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Research Paper / Araştırma Makalesi

Effect of Fermentation Time on the Ability of *Kluyveromyces marxianus* to Produce Bioaroma Compounds from Whey

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ABSTRACT

Whey is an important by-product of the food industry that must be recycled due to both the amount released and its environmental damage. Whey, an attractive substrate for various microorganisms due to its chemical composition, has a high potential for use in the production of sustainable bioaroma compounds through microbial biotechnology. Therefore, the potential of *Kluyveromyces marxianus* TGM66 to produce bioaroma compounds in a whey-based medium and the effect of fermentation duration (96 and 120 hours) on the bioaroma composition were investigated using GC-MS in this study. Results showed that *K. marxianus* TGM66 could produce valuable higher alcohols such as 2-phenylethanol and isoamyl alcohol, and valuable esters such as phenylethyl acetate and phenethyl propionate in whey-based medium. The relative abundances of some esters (ethyl octanoate, ethyl decanoate, and ethyl laurate) were significantly influenced by the fermentation time. In conclusion, results indicated that *K. marxianus* TGM66 strain has a high potential to improve the flavor profile of whey-based media and to produce natural and sustainable bioaroma compounds using these media.

Keywords: *Kluyveromyces marxianus*, Whey, Fermentation, Bioaroma, Waste management

Fermantasyon Süresinin *Kluyveromyces marxianus*'un Peynir Altı Suyundan Biyoaroma Bileşikleri Üretme Yeteneği Üzerindeki Etkisi

ÖZ

Peynir altı suyu hem açığa çıkma miktarı hem de çevreye zarar verme özelliği nedeniyle geri dönüştürülmesi gereken gıda sanayiinin önemli bir yan ürünüdür. Kimyasal kompozisyonu nedeniyle çeşitli mikroorganizmalar için cazip bir substrat olma özelliği de taşıyan peynir altı suyunun mikrobiyal biyoteknoloji ile sürdürülebilir biyoaroma bileşiklerinin üretiminde kullanılma potansiyeli yüksektir. Bu doğrultuda gerçekleştirilen bu çalışmada *Kluyveromyces marxianus* TGM66 suşunun peynir altı suyu içeren ortamda biyoaroma bileşiği üretme potansiyeli ve fermantasyon süresinin (96 ve 120 saat) biyoaroma kompozisyonuna etkisi GC-MS ile incelendi. Çalışmadaki bulgular, *K. marxianus* TGM66'nın peynir altı suyu temelli ortamda 2-feniletanol ve izoamil alkol gibi değerli yüksek alkolleri ve feniletil asetat ve feniletil propiyonat gibi değerli esterleri üretebildiklerini gösterdi. Ayrıca özellikle bazı esterlerin (etil oktanoat, etil dekanoat ve etil laurat) oransal bolluklarının fermantasyon süresinden önemli düzeyde etkilendiği gözlendi. Sonuç olarak, bulgular *K. marxianus* TGM66 suşunun peynir altı suyu temelli ortamların lezzet profilini iyileştirme ve dahası bu ortamlar kullanılarak doğal ve sürdürülebilir biyoaroma bileşikleri üretme potansiyelinin yüksek olduğuna işaret etmektedir.

Anahtar Kelimeler: *Kluyveromyces marxianus*, Peynir altı suyu, Fermantasyon, Biyoaroma, Atık yönetimi

