Valdez Castillo Mariana (Orcid ID: 0000-0002-3524-5660)
Brar Satinder (Orcid ID: 0000-0001-6633-4247)

Title: Production of aroma and flavor-rich fusel alcohols by cheese whey fermentation using the *Kluyveromyces marxianus* and *Debaryomyces hansenii* yeasts in monoculture and co-culture modes

Short title: Production of fusel alcohols by whey fermentation using yeasts

Mariana Valdez Castillo[1], Hamed Tahmasbi[2], Vinayak Laxman Pachapur[1], Satinder Kaur Brar[1,3*], Dajana Vuckovic[2], Dimitri Sitnikov[2], Sonia Arriaga[4], Jean-François Blais[1], Antonio Ávalos Ramirez[1,5]**

[1]Institut national de la recherche scientifique, Centre-Eau Terre Environnement, 490, Rue de la Couronne, Québec, Québec G1K9A9, Canada.

[2]Department of Chemistry and Biochemistry Loyola Campus, Concordia University, 7141 Sherbrooke Street West, Montreal, Québec, H4B 1R6, Canada.

[3]Department of Civil Engineering, Lassonde School of Engineering, York University, Toronto, Ontario M3J 1P3, Canada.

[4] Instituto Potosino de Investigación Científica y Tecnológica (IPICyT), División de Ciencias Ambientales, Camino a la Presa San José 2055, Lomas 4a Sección 78216, México.

[5]Centre National en Électrochimie et en Technologies Environnementales Inc. 2263, avenue du Collège, C.P. 610, Shawinigan (Québec) G9N 6V8, Canada.

Corresponding authors:

*Phone: 1 4167362100; E-mail: satinder.brar@ete.inrs.ca and Satinder.Brar@lassonde.yorku.ca

**Phone: 1 819 539 8508; E-mail: aaramirez@cnete.qc.ca

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jctb.6763

Abstract

Background: Whey is one of the main agro-industrial by-products generated in the production of cheese. It is a residue with low pH and high content of organic and inorganic compounds, such as lactose, proteins and minerals that can be used as nutrients in fermentation. The aim of the present study was to produce biomolecules with aroma and flavor properties by whey fermentation using *Kluyveromyces marxianus* (KM) and *Debaryomyces hansenii* (DH).

Results: These yeasts produced and accumulated in the culture broth aroma and flavor compounds, the main ones being ethanol, glycerol, propanoic acid, dihydroxyacetone, methionol, isopentanol and 2-phenylethanol (2PE). This last one was retained as the target biomolecule, because of its potential to be commercialized industrially. Both yeasts were able to metabolize L-phenylalanine (Lphe) to produce 2PE, in monoculture and co-culture modes. When yeasts were used under monoculture mode, KM produced the highest 2PE concentration 82±28 mg/L under aerobic fermentation, with yield of 0.16±0.08 g_{2PE}/g_{Lphe} and a productivity of 0.86±0.18 $mg_{2PE}/L*h$ at a fermentation time of 96 h. Whereas in co-culture mode the 2PE yield was 0.38 g_{2PE}/g_{Lphe}, twice as high as the maximum yield for monocultures. This yield corresponded to a productivity of 1.93±0.02 $mg_{2PE}/L*h$.

Conclusion: The whey fermentation using KM and DH in co-culture mode is technically feasible. The KM yeast is apparently dominant and the co-culture of both yeasts led to increase in the 2PE yield and the productivity. The faster kinetics of KM quickly induced substrate starvation triggering early production and accumulation of 2PE.

