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Yeast-*Lactobacillus* Co-Cultures as *in situ* Ethanol Producers for Flavor Ester Synthesis using Lipase in Fermented Milks

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Abstract

Background and Objective: Nowadays, novel biotechnological methods are preferred for flavoring productions since traditional methods include disadvantages. The aim of this study was to assess *in situ* biosynthesis of natural fruity flavors in fermented milks using microbial co-cultures and lipase enzyme.

Materials and Methods: Trans esterification of milk fats with ethanol was carried out to develop fruity flavors in ultra-high-temperature whole milks using lipase of Palatase coupled with ethanol fermentation. *Kluyveromyces marxianus* NCYC 1425 was used to produce *in situ* ethanol in co-cultures with *Lactobacillus fermentum* PCC or *Lactobacillus paracasei* L26. Effects of co-culturing on cell viability and fermentation progress were assessed using enumeration of viable cells and measurement of pH in samples at 0, 24 (Palatase addition) and 48 h (end of fermentation). Headspace solid phase microextraction-gas chromatography (SPME)-MS/FID was used for ester, ethanol and free fatty acid analyses at the beginning and end of the fermentation. Standard curve of ethanol was used to assess the products in terms of being Halal.

Results and Conclusion: *Kluyveromyces marxianus* included synergistic effects on *Lactobacillus paracasei* growth as well as antagonistic effects on *Lactobacillus fermentum* growth. Antimicrobial effects were seen in *Kluyveromyces marxianus-Lactobacillus paracasei* co-cultures when Palatase was added. Palatase significantly increased ester levels of the fermented samples. The co-cultures did not include significant differences in shorter chain ester levels (esters of 4-7 carbon chain fatty acids); in contrast, *Kluyveromyces marxianus- Lactobacillus fermentum* resulted in higher levels of longer chain esters. Although the *Kluyveromyces marxianus* cultures resulted in higher ester levels compared to that its co-cultures did, the cultures can be used as appropriate adjunct cultures with *Lactobacillus* cultures to boost flavor ester synthesis. This flavor synthesis can be an appropriate alternative for artificial flavoring agents.

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1. Introduction

Understanding of consumer preferences is the key to success in food marketing. Nowadays, people mostly choose foods with no additives. However, they still prefer foods containing natural additives over synthetic ones [1]. There are chemophobia attitudes in use of artificial flavorings, which are even nature identical. Extraction from natural sources is an expensive method since flavor substances are present in low quantities in natural sources. However, biotechnology is a preferred method currently with the advantage of "natural" labeling that definitely draws consumer attention. *De novo* microbial production (fermentation) and enzymatic synthesis are two biotechnological ways to achieve bioflavors. Enzymatic flavor production is commonly preferred over fermentation due to its high

production yields [2,3]. Esters of fatty acids are flavor compounds, which can be produced using enzymatic methods [4]. Lipases are enzymes that produce esters via esterification, alcoholysis, acidolysis and interesterification processes [5]. Palatase 20000 l, a lipase from *Rhizomucor miehei* produced by genetically modified *Aspergillus oryzae*, is a commercial lipase developed for use in foods that includes capability of ester biosynthesis via transesterification in aqueous environments of foods [6-8]. Palatase preference for short chain fatty acids [9] is an advantage in flavor ester production since long chain esters include soapy, tallow, and waxy tastes. In contrast, the short chain fatty acid esters include pleasant fruity tastes [10].

Milk fats containing significant quantities of short chain fatty acids and medium chain fatty acids (C4-C12) [11] are favorable sources for fruity flavor production. Researchers have studied lipase catalyzed ester production in dairies. Production of natural fruity flavors in milks, creams and cheeses by Liu et al. [12] and modification of the flavor profiles in recombined skim milks by Zhang et al. [13] are two studies carried out in this area. Ethanol is an alcoholic precursor with frequent uses in flavor ester synthesis [14]. It can be added to reaction media either manually or by *in situ* production through microbial fermentation. However, ethanol residues in products must include the standard of halal for the Islamic countries [12]. Flavor ester synthesis in coconut creams by Sun et al. [14] was carried out using *Saccharomyces* (*S*.) *cerevisiae* to produce *in situ* ethanol. To the best of the authors' knowledge, alcohol-producing microbial cultures have not been studied in ester production systems combined with Palatase in dairy products. *Kluyveromyces* (*K*.) *marxianus* is a dairy yeast that is capable of assimilating lactose and metabolizing it to ethanol [15]. It is generally recognized as safe [16] and could be a cell factory to increase ethanol and thus flavor ester quantities in fermented dairy systems with lipase use. *Lactobacillus* spp. are bacteria, which are widely used in dairy products [17]. This study was carried out to assess effects of co-cultures of *K. marxianus* with *Lactobacillus* (*L*.) *fermentum* and *L. paracasei* on cell viability, ethanol production and flavor ester synthesis in fermented milks as well as lipase biocatalysis. Based on the authors' knowledge, these co-cultures have not previously been used with lipase Palatase. As it has been hypothesized that lactic acid produced by lactobacilli could be converted into ethyl lactate with a creamy fruity flavor; thus, co-cultures of yeast and lactobacilli were used in this study. Enumeration of viable cells and measurement of pH values were carried out to monitor the fermentation. Since sensory analysis is linked to individuals, a further precise method, headspace solid phase microextraction-gas chromatography (SPME-GC), was used to analyze the produced esters, ethanol and free fatty acids (FFA).