INTRODUCTION

Some waste and by-products of the food industry are substrates that have the potential to be used in the production of various value-added compounds, as they can provide both environmentally friendly, low-cost, sustainable production and a good growth medium for microorganisms [1]. In this context, whey, which is an important by-product of cheese production and is rich in lactose, stands out both because of its release amount and its chemical composition [2]. In addition, the fact that approximately half of the whey produced in the world is released into the environment without being processed can cause various ecological problems [3], which makes the processing of whey a necessity. Although whey can be processed in various ways in the food industry, fermentation of whey with various microorganisms is promising as a sustainable alternative method [3]. Whey can be used as a growth medium for various microorganisms, especially some yeasts and lactic acid bacteria that can use lactose as a carbon source [4]. Yeasts stand out compared to bacteria due to their rapid growth rates on inexpensive substrates derived from by-products such as whey, as well as their capacity to produce a wide range of high value-added biotechnological products [5]. Among yeasts, *Kluyveromyces marxianus* emerges as a more suitable candidate for whey valorization than other conventional yeast species. While *Saccharomyces cerevisiae*, the most widely used industrial yeast, lacks the natural ability to metabolize lactose, *K. marxianus* can directly utilize lactose thanks to its ability to produce the β-galactosidase enzyme [6]. Furthermore, the "generally recognized as safe" (GRAS) status of *K. marxianus*, combined with its inherent capacity for the de novo synthesis of various aromatic compounds, makes it a potential cell factory for producing high value-added bioaroma compounds, such as 2-phenylethanol, in whey-based media [7]. Bioaroma compounds obtained through the utilization of food by-products, such as whey, as substrates by microorganisms are considered "natural" from a regulatory perspective, in addition to being produced in an environmentally friendly and sustainable manner. Furthermore, they hold significant potential to meet the increasing consumer demand for natural products in recent years (Figure 1) [8].

In our previous study [9], where we used sugar beet molasses as a fermentation medium, we observed that the yeast extract we obtained from *K. marxianus* TGM66 strain had a high potential to produce bioaroma compounds. Additionally, there are various studies in the literature examining the potential of *K. marxianus* to produce bioaroma compounds [10, 11]. However, there are limited studies in the literature investigating the effect of fermentation time on the bioaroma compounds produced by *K. marxianus* during whey fermentation. In particular, comparative data on how prolongation of fermentation time alters the relative abundances of different volatile compound groups, such as higher alcohols, esters, aldehydes, and ketones, are scarce. In this context, the present study aims to comparatively evaluate, using a time-dependent approach, the major volatile compound profiles formed during whey fermentation by *K. marxianus* TGM66 at two different fermentation times (96 and 120 h). The data obtained in this study are expected to elucidate the role of fermentation time on the relative distribution of volatile compounds in whey-based fermentations and to contribute to a better understanding of the time-dependent behavior of *K. marxianus* in bioaroma production.