Keywords: aerobic whey fermentation, yeasts, biotransformation, L-phenylananine, 2-phenylethanol

Nomenclature:

1KM:5DH	Co-culture of KM and DH yeasts at a of ratio 1:5

1KM:2DH	Co-culture of KM and DH yeasts at a of ratio 1:2
1KM:1DH	Co-culture of KM and DH yeasts at a of ratio 1:1
2Hfuranone :	2(3H)-furanone,dihydro-4-hydroxy
2KM:1DH	Co-culture of KM and DH yeasts at a of ratio 2:1
2PE :	2-phenylethanol
3D6Mpyranone :	3,5-dihydroxy-6-methyl-2,3-dihydropyran-4-one
4HDfuranone :	4-hydroxy-2,5-dimethyl-3(2H)-furanone
5Hfurfural :	5-hydroxymethylfurfural
5KM:1DH :	Co-culture of KM and DH yeasts at a of ratio 5:1
AA :	Amino acid
BA :	Butanoic acid
CWP :	Cheese whey powder
DH :	*Debaryomyces hansenii* yeast strain Y-1408
DHA :	Dihydroxyacetone
GLY :	Glycerol
KM :	*Kluyveromyces marxianus* yeast strain Y-1109
LM :	Lactose medium
Lphe :	L-phenylalanine
MRP :	Maillard reaction products
PA :	Propanoic acid
W :	Cheese whey powder diluted in water to reach a lactose concentration of 45 g/L
WY :	Cheese whey powder diluted in water to reach a lactose concentration of 45 g/L enriched with yeast extract and peptone
μ :	Growth rate

Introduction

Canada is one of the ten main cheese producer countries, reporting $5.2x10^5$ tonnes of cheese in 2019 [1]. Cheddar and mozzarella are the most common types of cheese in Canada. For example, 164 million kg of cheddar cheese was produced in 2019, representing 31% of the dairy cheese market. The consumption of cheese increases, causing an increase in by-products generation.

Whey is a liquid residue derived from the milk proteins coagulation which is promoted by the use of acids, starter microorganisms-cultures or an enzymatic action [2–4]. In general, the production of 1 kg of cheese generates 9 kg of whey [4]; therefore, in Canada in 2019 we can estimate the production of whey at approximately $4.7x10^6$ tonnes [5,6], representing 2.04 % of the annual whey production [5,6]. Whey contains up to 55% of total solids derived from milk, which could be considered as economical waste for the dairy industry. Whey contains lactose 45 g/L, proteins from 2.42 to 10 g/L, inorganic nitrogen from 1.3 to 1.19 mg/g and phosphate from 1 to 4.5 g/L, representing a chemical oxygen demand (COD) of 60 – 80 g/L [2–4]. It has a pH of 3.8 to 5.6, with low alkalinity (lower than 0.22 g as $CaCO_3$/L) [2,7]. Whey is an effluent with a high organic and inorganic load, representing an environmental risk when released without treatment. It would contribute to the eutrophication of water bodies, the agglomeration of soil structures and the increase of salts in environmental matrixes [3].

Currently, whey is used as feedstock for the farm animals, however, this can cause stomach diseases in the animals [8]. Biotechnological processes have been developed to valorize the whey. Among them, the biorefinery is a current process in which the fermentation is used to transform the whey into high value-added compounds, such as prebiotics, food biocolorants, bacterial cellulose, foaming agents, emulsifiers, microgels, microspheres, hydrogels, aromas and flavors [8–10]. Whey fermentation has been carried out using lactic acid bacteria, fungi and yeasts [11–14]. Of those, yeasts present advantages in fermentation because they tolerate low pH and they can directly consume whey lactose as a source of carbon. They can also transform amino acids into alcohols with high molecular weight (fusel alcohols), by the Ehrlich pathway [10,15]. The fusel alcohols compounds are in high demand as aroma and flavor additives in the food, cosmetic and pharmaceutical sectors. Among the fusel alcohols, the 2-phenylethanol (2PE) is a compound of interest because of its aroma (rose-honey) and antimicrobial properties. It is

currently used as an additive in pesticides, preservative in foods and aroma in personal care products. It is a high valued compound, with a current market price around $100/kg [16,17].

The biological production of 2PE is limited by the use of L-phenylalanine (Lphe) as a precursor and the 2PE yield ranging from 0.08 to 0.78 g_{2PE}/g_{Lphe} depending on the operating conditions [10,15,18]. Thus, the objective of the present research was to study whey fermentation using *K. marxianus* and *D. hansenii* yeasts to produce aromas and flavors. The study was performed in two stages. First, the use of KM and DH to produce aromas and flavors was validated, analyzing the main biomolecules that can be obtained. Second, the effect of the yeast culture mode (mono- and co-culture) and media composition on 2PE production was investigated. To our knowledge, there are no previous studies about the co-culture of these two yeasts using whey as substrate to produce 2PE.