2. Materials and Methods

2.1. Materials and microorganisms

Lipase Palatase 20000 l (Novozymes, Bagsværd, Denmark), MRS (de Man Rogosa Sharpe) broth, potato dextrose agar (PDA), yeast extract and bacteriological peptone (Oxoid, Basingstoke, UK), malt extract, MRS agar, HCl (Merck, Darmstadt, Germany), dextrose, chloramphenicol and saturated alkane standards (Sigma-Aldrich, St., MO, USA) and natamycin (50 g natamycin 100 g^{-1}) (Danisco A/S, Grindsted, Denmark) were purchased from the respective manufacturers. Ultra-high-temperature sterilized whole milks (Devondale, Melbourne, Australia) were purchased from supermarkets in Singapore. The *L. fermentum* PCC (Chr. Hansen A/S, Horsholm, Denmark), *L. paracasei* L26 (Lallemand, Ontario, Canada) and *K. marxianus* NCYC 1425 (National Collection of Yeast Cultures, Norwich, UK) were provided as pure cultures in freeze-dried forms.

2.2. Propagation of microorganisms

Yeast malt broth (10 g l^{-1} dextrose, 3 g l^{-1} yeast extract, 3 g l^{-1} malt extract and 5 g l^{-1} bacteriological peptone with pH adjusted to 5.0 using 1 M HCl) and MRS broth were used for the cultivation of *K. marxianus* and lactobacilli, respectively. Freeze-dried cultures were transferred into fresh broths and propagated at 30 and 37 °C for 48 h for yeast and lactobacilli, respectively. Propagated cultures were dispensed into cryovials and stored at -80 °C as stock cultures [18].

2.3. Inoculum preparation

Yeasts and lactobacilli were sub-cultured by inoculating 5% (v v^{-1}) of the stock cultures into fresh Yeast malt broth and MRS broth, respectively. Sub-cultures were incubated at 30 and 37 °C for 24 h for yeast and lactobacilli, respectively. After two consecutive sub-cultures, microbial cell pellets were collected by centrifuging at 8000 g for 10 min at 4 °C. Pellets were washed two times using normal saline (8.5 g l^{-1} NaCl) and centrifuging at the highlighted conditions. The cell pellets were re-suspended in 30 ml of normal saline to produce the inoculums [18].

2.4. Milk fermentation conditions

Yeast and bacterial mono-cultures were prepared by adding 1% (v v^{-1}) of the respective cultures into ultra-high-temperature whole milks. Moreover, yeast-bacterial co-cultures were prepared by inoculating milks with 1% (v v^{-1}) of *K. marxianus* culture and 1% (v v^{-1}) of either *L. fermentum* or *L. paracasei*. Inoculated milks were distributed into sterile 50-ml centrifuge tubes in 30-ml aliquots and incubated at 30 °C for 48 h [18]. Sterile-filtered undiluted Palatase was added at 0.1% (v v^{-1}) concentrations into tubes after 24 h of fermentation (Palatase quantity and enzyme addition time were selected based on previous experiments) [19]. The incubation temperature was set at 30

°C based on optimum temperature of Palatase to allow growth of the microorganisms. Uninoculated milks, mono-cultures and co-cultures with no Palatase addition as well as uninoculated milks with lipase served as controls.

2.5. Viable cell counts and pH

To assess effects of microbial co-culturing and lipase addition, viable cell count and pH measurement of individual tubes were carried out at 0 (beginning of fermentation), 24 (Palatase addition time) and 48 h (end of fermentation). Cell counts of lactobacilli were carried out using MRS agar supplemented with 0.25 g l^{-1} natamycin and pour plate method. Furthermore, cell counts of yeasts were carried out using PDA supplemented with 0.1 gl^{-1} chloramphenicol and spread plate method. The MRS agar and PDA plates were respectively incubated at 37 and 30 °C until colonies appeared (24-48 h). The pH values of samples were measured using calibrated pH meter (Metrohm, Herisau, Switzerland) [18,20].