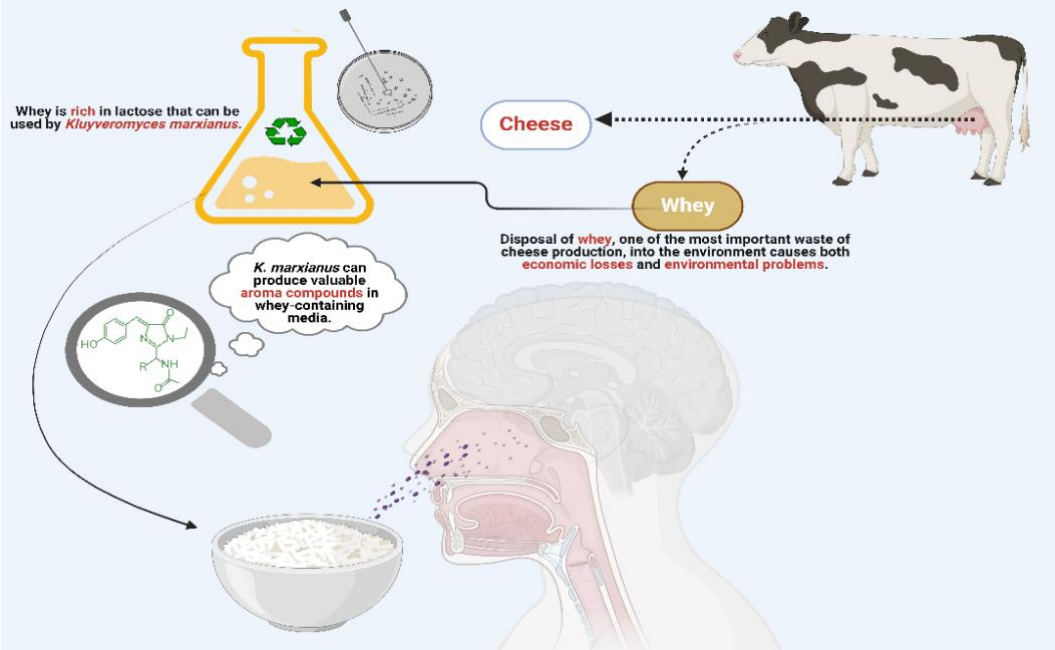

Figure 1. Production of bioaroma compounds by *K. marxianus* from whey

MATERIALS and METHODS

Yeast Strain and Medium

K. marxianus TGM66 culture was obtained from Yıldız Technical University, Department of Food Engineering Microbial Culture Collection (Türkiye). Stock cultures were kept at -80°C in malt extract broth (LAB M, UK), including 25% sterile glycerol (Riedel-de Haën, Seelze, Germany) until use. High-protein whey powder used in the study was obtained from Malkara Alliance Milk and Milk Products Inc. (Türkiye). The specification values of whey powder provided by manufacturer are as follows: Moisture content 1.60%, protein 11.29%, lactose 81.26%, fat 1.00%, ash content 4.85% and pH (after autoclaving, 10% solution at 25°C) 5.56. Whey powder was stored at 4°C until use.

Whey Fermentation

For fermentation experiments, the media containing 200 g each of 10% (w/w) whey powder was prepared with distilled water in Erlenmayer flasks (0.5 L) and autoclaved at 121°C for 15 min. Yeast culture was grown in malt extract broth for 48 h at 30°C, based on preliminary experiments conducted to obtain sufficient viable cells for fermentation inoculation. After incubation, cell numbers were determined by the spread plate technique, and 100 µL of yeast culture (10^5-10^6 CFU/mL) was inoculated into the fermentation medium. Whey fermentation was carried out at an agitation rate of 120 rpm, 30°C temperature, for 96 and 120 h in a sealed flask. Samples were taken under aseptic conditions every 24 h during fermentation, and cell counts were performed using the spread plate technique.

Analysis of Volatile Compounds

Volatile compound analyses (extraction and determination) of the samples were carried out by slightly modifying the methods suggested by Demirgül et al. [9] and Kıvançlı and Elmacı [12]. Volatile compounds in the samples were extracted by the solid phase microextraction (SPME) method coupled with an AOC-5000 plus multifunctional autosampler (Shimadzu, Japan) operating connected to a gas chromatography-mass spectrometry (GC-MS) system. The sample taken from flasks under aseptic conditions was weighed, transferred to vials (15 mL), and vortexed. In the volatile compound extraction, divinylbenzene/carboxen/ polydimethylsiloxane (DVB/CAR/PDMS, 30/50 µm) SPME fiber (Supelco, USA) was used, which was preconditioned at 250°C for 30 min before extraction. The samples in vials were preheated at 60°C for 10 min. After the extraction process was carried out at 58°C for 55 min on the samples, a silica capillary column (30 m × 0.25 mm × 0.25 µm, Restec Rtx-5MS, USA) was used to separate the volatile compounds, and helium (1.45 mL/min) was used as the carrier gas. The oven temperature was initially held at 40°C for 3 min, then increased to 100°C (at 8°C/min) and then increased to 200°C (at 5°C/min). After the oven temperature was increased to 250°C at a rate of 10°C per minute, it was maintained at this temperature for 10 min. The injection temperature was also adjusted to the final oven temperature (250°C). MS conditions were set as follows: ionization energy 70 eV, mass range 50-400 amu, interface temperature 280°C. Identification of volatile compounds in the samples was performed using WILEY Registry of Mass Spectral Data and National Institute of Standards and Technology (NIST) libraries. Unfermented whey without culture inoculation was used as a control.