2. Materials and Methods

2.1 Culture media

Cheese whey powder (CWP) was used as a substrate (CRINO/Agropur, Canada). The CWP composition (% w/w) was: crude proteins 2, moisture 5, lactose 80, and ash 9. Yeast extract (Fisher Scientific, Canada) and peptone (Organotechnie S.A.S, Canada) were used as a source of nitrogen supplements. Lactose (Sigma-Aldrich, Canada) was used as a reference carbon source. Yeast malt extract medium (YME) containing glucose 10 g/L, malt extract 3 g/L, peptone 5 g/L, and yeast extract 3 g/L was prepared to revive the yeasts.

Phenylalanine and mobile phase additives were purchased from Millipore Sigma (Oakville, ON, Canada) and phenylalanine(d5) from CDN Isotopes (Pointe-Claire, QC, Canada). LC-MS grade solvents were purchased from Fisher Scientific Inc. (Ottawa, ON, Canada).

Ethanol, propanoic acid and butyric acid at 98% of purity were purchases from Sigma-Aldrich, Canada. 2PE and dihydroxyacetone at 99% purity were purchased from Fisher Scientific, Canada.

Lactose medium (LM) was used as a reference; it contained lactose 20 g/L, yeast extract 10 g/L, and peptone 20 g/L. To study the effect of an external nitrogen source, two media were prepared as follows: one was prepared with 54 g/L of CWP in water which contained lactose at a concentration of 45 g/L (medium "W"), the other was similar to W, but supplemented with 10 g/L of yeast extract and 20 g/L of peptone (medium "WY"). The pH was adjusted to 4.5 with a 6M HCl solution.

2.2 Yeasts and inoculum

Kluyveromyces marxianus NRRL Y-1109 (KM) and *Debaryomyces hansenii* NRRL Y-1448 (DH) were selected for their capacity to metabolize lactose and proteins and to produce aroma and flavor biomolecules. The yeasts were conserved in Petri plates with YME solidified with 20 g/L of agar and following the instructions of collection cultures where they were acquired (United States Department of Agriculture; USDA). One loop of grown-up yeast colonies was transferred to 30 mL of LM medium contained in 125 mL flask and incubated at 25 °C and 200 rpm during 20 h to prepare the inoculum.

2.3 Fermentation conditions

In the first stage, anaerobic and aerobic fermentations were carried out in 125 mL serum bottles and flasks filled with 30 mL of medium, respectively. The LM, W, WY were added to containers and autoclave sterilized at 121 °C for 20 min. To study the KM or DH monoculture effect, the inoculum was added to the containers using a concentration of $1.5x10^7$ CFU/mL of each yeast strain, which corresponded to an optical density (OD_{600}) of 0.1. The OD_{600} was measured using a UV-VIS spectrophotometer (Epoch microplate spectrophotometer 2666795,

BioTek, Vermont, U.S.A.). Assays were performed in duplicates and the fermentation lasted 108 h. Temperature and agitation were set at 25 °C and 200 rpm respectively. Samples of 1 mL were taken at 0, 4, 8, 24, 32, 48, 54, 72 and 96 hours for biomass analysis. Additionally, the samples taken at 0, 24, 48 and 96 h were used for yeast growth and metabolite analysis and those taken at 0, 14, 32, 54, 72 and 108 h for Lphe analysis. Aerobic and anaerobic controls of media incubated under the same conditions were carried out to corroborate sterility and determine the molecules naturally present in each medium.

In the second stage, five co-culture ratios of KM and DH were tested. For the co-culture assays, the inoculum of each yeast was added to have five mixture ratios of KM and DH in the culture broth at time 0 h according to the OD_{600}. The KM:DH mixture ratios were: 0.1:0.5, 0.1:0.2, 0.1:0.1, 0.2:0.1 and 0.5:0.1 and were identified as 1KM:5DH, 1KM:2DH, 1KM:1DH, 2KM:1DH and 5KM:1DH respectively. The co-culture assays were performed under aerobic fermentation of WY during 96 h at 25 °C and 200 rpm. The samples were taken at the same fermentation time as monoculture assays.