2.6. Ester, ethanol and free fatty acid analyses

Samples were analyzed for short chain ethyl esters, FFAs and ethanol using SPME-GC. Briefly, 2.2 g of the samples and 2 ml of saturated NaCl aqueous solutions were mixed in a 20 ml glass headspace vial sealed with PTFE septum. Samples in the vials were equilibrated at 250 rpm for 20 min at 60 °C. The volatile esters in the headspace were extracted for 30 min under the similar temperature and agitation conditions using 85-µm carboxen/polydimethylsiloxane SPME fiber (CAR/PDMS, Supelco, Munich, Germany) equipped with Combi Pal Autosampler (CTC Analytics, Zwingen, Switzerland). The SPME fiber was desorbed at 250 °C for 3 min after insertion into the injection port of the GC. A gas chromatograph (Agilent, 7890A, CA, USA) with DB-FFAP capillary column (60 m length, 0.25 µm i.d., 0.25 µm film thickness) (Agilent, VA, USA) connected to Inert Mass Selective Detector (MSD) and flame ionization detector (FID) (Agilent 5975C, Agilent, VA, USA) was used. Oven temperature was initially 50 °C and after 5 min increased gradually up to 230 °C by 5 °C min^{-1} held for 30 min. The carrier gas was helium with a flow rate of 1.2 ml min^{-1}. The MS detector was adjusted in electron ionization mode (70 eV) and the ion source temperature was 230 °C. Peaks from the MS detector were identified based on the NIST 05 Database and peak areas of the corresponding FID peaks were used for quantitative assessment. Linear retention indices (LRI) of the MS peaks were compared to data of NIST WebBook to verify identifications. Retention times of C7-C40 saturated alkane standards were used to derive LRI values [18].

2.7. Statistical analysis

Data were reported as mean values ±SD (standard deviations) of three independent experiments (n = 3). One-way analysis of variance, Duncan's multiple range test and SPSS Software v.16.0 (IBM Analytics, USA) were used to calculate significant differences ($p < 0.05$).

2.8. Ethical considerations

This study did not contain human or animal experiments.

3. Results and Discussion

3.1. Viable cell counts and pH

Table 1 illustrates changes in viable cell counts (log CFU ml^{-1}) and pH values of *K. marxianus-L. paracasei* and *K. marxianus-L. fermentum* co-cultured milk samples during 48 h of fermentation with and without Palatase addition. All samples included similar bacteria or yeast counts and pH values at the beginning of fermentation (no significant differences were seen, statistical analyses not shown). Furthermore, each microbial culture included similar cell counts and pH values during the fermentation before Palatase addition (0 and 24 h). This was expected as the yeast and bacteria were inoculated into milk samples in similar quantities. Based on the previous experiments, Palatase addition at earlier stages of fermentation inhibited yeast and lactobacilli growth whereas its addition after 24 h of fermentation resulted in better cell growth, compared to control samples with no Palatase [19].

Table 1. Changes in microbial counts (log CFU ml^{-1}) and pH values during 48 h of milk fermentation by *Kluyveromyces marxianus* and *Lactobacillus* co-cultures with and without Palatase

Parameter	Sampling time (h)	*K. marxianus-L. paracasei* with P	*K. marxianus-L. paracasei*	*K. marxianus-L. fermentum* with P	*K. marxianus-L. fermentum*
K. marxianus count	0	5.80 ± 0.02^{a}	5.78 ± 0.05^{a}	5.74 ± 0.07^{a}	5.72 ± 0.05^{a}
	24	7.37 ± 0.02^{c}	7.40 ± 0.01^{c}	$7.49 \pm 0.13^{b,c}$	7.43 ± 0.02^{b}
	48	6.76 ± 0.10^{b}	7.33 ± 0.07^{c}	$7.54 \pm 0.10^{b,c}$	7.63 ± 0.13^{c}
Lactobacillus count	0	7.33 ± 0.05^{a}	7.30 ± 0.03^{a}	7.21 ± 0.05^{a}	7.19 ± 0.04^{a}
	24	9.34 ± 0.02^{c}	9.32 ± 0.02^{c}	7.79 ± 0.18^{b}	7.79 ± 0.17^{b}
	48	8.88 ± 0.06^{b}	9.48 ± 0.02^{d}	8.15 ± 0.15^{c}	7.82 ± 0.04^{b}
pH	0	6.58 ± 0.04^{d}	6.58 ± 0.03^{d}	6.62 ± 0.04^{d}	6.64 ± 0.05^{d}
	24	4.99 ± 0.08^{c}	4.94 ± 0.06^{c}	5.80 ± 0.11^{c}	5.79 ± 0.10^{c}
	48	4.12 ± 0.05^{b}	3.97 ± 0.06^{a}	5.03 ± 0.06^{a}	5.57 ± 0.10^{b}

Mean values with different lower case letters are significantly different ($P < 0.05$). Values are expressed as the mean ± SD of three independent experiments (n = 3). Each parameter and each co-culture (with and without Palatase) analyzed separately. P= Palatase, CFU= Colony forming unit, *K.* = *Kluyveromyces*, *L.*= *Lactobacillus.*

In this study, Palatase was added after 24 h of fermentation to mitigate inhibitory effects of the lipase. In a study by Sun et al. [14], Palatase addition at the earlier stages of fermentation included higher effects on decreases of *S. cerevisiae* viable cell counts although overall reductions of cell counts were negligible and Palatase did not affect fermentation processes. Decreases in viable cell counts after Palatase addition could occur to fatty acids released from milk fats by lipase. Palatase can decrease toxicity of FFAs by esterifying them with ethanol produced by microorganisms. In general, composition of fermentation media, environmental conditions and type of yeast strains are factors that affect stress tolerance levels [14]. Effect on cell membrane, disruption of electron transport chains and oxidative phosphorylation, increasing cytosolic concentration of protons, increasing permeability of cells, inhibition of enzyme activity, impairment of nutrient uptake, peroxidation and auto-oxidation are possible reasons for the antimicrobial activity of FFAs [21,22].