Statistical Analysis

Statistical analysis was performed by using SPSS Version 23 (USA) statistical program. All experiments in the study were performed in triplicate, and the data obtained were expressed as means±standard deviations (SD). The assumptions of normality and homogeneity of variance of the data were tested. Microbial count result data were evaluated by one-way analysis of variance (ANOVA), and then multiple comparisons were made with the Tukey test to determine the differences between the groups. Volatile compound data were evaluated using an independent samples t-test. In all analyses, differences obtained at the 95% confidence level ($p \leq 0.05$) were considered statistically significant. In the statistical evaluation of volatile compounds, values that could not be detected were excluded from the evaluation.

RESULTS and DISCUSSION

Determination of Cell Numbers throughout Fermentation

Yeast cell numbers of samples taken from the fermentation medium at 24 h intervals during 120 h of whey fermentation are shown in Table 1.

Table 1. Number of *K. marxianus* TGM66 cells (log CFU mL^{-1}) at different fermentation times

Fermentation time (h)	TGM66
0	$5.40 \pm 0.18^{a*}$
24	5.17 ± 0.25^{a}
48	7.55 ± 0.55^{b}
72	7.87 ± 0.41^{b}
96	7.94 ± 0.36^{b}
120	8.31 ± 0.52^{b}

*Different superscripts indicate the difference between two values is statistically significant ($p \leq 0.05$). CFU: colony forming unit. Results were expressed as mean value ± SD (n = 3).

In the cell count performed immediately after the inoculation of *K. marxianus* TGM66 culture into the fermentation medium, the number of *K. marxianus* TGM66 was 5.40 log CFU/mL. A statistically insignificant ($p > 0.05$) decrease in the number of *K. marxianus* TGM66 occurred in the first 24 h of fermentation. With 48 h of fermentation, a significant increase ($p \leq 0.05$) occurred in the number of *K. marxianus* TGM66 (7.55 log CFU/mL), and this increase continued until the end of fermentation (120 h) at a statistically insignificant ($p > 0.05$) level. At the end of fermentation, the number of *K. marxianus* TGM66 in the medium increased by approximately 3 logs compared to the beginning and reached 8.31 log CFU/mL. These results show that *K. marxianus* TGM66

strain can grow effectively in a medium containing 10% high-protein whey powder.

Major Volatile Compound Profile

A total of 20 important volatile compounds, including four higher alcohols, 12 esters, three aldehydes, and one ketone, were determined in the samples obtained by fermenting whey for 96 and 120 h by the *K. marxianus* TGM66 strain. The relative abundances (%) of volatile compounds determined in fermented samples and unfermented whey (control) are summarized in Table 2.

Table 2. Effect of fermentation time on the major volatiles in whey fermentation by *K. marxianus* TGM66