2.4 Kinetics of yeast growth and metabolite production

The growth rate was calculated for biomass concentration (dry basis) and cell density by counting plates [19]. Biomass concentration was used for the monocultures and cell density for the co-cultures. To determine the Lphe consumption rate, data were fitted using zero-order reaction kinetics [19,20].

The 2PE productivity was calculated by dividing the concentration of 2PE at 96 h of fermentation. Finally, the production yield was calculated as the ratio of the mass of 2PE to the mass of Lphe consumed.

2.5 Sample handling

Samples of monoculture and co-culture assays were handled as follows: sample volumes of 1 mL were taken at the aforementioned times. Each sample was transferred to microtubes and centrifuged (Minispin plus, Eppendorf) at 9660*x g* for 2 min. The supernatant was stored at -20 °C until analysis. Pellets were dried at 60 °C for 24 h to quantify biomass.

To determine the cellular density during co-culture assays, serial dilutions (from 10^{-1} to 10^{-8}) from samples of 1 mL were prepared and cultured in plates containing solid LM with 20 g/L of agar.

2.6 Preliminary identification of aroma and flavor metabolites using GC-MS

In the first stages, the identification of metabolites was performed at 72 h of fermentation using gas chromatography (Trace 1310, Thermo Scientific), coupled with a mass spectrometry detector (ISQ Thermo Scientific) (GCMS). The identification method was adapted from Leclercq-Perlat et al., (2004) [18]. The GCMS was equipped with a CP-Wax 57 CB (Agilent Technologies Inc.) column with a length of 25 m, an internal diameter of 0.25 mm, and a film thickness of 0.20 µm. The GC temperature for the injector and detector were both set to 240 °C. 0.7 µl of the undiluted sample was injected in the splitless mode. The oven temperature was held at 70 °C for 3 min, then it increased to 200 °C, at 5 °C/min, and finally held at 200 °C for 6 min. Helium at 103 kPa was used as a gas carrier, at a flow of 1 mL/min. To identify the biomolecules, the match factor, reverse match factor and probability in the library of the mass spectral database were calculated (Agilent Technologies, Inc).

2.7 Quantification of lactose, Lphe and metabolites

Lactose was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS, using a Surveyor Instrument, Thermo Scientific, Mississauga, ON, Canada) using the method reported earlier by Osorio-González, et al., 2019 [21]. In brief, 20 µL of the sample was injected into a Shodex HILICpak VG-50 2D (150×2 mm, 5 µm; Canadian Life Science) column at

40°C. A solution of acetonitrile:water (89:11) was used as mobile phase and samples were eluted at a flow rate of 0.3 mL/min. Glucose-D2 98% purity (CDN Isotope, Pointe-Claire, Québec) and Lactose 99% purity (Sigma Aldrich, Canada) were used as the internal standard for CIP detection and for the calibration curve, respectively. The concentration was calculated based on the analyte areas and the calibration curve [21].

To determine the Lphe concentration, the samples of culture broth were analyzed by Liquid chromatography – high resolution mass spectrometric (LC-HRMS). LC-HRMS analysis was executed on an Agilent 1290 Infinity II liquid chromatograph connected to QTOF 6545 (Agilent Technologies, ON, Canada) 2 x 150 mm, mixed-mode Scherzo SM-C18 column with 2 x 5 mm guard packed with 3 µm particles, (Imtakt, Portland, USA) at 35 °C and a flow rate of 0.2 mL/min were used for the analysis. The injection volume was 10 µL for all the analyses. The gradient was generated using mobile phase A consisting of 5 mM ammonium formate in water and mobile phase B, consisting of 0.5% (v/v) formic acid in methanol. The run began with 2 min isocratic 100% A, followed by a linear increase to 24% B in 6 min and a further increase to 90% B in 0.1 min. 90%B was held for 3.9 min and the column was then re-equilibrated for 8 min to starting conditions of 100% A. Positive electrospray ionization was used for the analysis, with the capillary voltage set to 3500 V, nozzle voltage to 800 V, fragmentor voltage to 175 V, drying gas temperature to 250 °C and sheath gas temperature to 275 °C. Data were acquired in the 50-1000 m/z range at an acquisition rate of 3 spectra/s. The column output was directed into waste during two segments: (i) between 0 and 2 min and (ii) between 8.5 and 14.5 min to reduce contamination of the source. Phenylalanine quantitation was executed using Mass Hunter Quantitative Analysis (v. 10) software and weighted (1/x) calibration curves were built using the peak areas extracted with a mass accuracy window of 20 ppm. To ensure the desired mass accuracy of recorded ions, continuous internal calibration was performed throughout the sample analysis using signals at m/z 121.0509 (protonated