Although Palatase addition decreased *K. marxianus* counts co-cultured with *L. paracasei*, it included no significant effects on the yeast counts when combined with *L. fermentum*. Palatase addition to co-cultured samples included adverse effects on *L. paracasei* growth whereas cell counts unexpectedly increased for *L. fermentum*. With no Palatase addition, pH values of co-cultures containing *L. paracasei* were lower than those containing *L. fermentum* co-cultures (3.97 ±0.06 compared to 5.57 ±0.10, respectively), possibly due to the great quantity of LA produced by a higher population of lactobacilli as well as homofermentative characteristics of *L. paracasei* [23]. In *K. marxianus*-*L. fermentum* samples, Palatase addition decreased pH values due to released FFAs. In contrast, pH values of *K. marxianus*-*L. paracasei* samples did not decrease after Palatase addition similar to control samples with no Palatase, possibly due to the inhibition of yeast and lactobacilli growths. These results were similar to those of coconut cream fermentation by *S. cerevisiae* with Palatase addition reported by Sun et al. [14]. They stated that decreased formation of carbon dioxide and acids during fermentation by the yeasts was the major reason of pH increases. The pH decrease of *K. marxianus*-*L. paracasei* samples due to fermentation was so high that it caused pH decreases by Palatase. Yeast cell decreases were unlikely due to pH decrease only, since pH values of all samples were in a range; in which, *K. marxianus* cells could grow [24]. Undissociated LA and FAs included inhibitory effects on cell growth of the yeasts.

To compare co-cultures with mono-cultures, milk samples fermented for 48 h by mono-cultures of each microorganism were assessed (statistical analyses not shown). Table 2 shows viable cell counts (log CFU ml^{-1}) and pH values of mono-cultured milk samples after 48 h of fermentation with and without Palatase addition. With no Palatase addition, *L. paracasei* showed antagonistic effects on *K. marxianus* cells, compared to *K. marxianus* mono-cultures with a decrease of 0.38 log CFU ml^{-1}. This decrease could be attributed to LA, diacetyl, and hydrogen peroxide produced by the *Lactobacillus* strains [25]. In contrast, *K. marxianus* stimulated the growth of *L. paracasei* as shown by a 0.6 log CFU ml^{-1} higher cell count in the co-culture. Cell counts of *L. fermentum* in co-cultured samples were lower than those in mono-cultures of *L. fermentum* with no Palatase addition. However, *L. fermentum* demonstrated no significant effects on *K. marxianus* counts. Inhibitory and stimulatory effects caused by the primary and secondary metabolites have previously been reported in microbial co-cultures. In previous studies on lactic acid bacteria, it has been suggested that the enhanced growth could be resulted from enzymatic activity of a microbial strain providing substrates for another strain, decrease of growth inhibiting substances by one strain and decrease of available oxygen by aerobic microorganisms that improve the environment for anaerobic or microaerophilic strains [26]. Furthermore, cooperative metabolism (production of molecules that no microorganisms in mono-cultures could produce), protein secretion and gene transfer can be involved in cell increases [27]. Increased viable cell counts of *L. paracasei* co-cultured with *K. marxianus* in the present study was possibly linked to such effects such as excretion of amino acids by yeasts for the benefits of lactobacilli.

Table 2. Microbial counts (log CFU ml^{-1}) and pH values of the milk samples during 48 h of fermentation using mono-cultures of each microorganism with and without Palatase

Parameter	*K. marxianus* with P	*K. marxianus*	*L. fermentum* with P	*L. fermentum*	*L. paracasei* with P	*L. paracasei*
Yeast count	7.59 ± 0.05	7.71 ± 0.06	-	-	-	-
Lactobacillus count	-	-	8.50 ± 0.45	8.62 ± 0.11	8.84 ± 0.07	8.88 ± 0.06
pH	5.33 ± 0.2	5.76 ± 0.06	5.22 ± 0.36	5.95 ± 0.14	5.24 ± 0.33	6.08 ± 0.19

Values are expressed as the mean ± SD of three independent experiments (n = 3). P= Palatase, CFU= Colony forming unit, *K.* = *Kluyveromyces*, *L.*= *Lactobacillus.*

Inhibition of yeast growth could occur due to a higher LA production as a result of higher cell counts of *L. paracasei* (9.48 log CFU ml^{-1}), compared to *L. fermentum* (7.82 log CFU ml^{-1}) in co-cultured samples. In addition, homofermentative metabolism of *L. paracasei* inherently produced higher quantities of LA, compared to heterofermentative metabolism of *L. fermentum*. Similar results have been reported by Basso et al. [23] when *S. cerevisiae* CAT-1 was co-cultured with *L. plantarum* and *L. fermentum* in sugar cane based ethanol fermentation. The *L. plantarum* caused a greater yeast cell decrease since it was homofermentative. Yeast inhibition by LA was linked to the antimicrobial characteristics of its undissociated form and decreased pH values [28]. Media composition, strain type and competition for the nutrients by lactobacilli and yeasts are effective in yeast resistance to LA [29]. With no Palatase addition, co-cultures included lower pH values compared to that each mono-culture did, especially for *K. marxianus-L. paracasei* co-cultures. Similar to the current findings, co-culturing *Bifidobacterium animalis* with yeast species by Toh and Liu [18] resulted in further decreases in pH, compared to *B. animalis* mono-culture possibly due to the better growth of lactobacilli facilitated by the yeasts.