Major Volatile Compounds	*K. marxianus* TGM66 96h	*K. marxianus* TGM66 120h	Control[1]
Higher alcohols			
Phenethyl alcohol (2-phenylethanol)	9.89[2] ± 1.31[a*]	10.66 ± 1.91[a]	-
2-Methyl-1-propanol (isobutanol)	4.44 ± 0.50[a]	4.65 ± 0.72[a]	-
3-Methyl-1-butanol (isoamyl alcohol)	8.47 ± 1.03[a]	9.24 ± 1.74[a]	-
2-Methyl-1-butanol	5.14 ± 0.75[a]	3.68 ± 0.30[b]	-
Esters			
Ethyl acetate	0.79 ± 0.09[a]	1.04 ± 0.21[a]	-
Ethyl octanoate	0.58 ± 0.31[a]	1.85 ± 0.28[b]	-
Isoamyl acetate	0.44 ± 0.10[a]	0.39 ± 0.04[a]	-
Phenylethyl acetate	6.01 ± 1.30[a]	7.89 ± 1.86[a]	-
Phenethyl propionate	3.04 ± 0.89[a]	2.39 ± 0.20[a]	-
Ethyl decanoate	0.52 ± 0.31[a]	1.92 ± 0.73[b]	-
Phenethyl isobutyrate	2.75 ± 0.40[a]	2.65 ± 0.22[a]	-
Ethyl laurate	0.57 ± 0.11[a]	1.31 ± 0.26[b]	-
Ethyl hexanoate	0.23 ± 0.06[a]	0.30 ± 0.05[a]	-
Isoamyl decanoate	0.16 ± 0.04[a]	0.35 ± 0.07[b]	-
Phenylethyl butyrate	0.22 ± 0.06	-	-
Ethyl 9-decenoate	-	0.64 ± 0.31	-
Aldehydes			
3-Methylbutanal	0.32 ± 0.09[a]	-	0.74 ± 0.25[b]
2-Methylbutanal	0.19 ± 0.03[a]	-	0.56 ± 0.07[b]
Phenylacetaldehyde	0.29 ± 0.11[a]	-	0.96 ± 0.38[b]
Ketone			
Acetoin	-	0.41 ± 0.08	-

*Different superscripts in the same row indicate the difference between two values is statistically significant ($p \leq 0.05$).Values were expressed as mean value ± SD (n = 3). [1]The control (unfermented whey) sample was taken at time zero, kept at 4 °C, and analyzed together with the 96 and 120 h samples, the results were found to be identical. [2]Relative abundances were expressed as percentages of the total peak area.

Higher alcohols are among the most abundant volatile compound groups produced in fermentation by yeasts and contribute to the basic aroma in fermented foods [13]. In both 96 and 120 h fermentations carried out by *K. marxianus* TGM66, higher alcohols (2-phenylethanol, isobutanol, isoamyl alcohol, and 2-methyl-1-butanol) were present in the highest relative abundance with 27.94% and 28.23%, respectively. Among higher alcohols, 2-phenylethanol, characterized by floral aroma notes [14], was the compound found in the highest relative abundance. Although there was some increase in 2-phenylethanol abundance with fermentation time, this increase was statistically insignificant ($p>0.05$). 2-Phenylethanol is one of the most used aroma compounds in the food and cosmetic industries to increase the organoleptic properties of final products [15], and some yeasts can convert the amino acid L-phenylalanine to 2-phenylethanol via the Ehrlich metabolic pathway [16]. *K. marxianus* is one of the best 2-phenylethanol-producing yeast species, and promising results were obtained with the use of *K. marxianus* in 2-phenylethanol production in many studies [7, 17, 18]. After 2-phenylethanol, the higher alcohol produced in highest relative abundance by *K. marxianus* TGM66 in whey medium was isoamyl alcohol, characterized by a banana-like aroma [19]. The relative abundance of isoamyl alcohol did not show any significant ($p>0.05$) change with fermentation time. Similarly, the relative abundance of isobutanol, characterized by a pine-like aroma [20], was not significantly affected by fermentation time ($p>0.05$). However, the relative abundance of 2-methyl-1-butanol, which is characterized by whisky-like aroma [21], decreased at a statistically significant ($p\leq0.05$) level at 120 h of fermentation compared to 96 h of fermentation. The reason for these decreases needs to be elucidated by further studies.

Esters are important aroma compounds that can be produced by fermentation by various yeasts or other microbial groups and contribute to the floral and fruity notes of fermented beverages such as beer, wine, and sake, and are widely used as additives in fruit-flavored products [22]. In the present study, the compounds produced in the highest relative abundance (15.31% at 96 h fermentation and 20.73% at 120 h fermentation) after higher alcohols were esters. Among the esters