purine) and m/z 922.0098 (protonated hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine (HP-921)).

The quantification of metabolites was determined using gas chromatography (Thermo Scientific), equipped with flame ionization detection (GC-FID).The quantification method was adapted from Dragon et al. 2009 [14]. The GC-FID was equipped with a Split/Splitless injector capillary column CP-Wax 57 CB (25 m, 0.25 mm and film thickness of 0.20 µm; Agilent Technologies Inc.). The temperature of the injector and detector was set to 250 °C. The oven temperature was held at 50 °C for 5 min, then it increased to 220 °C at 3 °C/min, and held at 220 °C for 10 min. Helium at 125 kPa was used as the gas carrier. 2 µl of the sample was injected in the splitless mode (vent time, 15 s); isobutanol (internal standard) was added to the sample at a concentration of 1 mg/L [14]. The volatile compounds were identified by comparing the retention times with those of standard compounds (ethanol, propanoic acid, butyric acid, 2PE and dihydroxyacetone), and the quantification was performed using GC Chromeleone Agilent FID software.

2.8 Statistical analysis

Two-way ANOVA analysis was performed to determine if the 2PE production was a function of media composition and fermentation conditions (aerobic or anaerobic). Whereas for the co-cultures, an ANCOVA of one-factor analysis was performed to determine if the 2PE production was a function of co-culture ratios. Both ANOVA and ANCOVA analyses were run in R software using the concentration of 2PE as the response variable.

3. Results and discussion

The first stage of the study was the screening and identification of compounds with aroma and flavor properties produced for the whey fermentation.

3.1 Effect of the media composition on biomolecules production under monoculture fermentation mode

The analysis of aroma and flavor molecules of control helped distinguish the molecules produced by yeasts from those that were initially present in the culture media. The GC-MS method allowed semi-quantitative determination of each compound, using the relative area obtained by chromatogram integration and expressed with arbitrary units of counts*min. The spectrum that presented a probability higher than 80% for a specific compound was retained as the most likely compound.

3.1.1. Compounds present in the culture medium

The compounds identified in controls and culture broth for both aerobic and anaerobic fermentation are shown in Table 1. The controls for all culture media contained acetic acid, 5-3,5-dihydroxy-6-methyl-2,3-dihydropyran-4-one (3D6pyranone) and 5-hydroxymethylfurfural (5Hfurfural). The last one was the most abundant compound in LM and W media controls, presenting the highest relative area of $39.5x10^7$ counts*min in the W-aerobic control. Maltol was only found in W and WY media with relative areas of $0.2\pm0.03x10^7$ and $4.1\pm1.3x10^7$ counts*min, respectively; and 3D6pyranone was the most abundant in WY media with a relative area of $10.7\pm0.8x10^7$ counts*min.