3.2. Ester and ethanol analyses

Table 3 shows the FID peak areas ($\times 10^6$) of ethanol produced by each culture as well as ethyl esters of milk FAs synthesized by Palatase. With no Palatase addition, *K. marxianus-L. paracasei* co-cultures produced significantly higher quantities of ethanol, compared to that the *K. marxianus-L. fermentum* co-cultures did. However, in previous experiments on mono-cultures with no Palatase, the FID peak area ($\times 10^6$) of ethanol included 467.31 ±42.58 for *K. marxianus* instead of 8.15 ±3.32 for *L. paracasei* and 4.21 ±1.58 for *L. fermentum* [19]. Since *K. marxianus* produced much higher quantities of ethanol and included significantly lower counts when co-cultured with *L. paracasei* compared to *L. fermentum* (statistical analyses not shown), differences in ethanol level should be linked to factors other than cell counts. In a study by Bayrock and Ingledew [29], although *S. cerevisiae* was inhibited by LA produced by lactic acid bacteria , increased LA concentrations of 3.5% (m v^{-1}) and more resulted in higher glucose consumption and ethanol production on per surviving cell basis. This showed that although yeast viable cells decreased, the rest of cells began to produce a higher ethanol level. It was suggested that increases in ethanol production were due to the increased energy demands of yeast cells to expel excess intracellular hydrogen ions following LA influx to major the pH gradient. In the current study, this phenomenon could occur that resulted in a higher ethanol level produced by *K. marxianus* in *K. marxianus-L. paracasei* co-cultures.

Table 3. Ethanol and ester levels (FID peak areas, $\times 10^6$) of the samples during 48 h of fermentation by various microbial cultures with and without Palatase.

Microbial culture	*K. marxianus-L. paracasei*		*K. marxianus-L. fermentum*		without microorganism		*K. marxianus*	*L. fermentum*	*L. paracasei*	LRI
Palatase addition	24 h	Without P	24 h	Without P	24 h	Without P	24 h	24 h	24 h	
E alcohol	175.24 ± 7.57^{b}	583.23 ± 35.55^{e}	293.01 ± 87.91^{c}	473.93 ± 108.20^{d}	1.80 ± 0.32^{a}	nd	405.48 ± 84.69^{d}	6.58 ± 4.08^{a}	1.35 ± 0.09^{a}	---
E acetate	18.92 ± 5.47^{b}	111.63 ± 7.44^{d}	29.13 ± 2.50^{c}	122.72 ± 13.45^{e}	1.86 ± 0.40^{a}	0.60 ± 0.04^{a}	16.15 ± 5.36^{b}	1.65 ± 0.30^{a}	0.51 ± 0.01^{a}	---
E butanoate	177.84 ± 58.87^{b}	8.03 ± 2.75^{a}	235.22 ± 53.48^{b}	8.36 ± 0.35^{a}	5.92 ± 1.60^{a}	nd	321.05 ± 82.19^{c}	22.35 ± 8.72^{a}	6.84 ± 0.68^{a}	1037.46
E pentanoate	2.31 ± 0.6^{b,c}	nd	3.06 ± 0.87^{c,d}	nd	nd	nd	3.28 ± 0.93^{d}	1.42 ± 0.78^{b}	0.20 ± 0.01^{a}	1129.29
E hexanoate	251.33 ± 45.73^{b}	6.58 ± 0.45^{a}	268.36 ± 57.41^{b}	4.66 ± 0.31^{a}	4.79 ± 1.40^{a}	nd	312.61 ± 78.09^{b}	15.93 ± 8.33^{a}	2.62 ± 0.21^{a}	1235.18
E heptanoate	3.23 ± 0.34^{c}	0.54 ± 0.20^{b}	3.19 ± 0.21^{c}	nd	nd	nd	3.90 ± 0.12^{d}	0.30 ± 0.07^{a,b}	0.16 ± 0.02^{a}	1331.71
E octanoate	196.79 ± 12.58^{b}	3.87 ± 0.47^{a}	227.96 ± 15.96^{c}	2.86 ± 0.57^{a}	3.70 ± 0.52^{a}	nd	283.06 ± 9.87^{d}	9.52 ± 2.64^{a}	2.75 ± 0.13^{a}	1439.34
E nonanoate	4.89 ± 0.12^{b}	nd	5.63 ± 0.21^{c}	nd	nd	nd	6.48 ± 0.74^{d}	nd	0.41 ± 0.03^{a}	1536.48
E decanoate	313.91 ± 54.59^{b}	1.34 ± 0.45^{a}	419.5 ± 36.18^{c}	2.56 ± 0.05^{a}	3.54 ± 0.17^{a}	nd	566.81 ± 58.50^{d}	8.06 ± 0.23^{a}	4.04 ± 0.17^{a}	1649.58
E dodecanoate	220.79 ± 5.2^{a}	nd	302.09 ± 53.77^{b}	nd	nd	nd	nd	nd	nd	1844.11