produced by *K. marxianus* TGM66, the ester produced in the highest relative abundance in both fermentation times was phenylethyl acetate (rose-like floral aroma notes) [15]. Phenylethyl acetate is used in various products such as shampoo and soap, as well as soft drinks [15]. The esters found in the highest relative abundances after phenylethyl acetate in the fermentation medium were phenethyl propionate, which is generally characterized by rose-like aroma notes [23], and phenethyl isobutyrate, which is characterized by fruity/rosy-like aroma notes [24], and the relative abundances of both esters decreased statistically insignificant level ($p>0.05$) with increasing fermentation time. Similarly, the relative abundances of isoamyl acetate, which is characterized by apple/banana-like aroma notes and is one of the most used aroma compounds in the food industry [22, 25], and phenylethyl butyrate, which is characterized by floral and fruity aroma notes [26], also decreased as fermentation time extended. We think that one of the possible reasons for the decrease in the relative abundance of these esters is esterase activity. However, the relative abundances of ethyl octanoate (apple and pineapple-like aroma), ethyl laurate (fruity aroma), ethyl decanoate (grape-like aroma), and isoamyl decanoate (waxy and fruity aroma) increased significantly ($p\leq0.05$) as fermentation time increased, while ethyl acetate (orange and fruit-like aroma), ethyl hexanoate (apple, anise and strawberry-like aroma), and ethyl 9-decenoate (floral aroma) increased slightly [22, 25, 27, 28, 29]. Similar to our results, previous studies [19, 30, 31] reported that *K. marxianus* can synthesize various esters in whey.

Aldehydes such as 3-methylbutanal and 2-methylbutanal are important aroma compounds in both various fermented foods and heat-treated unfermented foods, but in some foods, the aroma imparted by aldehydes is undesirable. For example, the amount of aldehydes in fresh beer is generally low, but their amount can increase as the beer is stored for longer, and in aged beer, the aromas given by 2-methyl butanal, 3-methyl butanal, and phenylacetaldehyde are sometimes undesirable [32]. In this study, the relative abundances of aldehydes such as 3-methylbutanal, 2-methylbutanal, and phenylacetaldehyde were statistically significantly ($p\leq0.05$) higher in the unfermented control sample compared to the fermented samples. During fermentation, these aldehydes can be reduced to higher alcohols by yeast [13]. In the unfermented control sample, since there is no microbial activity, these aldehydes remain without converting to higher alcohols, and these compounds can also form during heat treatments (such as autoclaving) applied to the fermentation medium [33].

In this study, only one ketone (acetoin) was detected at 0.41% relative abundance in the sample fermented for 120 h. Characterized by a pleasant yoghurt odor and a fatty, creamy buttery taste, acetoin is used as an additive in the production of various foods to enhance palatability [34]. Although it can be said that the ability of yeasts to produce acetoin is generally weak [34], the study conducted by Roy et al. [35] reported that *K. marxianus* can produce acetoin, similar to the findings in our study.

CONCLUSION

The results of this study showed that the volatile compound profiles of fermented samples were significantly different from the unfermented control sample and that *K. marxianus* TGM66 strain was capable of producing bioaroma compounds such as various higher alcohols, esters, and ketones by whey fermentation. In general, higher alcohols were insignificantly affected by fermentation time, with one exception (2-methyl-1-butanol). However, the relative abundances of some esters such as ethyl octanoate, ethyl decanoate, and ethyl laurate increased significantly with increasing fermentation time. On the other hand, the decrease in the relative abundance of aldehydes in the unfermented control sample in the fermented samples indicates that these compounds were converted to higher alcohols or esters by yeast activities. These results demonstrate that fermentation time is a critical process parameter for modulating the volatile aroma profile in whey-based fermentations and for optimizing the production of specific target aroma compounds. In conclusion, it can be said that *K. marxianus* TGM66 culture has a high potential to produce valuable bioaroma compounds such as 2-phenylethanol and phenylethyl acetate from whey, an important by-product of the dairy industry. In this context, the present study provides a scientific basis for the sustainable valorization of dairy industry by-products and offers practical insights for the development and scale-up of industrial fermentation processes aimed at natural aroma production in the food industry.

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