All the compounds shown in Table 1 have aroma and/or flavor properties. Specifically, the compounds identified in the controls are commonly associated with dairy products and they confer buttery, nutty or caramel flavors [22]. These compounds are commonly produced by the Maillard reaction, which occurs at temperatures from 100 °C to 149 °C. The generation of Maillard reaction products (MRP) occurs when lactose reacts with the amino groups of glycine, proline, lysine, arginine or histidine to form deoxy-ketoses. Subsequently, reduction reactions form enol groups (alkenes with a hydroxyl group) in the carbonyl structure, resulting in furfural

and pyranosyl type compounds [22]. Huffman and Ferreira (2011) [23] reported that the cheese whey naturally contains lactose and amino acids, such as lysine, arginine and histidine. In the present study, the concentration of the amino acids in the medium was increased by adding yeast extract and peptone. The enrichment in amino acids could increase the production of MRP, which was a function of media composition and sterilization conditions. According to Table 1, the relative area of acetic acid, 3D6pyranone, maltol and 5Hfurfural is higher under aerobic vs anaerobic sterilization conditions (in hermetic serologic bottles). This suggests that the content of oxygen increases the production of MRP. For example, Munanairi et al. (2007)[24] observed that the production of MRP from ribose 5-phosphate was higher when it was transformed through aerobically thermochemical process [24].

3.1.2. Compounds produced by fermentation

At 72 h of aerobic and anaerobic fermentation, the culture broth was analyzed by GC-MS to determine which compounds had been produced or consumed by the yeasts. At this point, all the fermentation assays were in the stationary phase of the cellular growth of yeasts, meaning that both primary and secondary metabolites should be present. According to Table 1, the most important compounds identified in the culture broth at 72 h were as follows:

Acetic acid. After anaerobic fermentation, the relative amount of acetic acid was higher than in aerobic fermentation for all media, except for WY with KM. In the pathway to produce acetic acid, yeasts consume lactose and transform it into glyceraldehyde-3-phosphate, then to pyruvate by glycolysis, and finally to ethanol and acetic acid. Under stressful conditions, such as limited oxygen, low pH and/or the presence of inhibitors (for example ethanol and MRP), the acetic acid is produced and accumulated in the culture media. Christensen et al. (2011) [25] observed an accumulation of ethanol up to 24 g/L when KM was used to ferment whey at 32 °C

for 50 h. This would cause the production of acetic acid, which accumulated at a concentration of 1 g/L [25].

5Hfurfural. For LM and WY fermentations, 5Hfurfural was detected only for WY anaerobic fermentation with DH. The relative area of 5Hfurfural was similar to the respective control. For the W medium fermentation assays, it was smaller than in the controls. It is known that furfurals are inhibitors for both KM and DH yeasts [26,27]. Nevertheless, as a stress-response, these yeasts produce enzymes able to attack pentoses present in the structure of furfurals [26,27]. According to Table 1, both yeasts could remove 5Hfurfural, which disappeared or decreased in concentration at 72 h for all fermentations.

Pyranosyl type compounds. 3D6Mpyranone is a pyranosyl type compound which was found in all assays. Its relative amount after fermentation was higher than in the controls for all assays using DH, except for WY media. Similarly, the relative amount of maltol, another pyranosyl type compound, was higher for W and WY anaerobic fermentation than in controls also using DH. DH could produce the homodimeric type III polyketide synthase. This enzyme is produced by means of the polyketide pathway and triggered by the presence of the lactose. The enzyme catalyzes the formation of multiple ketene groups and cyclization reactions of organic carbon structures. The polyketide synthase carboxylates the acetyl-coenzyme A to transform it into malonyl coenzyme A. Then, condensation chain reactions are initiated to cycle the malonyl coenzyme A. The final step involves enolization reactions to form structures such as 3D6Mpyranone and maltol [28,29].

Propionic (PA) and Butanoic (BA) acids. Both yeasts produced PA, and BA under aerobic conditions, with PA being more abundant than BA in WY fermented by KM ($4.5x10^7$ counts*min). They are produced by the degradation of pyruvate by the acetyl CoA carboxylase [30,31]. Both PA and BA are flavor and aromatic compounds commonly found on the cheese

surface during ripening. To the best of our knowledge, this is the first study reporting that DH and KM can specifically produce these acids.