Each ester analyzed separately. Mean values in the same row with different lower-case letters are significantly different ($p < 0.05$). Values are expressed as the mean ±SD of three independent experiments ($n = 3$). nd = not detected, LRI = linear retention index, P = Palatase, E = ethyl, FID = flame ionization detector, *K. = Kluyveromyces, L.= Lactobacillus.*

Thomas et al. [30] reported decreases in ethanol when co-culturing *S. cerevisiae* with *L. paracasei*, *L. fermentum* and two other lactobacilli, compared to mono-culturing *S. cerevisiae* in corn mash fermentation. These decreases in ethanol contents were not observed in the present study, including *K. marxianus* monocultures with FID peak areas ($\times 10^6$) of 467.31 ±42.58. This difference was possibly due to different behaviors of *S. cerevisiae* and *K. marxianus*. Nevertheless, other researchers achieved higher ethanol levels in *Zymomonas mobilis* and *Saccharomyces* co-cultures, compared to those they achieved in mono-cultures of each microorganism [26].Similarly, ethanol levels increased in *K. marxianus-L. paracasei* co-cultures in the absence of Palatase in the present study, compared to mono-cultures.

In this study, ethanol was not detected in uninoculated samples due to absence of microbial activities. However, Palatase addition to these samples increased their ethanol contents, possibly as a result of lipase catalyzed hydrolysis of ethyl esters previously available in milk media. The liberated ethanol then caused ester synthesis in uninoculated samples containing Palatase. Ethanol decreases after Palatase addition occurred due to ethanol entering into esterification and transesterification reactions catalyzed by the Palatase. As shown in Table 3, ethyl acetate was the only ethyl ester detected in whole milks with no Palatase and microorganisms. Low levels of ethyl acetate were most likely produced *de novo* by the microorganisms such as yeasts, lactobacilli or acetic acid bacteria [31,32] present in milk samples before sterilization. In mono-culture samples with Palatase addition, ethyl acetate level significantly increased only for *K. marxianus*, compared to that for uninoculated samples. This was linked to higher levels of ethanol, one of the esterification precursors produced by the yeasts. Ethyl acetate levels in *K. marxianus* mono-culture samples included 90.05 ±15.14 after 48 h of fermentation in absence of Palatase, while Palatase addition decreased the level of this ester. Such a decrease was also observed in co-cultured samples after Palatase addition. Co-culturing with no Palatase addition dramatically increased ethyl acetate levels, compared to that the mono-culturing did. However, Palatase addition included significant effects on ethyl acetate synthesis in co-culture samples. It was possibly due to the hydrolytic activity of Palatase against this ester that converted the ester into ethanol and acetic acid. Another reason could be repressive effects of Palatase on *de novo* ethyl acetate synthesis by the microorganisms. Levels of other esters in mono-culture samples increased, compared to those in uninoculated samples especially for *K. marxianus* with higher ethanol levels.

In this study, quantity of shorter chain ethyl esters was not significantly different between the two co-cultures containing Palatase. However, longer chain ethyl esters were significantly higher in *K. marxianus-L. fermentum* samples despite lower ethanol levels before addition of Palatase. This selectivity could be due to the pH of the samples, which affected Palatase activity. Various pH values led to various substrate selectivity rates of Palatase as a result of conformation alterations as well as decreased quantity of ionized FFAs that inhibited enzyme activity [33]. Naturally, the working pH range of Palatase is 5.0-9.5 with an optimum of 6.5-7.5 [34]. The pH values of *K. marxianus-L. paracasei* samples were lower than those of *K. marxianus-L. fermentum* samples and both co-culture samples with Palatase included pH values lower than those the *K. marxianus* mono-culture samples did (5.33 ±0.2 after 48 h of fermentation). Only samples inoculated with *K. marxianus-L. paracasei* included pH values lower than 5.0. These low pH values most likely included inhibitory effects on esterification reactions by Palatase. The lower ester levels in *L. paracasei* co-culture could also be due to higher ethanol levels of *K. marxianus-L. paracasei* samples (FID peak area ($\times 10^6$) of 583.23 ±35.55) that partially inhibited Palatase, compared to *L. fermentum* co-culture samples [35]. Ethanol decreases after Palatase addition in *K. marxianus-L. paracasei* co-cultures were higher than those in *K. marxianus-L. fermentum* samples (FID peak areas ($\times 10^6$) decrease of 407.99 compared to 180.92). Therefore, ethanol might enter into other reactions. Between the mono-cultures with Palatase, *K. marxianus* produced the highest levels of esters. Levels of most esters decreased as ethanol decreased in co-culture samples with Palatase, compared to *K. marxianus* mono-culture samples.

