^{A}	102.5±5.5^{AB}	98.5±1.5^{AB}	90±3.0^{B}	64.5±3.5^{C}	69.5±2.5^{C}	228.6

SE standard error

$^{A-G}$ Means followed by different uppercase letters represent significant differences for fermentation time in each aroma compound ($P \leq 0.01$).

*Results predicted for the same medium composition according to statistical design for 24 h cultivation.

Here, it was important to state oxygen was rapidly consumed by the cells after 3 h (data not shown) and there was only mechanical agitation (120 rpm) during the production. Previous findings have related the increase in the ethyl ester production (ethyl hexanoate, octanoate and decanoate) with the limited oxygen supply (Mantzouridou & Paraskevopoulou, 2013). When the culture was shifted from aerobic to oxygen-limited cultivation, the specific production rate of ethyl acetate increased more than 10-fold stated also by Passoth *et al.* (2006). Contrary to the mentioned results, Rojas *et al.* (2001) demonstrated that low levels of acetate esters were found when non-*Saccharomyces* yeast strains were grown under minimally aerobic conditions. The maximum concentration of ethyl acetate was reached at 6 h of cultivation with 1607.5 µg L^{-1} (Table 3), was 2 to 8 times higher compared to the values obtained before with longer fermentation periods (Medeiros *et al.* 2000; Wilkowska *et al.* 2014; Loughlin *et al.* 2015). Ethyl acetate is derived from carbon

metabolism (Morrissey *et al.* 2015). As might be expected, the stimulating effect of glucose on the production of ethyl acetate was also observed by this study during the exponential phase of cells. In the following three hours (until 9 h), although the level of ethanol known as a substrate for ethylacetate synthesis increased 4 times, the level of ethyl acetate rapidly dropped to 913 µg L^{-1} and continued to its decrease through the fermentation. This is probably a consequence of high volatility of ethylacetate and hence inevitably discharged from the aerated system (Urit *et al.* 2013a; 2013b).

The isoamyl alcohol was the major compound produced in the bioreactor which was also the same in flask cultures. Then, 2-PE and 2-PEA were following volatile compounds with high concentrations. The production of fusel alcohols were significantly enhanced between 6 - 9 h cultivation and then continued by a slight increase until 12 h cultivation (1809 µg/L). The production of fusel alcohols is known as growth-associated but the process is subject to product inhibition by the higher alcohols (Etschmann *et al.* 2003; Yilmaztekin *et al.* 2013). After 12 h cultivation, the concentration of 2-PE decreased slightly until end of the fermentation. According to CCD, as shown in Table 3, predicted 2-PE amounts in the fermentation broth was 1360 µg L^{-1} after 24 h cultivation. However, the productivity for 2-PE was higher than this value in bioreactor. Although, the production of acetate esters is not only depend on the concentration of fusel alcohols in the medium, it was surprising to have very low levels of isoamyl acetate and phenylethyl acetate in bioreactor compared to expected values. This situation was also similar for *D. hansenii* grown on pepper pomace (Guneser *et al.* 2015). Acetate esters are recognized as important flavor compounds in fermented beverages. The characteristic fruity flavors of wine and other grape-derived alcoholic beverages are primarily due to a mixture of those esters. Yeast strain and fermentation conditions have significant effects on levels of higher alcohols and acetate esters (Rojas *et al.* 2001). Under semiaerobic conditions, concentrations of acetates peaked and then decreased, probably as a result of their hydrolysis under the action of cellular esterases, the activity of which increases at the end of fermentation (Yilmaztekin *et al.* 2013). Confirming this research, the concentrations of all acetate esters maximized at the end of exponential growth under minimally aerobic conditions and then decreased through the fermentation in this study.