Glycerol (GLY). Glycerol was only identified in W and WY fermented by KM. Glycerol was the most abundant metabolite, with relative areas in a range of $11.5\pm0.6x10^7$ to $24.4\pm3.2x10^7$ counts*min under aerobic and anaerobic conditions, respectively. Its production is caused by an osmotic stress-response of KM. Glycerol is a cell-protector compound produced during the transformation of glycerol-3-phosphate by glycerol-3-phosphate dehydrogenase [32]. In the present study, the osmotic stress could be caused by the high initial lactose concentration in W and WY media (45 g/L), which was 2.25 fold times than the lactose concentration of LM (20 g/L). Also, the salts naturally present in the CWP contributed to increasing the osmotic pressure. Some studies showed that increasing the initial lactose concentration from 50 g/L to 100 g/L induced the production of GLY by KM, which accumulated at a concentration of 2.2 g/L and 2.7 g/L, respectively [32,33].

Dihydroxyacetone (DHA). This compound was only produced by DH at an average relative area of $0.70\pm0.1x10^7$ counts*min for the assays with W and WY media. Some studies have reported that DHA is a by-product of GLY transformation. Similarly to KM, DH can produce and accumulate GLY to equilibrate the osmotic pressure of the environment. In the case of whey fermentation, DH could accumulate the GLY intracellularly as a response to the osmotic pressure produced by lactose and salts [34]. In contrast with KM, DH can transform GLY when it is over accumulated, stimulating the transcription of four key enzymes: glycerol-3-phosphate dehydrogenase, dihydroxyacetone kinase, glycerol dehydrogenase and a phosphatase. The first enzyme catalyzes the production of glycerol-3-phosphate from dihydroxyacetone phosphate, which can be transformed into dihydroxyacetone by the dihydroxyacetone kinase. Then, the glycerol-3-phosphate is transformed into GLY by the phosphatase, and simultaneously the glycerol-dehydrogenase along with the kinase transform the intracellular accumulated GLY

into DHA and dihydroacetone phosphate [34,35]. This allows the production of dihydroxyacetone and prevents the intracellular accumulation or the excretion of GLY. This phenomenon was observed for the first time by Adler et al. (1985) [34] and later confirmed by Gori et al. (2007) [35] when the NaCl at 8 % (wt/v) promoted the accumulation of GLY and DHA in DH cells.

Fusel alcohols. The production of 2PE, isopentanol and methionol was stimulated under aerobic conditions. 2PE was identified in all the fermentation assays performed with KM, whereas for DH the 2PE was only produced when LM and WY media were fermented. Isopentanol and methionol were produced by KM under aerobic conditions. The production of fusel alcohols is limited by the availability of amino acids, which are transformed into their respective fusel alcohol by the Ehrlich pathway [36]. All the media tested (LM, W and WY) contained Lphe, leucine and methionine, which were provided by yeast extract, peptone and the CWP itself [23]. These amino acids can be transformed into 2PE, isopentanol and methionol, respectively. In the fusel alcohols pathway, the pyruvate is transformed into α-ketoglutarate during the Krebs cycle. In this way, the production of fusel alcohols is stimulated by the presence of oxygen. The α-ketoglutarate acts as an electron acceptor from the transamination of amino acids, and subsequent decarboxylation and final dehydrogenation are carried out to produce the respective fusel alcohols [15,36]. Wittman et al. (2002) [15] shown that the presence of α-ketoglutarate is necessary to produce 2PE from Lphe during glucose fermentation [15].

The first stage determined the compounds that can be produced by whey fermentation with KM and DH. For the next stages of the present study, Lphe consumption and the production of 2PE were monitored. Also, the isopentanol concentration through the kinetics was determined by GC method. Whereas, the production of methionol was not studied further because it was only identified in WY aerobic fermentation by KM. GLY is not considered either an aromatic compound or a flavor, thus its production was not followed further in this study.

3.2 Whey fermentation under monoculture mode

Yeast growth

Figure 1 shows the biomass concentration (dry basis) of KM and DH for aerobic and anaerobic fermentations under monoculture mode. For the aerobic assays, the exponential phase occurred from 8 h to 32 h, except for DH in WY, where the exponential phase ended at 72 h. The anaerobic fermentation did not present a lag phase and the exponential phase finished within 24 h. For both yeasts, Table 2 shows the kinetics parameters of growth rate (μ), Lphe consumption, maximum yield and maximum productivity of 2PE under the fermentation conditions. The μ for DH was always higher than for KM, except for LM-anaerobic, where it was nearly similar. Among all assays, the μ of DH (0.16 h^{-1}) calculated for WY medium fermentation was the maximum value observed.