Levels of esters in co-cultured samples were significantly higher than those in lactobacilli mono-cultures. This increase resulted from the much higher levels of ethanol produced by *K. marxianus*. Hence, *K. marxianus* can be used as an adjunct culture to increase ethanol and ester levels in lipase catalyzed flavor ester synthesis coupled with LA fermentations. It is noteworthy that ethyl esters detected in fermented samples with no Palatase were not produced *ex vivo*; however, they included metabolites of the microorganisms [36,3]. In the current study, ethyl lactate was expected to be synthesized by Palatase since LA was produced by the lactobacilli during fermentation [37]. Nevertheless, high hydrophilic characteristics of ethanol and LA could prevent ethyl lactate synthesis. Hydrophilic FAs such as acetic acid and butyric acid were transesterified from milk triglyceride sources. Therefore, esterification of these chemicals was easier than esterification of free LA.

Table 4. Free fatty acid production (FID peak area ($\times 10^6$)) in samples during 48 h of fermentation by *Kluyveromyces marxianus* and *Lactobacillus* co-cultures with and without Palatase

Microbial culture	*K. marxianus-L. fermentum*		*K. marxianus- L. paracasei*		Without microorganism	
Palatase addition	24 h	Without P	24 h	Without P	24 h	Without P
Acetic acid	3.68 ± 0.57^{b}	3.88 ± 0.23^{b}	9.90 ± 1.06^{c}	21.98 ± 0.93^{d}	1.22 ± 0.48^{a}	nd
Butanoic acid	47.00 ± 12.85^{b}	1.46 ± 0.04^{a}	83.68 ± 10.67^{c}	6.20 ± 0.47^{a}	117.97 ± 7.38^{d}	nd
Hexanoic acid	161.39 ± 28.87^{c}	6.11 ± 0.41^{a}	104.86 ± 22.49^{b}	25.01 ± 3.26^{a}	543.74 ± 21.90^{d}	1.93 ± 0.21^{a}
Octanoic acid	104.69 ± 36.04^{b}	6.68 ± 1.52^{a}	123.39 ± 29.18^{b}	11.88 ± 0.34^{a}	219.71 ± 24.20^{c}	2.45 ± 0.61^{a}
Decanoic acid	95.71 ± 28.91^{c}	3.91 ± 1.20^{a}	103.34 ± 17.60^{c}	5.85 ± 0.99^{a}	61.17 ± 13.55^{b}	nd

Each fatty acid analyzed separately. Mean values in the same row with different lower-case letters are significantly different ($P < 0.05$). Values are expressed as the mean ± SD of three independent experiments ($n = 3$). nd= not detected, P= Palatase, FID= Flame ionization detector, *K.* = *Kluyveromyces*, *L.*= *Lactobacillus.*

Liu et al. [6] showed that butanoic acid and ethanol could not be esterified via direct esterification, whereas tributyrin with a higher hydrophobicity was a better choice for ethyl butanoate production. In this study, ethanol concentrations were assessed based on the standard curve. In general, *K. marxianus-L. paracasei* samples included 16.88 mg g^{-1} (1.69%) ethanol with no Palatase and 6.83 mg/g (0.68 %) with Palatase. Concentrations for *K. marxianus-L. fermentum* samples included 13.65 and 8.31 mg g^{-1} (1.37 and 0.83%), respectively. Since ethanol concentrations below 1% are halal, the two co-cultures with Palatase included Islamic approval [14,38].

3.3. Free fatty acid analysis

Table 4 shows FID peak areas ($\times 10^6$) of the major FFAs from the milk samples fermented for 48 h using *K. marxianus-L. paracasei* and *K. marxianus-L. fermentum* co-cultures with and without Palatase addition. In uninoculated samples with no Palatase (whole milks), hexanoic and octanoic acids were detected that possibly were produced by microbial or milk lipases before sterilization [39]. With no Palatase addition, FFAs were produced by the micro-organisms. The *K. marxianus-L. paracasei* co-cultures produced more acetic acid than that the *K. marxianus-L. fermentum* co-cultures did, possibly due to the higher ability of *L. paracasei* than *L. fermentum*, higher viable cell counts of *L. paracasei* or synergistic effects of *K. marxianus-L. paracasei* co-cultures in acetic acid production. In this study, FFAs increased in uninoculated samples after Palatase addition. In contrast, FFAs increased in fermented samples after Palatase addition except for acetic acid. It could be due to inhibitory effects of Palatase on acetic acid production by microorganisms. In presence of Palatase, FAs of the fermented samples were less than of FAs of the uninoculated samples since FAs partially entered into esterification reactions with ethanol; however, fermentation increased decanoic acid, compared to uninoculated samples. Factors likely increased Palatase activity in decanoic acid hydrolysis from triglycerides. In a study on milk hydrolysis using Palatase and protease, FA liberation increased due to the synergistic effects of protease [40]. It seems that pH differences between the *K. marxianus-L. paracasei* and *K. marxianus-L. fermentum* samples did not affect Palatase hydrolytic activity. This result was similar to results by Sun et al. [14] that the synthetic activity of Palatase in coconut creams was affected by pH whereas the hydrolytic activity was not affected.