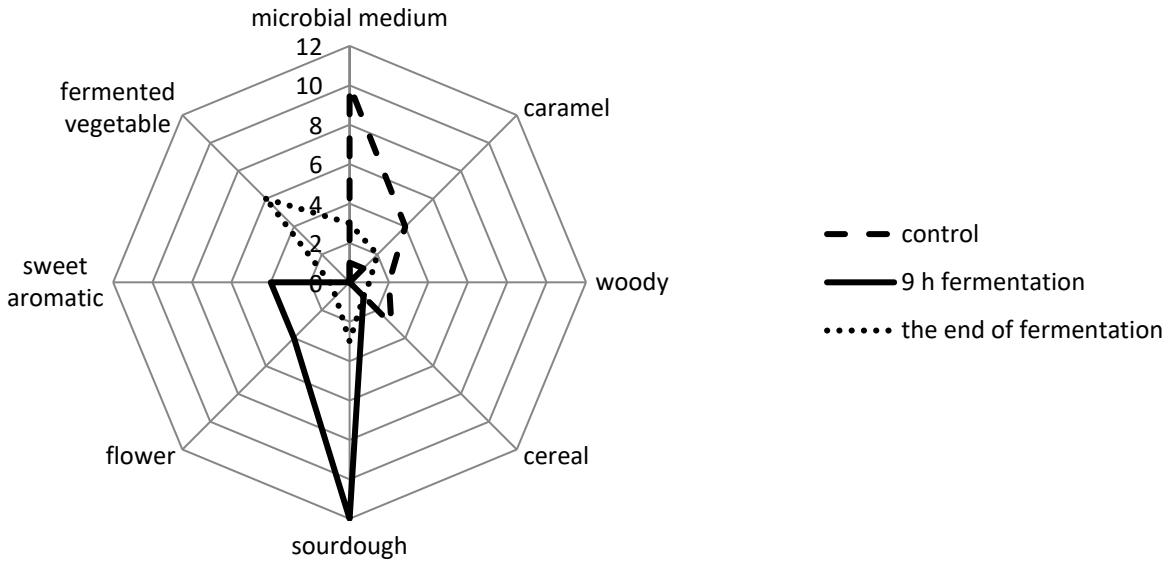

Figure 2. Descriptive aroma terms of volatile flavor and fragrance compounds produced in the bioreactor.

Sensory Analysis of Aroma Compounds Produced by K. marxianus During the Production in a Bioreactor

Sensory analysis is the most powerful tool for interpreting the relationship between quantity of flavor compound and its sensory perception (Guneser *et al.* 2015). Therefore, descriptive sensory analysis was also conducted in the present study. Totally eight descriptive aroma terms were developed by panelists. They were broth-like, cereal, caramel, woody, sourdough, flower, sweet aromatic, and fermented vegetable. Figure 2 shows the mean intensities of descriptive sensory terms for control sample and samples at 9 h fermentation time (at which the amount of volatiles were highest) and the end of fermentation. It was observed that there were significant differences between samples in terms of caramel, fermented, flower and sweet aromatic aromas ($p \leq 0.01$). Control sample had the higher broth-like, caramel, cereal aromas than other samples, whereas sample at 9 h had higher sourdough, flower and sweet aromatic aromas than control and the sample belonging to end of fermentation. It was also found that the sample belonging to end of fermentation had the highest fermented vegetable aroma than others. These results proved that the changes in aroma profiles owing to the production of volatile flavor compounds by *K. marxianus* during the production in bioreactor. It was also revealed that sweet aromatic and caramel aromas were related to isoamyl acetate and phenylethyl propanate by multidimensional scale analysis (data not shown).

Conclusion

In conclusion, the optimization of nitrogen and carbon sources in culture medium of *K. marxianus* led to obtain higher biomass productivities and the right balance between the growth of yeast and synthesis of volatile flavor compounds compared with previous studies. It was also important to note that the strain of *K. marxianus* studied in this work produced mainly ethyl acetate (fruity), isoamyl alcohol (banana), 2-phenylethyl acetate (floral) on a medium with a semi-defined composition. Results obtained during this work indicated that the growth of yeast and level of flavor components depended on the nature and the concentration of nutrients. It was revealed that ammonium sulphate which was generally used nitrogen source in previous researches was not necessary for the growth in the presence of yeast extract. Yeast extract seemed to be preferentially assimilated by the organism. This study also allowed to the regulation of the level of target volatile compounds according to the nutritional requirements of yeast. Furthermore, it was presented that highest productivity values for major volatile compounds were obtained during the exponential growth of the yeast. Therefore, further improvement in the level of flavor compounds may be obtained by employing suitable nutrient feeding strategies (fed-batch, repeated fed-batch, continuos systems) during the production in bioreactor. This work also demonstrated high product losses due to the stripping effect when the production scaled up from shaking flask to bioreactor. Hence, in situ recovery of the volatile products can improve the productivity of the bioprocess considerably. Because of its volatile compound profile (mainly acetate esters and fusel alcohols) and ability also to grow on a broad variety of substrates, at higher temperatures and rapid growth rates, *K. marxianus* can be used as starter cultures for various fermented foods especially beverages.

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