4. Conclusion

This study was carried out to assess *K. marxianus*-lactobacilli co-cultures as *in situ* ethanol producers for Palatase use coupled with fermentation systems of flavor ester synthesis. Co-culturing of *K. marxianus* with two *Lactobacillus* spp. resulted in various effects on viable cell counts. Palatase addition at 24 h included various effects on the cell growth of co-cultures, compared to that it included on the cell growth of mono-cultures. Palatase included significant effects on flavor ester synthesis in fermented milk samples. Co-culture samples included less ester levels, compared to that the *K. marxianus* mono-cultures did. However, *K. marxianus* can be used as an appropriate adjunct culture in *Lactobacillus*-Palatase flavor producing systems for increasing ethanol and ester levels. In acceptability, the final product includes fruity flavor and thus can be used as a natural fruity flavoring agent or flavor enhancer in formulations of other products. Furthermore, this product can be consumed directly if diluted with non-flavored milks or adjusted for microbial cultures and Palatase quantities to achieve milder tastes. Masking off-flavors such as goat-like and mutton-like flavors in dairy products can also benefit this flavoring agent. The flavor synthesis procedure can be an appropriate alternative for artificial flavoring agents.

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6. Conflict of Interest

The authors report no conflicts of interest.

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کشت همزمان مخمر-لاکتوباسیلوس به‌عنوان تولیدکنندگان درجا اتانول به‌منظور ساخت استر عطر و طعم دار با استفاده از لیپاز در شیر تخمیری

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چکیده

سابقه و هدف: امروزه، برای تولید طعم دهنده‌ها روش‌های نوین زیست‌فناوری ترجیح داده می‌شوند، زیرا روش‌های سنتی دارای معایبی هستند. هدف این مطالعه، ارزیابی بیوسنتز عطر و طعم‌های میوه‌ای طبیعی به صورت درجا در شیر تخمیری با استفاده از کشت‌های همزمان میکروبی و آنزیم لیپاز بود.

مواد و روش ها: ترانس استری‌کردن چربی شیر با اتانول برای تولید عطر و طعم‌های میوه‌ای در شیر کامل فرادما، با استفاده از لیپاز پالاتاز همراه با تخمیر اتانولی انجام شد. به‌منظور تولید درجا اتانول، از *کلایورومایسس مارکسیانوس* NCYC 1425 در کشت همزمان با *لاکتوباسیلوس فرمنتوم* PCC یا *لاکتوباسیلوس پاراکازئی* L26 استفاده شد. اثر کشت همزمان بر زنده‌مانی سلول و پیشرفت تخمیر، با استفاده از شمارش سلول‌های زنده و اندازه‌گیری pH نمونه ها در ساعت ۲۴،۰ (افزودن پالاتاز) و ۴۸ (پایان تخمیر) ارزیابی شد. از ریز استخراج با فاز جامد از فضای فوقانی-کروماتوگرافی گازی برای آنالیز استر، اتانول و اسیدهای چرب آزاد در ابتدا و انتهای تخمیر استفاده شد. منحنی استاندارد اتانول برای ارزیابی محصولات از نظر حلال بودن مورد استفاده قرار گرفت.

یافته‌ها و نتیجه‌گیری: *کلایورومایسس مارکسیانوس* دارای اثرات هم افزایی بر رشد *لاکتوباسیلوس پاراکازئی* و نیز اثر مهاری بر رشد *لاکتوباسیلوس فرمنتوم* بود. در صورت افزودن پالاتاز، اثرات ضدمیکروبی در کشت‌های همزمان *کلایورومایسس مارکسیانوس-لاکتوباسیلوس پاراکازئی* مشاهده شد. پالاتاز به‌طور معنی‌داری میزان استر نمونه‌های تخمیر شده را افزایش داد. در کشت‌های همزمان تفاوت معنی‌داری در میزان استرهای کوتاه زنجیرتر (استر اسیدهای چرب با ۴-۷کربن) نداشتند؛ در مقابل، *کلایورومایسس مارکسیانوس-لاکتوباسیلوس فرمنتوم* منجر به میزان بیشتر استرهای با زنجیره طولانی‌تر شد. هرچند کشت‌های *کلایورومایسس مارکسیانوس* دارای میزان بالاتر استر در مقایسه با کشت‌های همزمان آن بود، می توان از آن به عنوان کشت کمکی مناسبی به همراه کشت‌های *لاکتوباسیلوس* برای تقویت ساخت استرهای عطر و طعم دار استفاده کرد. این ساخت عطر و طعم می‌تواند جایگزین مناسبی برای مواد مصنوعی عطر و طعم دهنده باشد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

تاریخچه مقاله

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واژگان کلیدی

- شیر تخمیری
- استر عطر و طعم‌دار
- *کلایورومایسس مارکسیانوس*
- *لاکتوباسیلوس*
- لیپاز
- ریز استخراج با فاز جامد SPME

***نویسنده مسئول**

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