μ is a kinetics parameter defined by the strain and fermentation conditions. For WY-aerobic, the μ of both KM and DH strains was higher than for the rest of the assays. This shows that the aerobic fermentation of a culture medium enriched with organic nitrogen was favorable for KM and DH growth. The μ for DH fermenting glucose (20 g/L) was 0.21 h^{-1} [37,38], and for KM fermenting lactose (50 g/L) at 35 °C, was 0.55 h^{-1}. [32].

The μ obtained in this study was smaller than the μ of DH and KM of other studies from 0.21 to 0.55 h^{-1} [32,37,38]. However, the μ of the present study was not obtained under optimal conditions. The highest biomass concentration was observed for WY-aerobic, which can be explained by the enrichment of the medium with yeast extract and peptone as an organic nitrogen source.

For almost all fermentations, the biomass concentration after reaching the stationary phase was in the range of 5.25 to 12.35 g/L. However, for WY-aerobic using either KM or DH, the biomass concentration was higher, 22 g/L and 33 g/L respectively (shown in Figure 1). This

can be explained by the combination of a rich media and oxygen availability. The electrons donated by the oxidation of lactose are used to produce energy which is mainly used for cell synthesis [25,39], or store it under chemical bonds (example ATP). This suggests that under aerobic fermentation KM and DH used energy for cell synthesis.

In the case of WY-aerobic using DH, the highest biomass concentration of 33 g/L can be explained by the effect of a high concentration of lactose and salts in media. Generally, KM excretes the glycerol, while DH accumulates it inside the cells. For example, some studies show that DH could accumulate up to 0.34 $g_{GLY}/g_{biomass}$ when the concentration of NaCl was 16 % wt/v [34].

The DHA is also formed using the glycerol pathway, where DHA can be transformed into glycerol utilizing the glycerol dehydrogenase and the coenzyme NADP/NADPH. In the present study, the DHA concentration was lower than 82 mg/L except for the W fermentation using DH, where it was 1719±328 and 1876±114 mg/L under aerobic and anaerobic conditions respectively. The W medium was the only one not enriched with peptone and yeast extract. This suggests that the production of energetic molecules was limited, and as a consequence the DHA was not converted into glycerol, and was excreted [34,35].

Lphe consumption and alcohols production of 2PE and ethanol by *Kluyveromyces marxianus*

Figure 2 shows the production of 2PE and ethanol for the aerobic and anaerobic fermentation using KM. The aerobic condition stimulated the production of 2PE by KM, especially for WY fermentations. For this case, the highest production of 2PE was observed, 2.46±0.70 mg_{2PE} at 96 h (corresponding to a concentration of 82.12±16.88 mg/L). At this point, KM presented also a maximum 2PE yield of 0.16±0.03 mg_{2PE}/mg_{Lphe} and the highest Lphe consumption rate of 7.04 mg_{Lphe}/L*h (Table 2). In contrast to kinetics parameters such as productivity, the

production yield is not a good indicator of metabolite production for 2PE. For example, for W media, the yields for aerobic and anaerobic fermentations were 0.75 and 0.55 g_{2PE}/g_{Lphe}, respectively. However, the Lphe consumption rate for W-aerobic was the lowest value. Additionally, for W and WY aerobic and W-anaerobic fermentation, the Lphe was completely consumed, whereas, for the LM-aerobic, LM-anaerobic and WY-anaerobic, the Lphe was partially consumed, corresponding to 51.6, 35.5 and 29.3% of the initial concentration of Lphe 492.3±1.3 mg_{Lphe} (equivalent to a mass of 14.8±0.0 mg_{Lphe}), as expressed in Table 2.

The ethanol concentration was higher for the anaerobic assays than for the aerobic ones. In all cases, the highest ethanol concentration was observed around the end of the exponential growth phase. Maximum ethanol concentrations corresponded to WY-aerobic and anaerobic with 16.5±2.3 and 17.2±1.5 $g_{ethanol}/L$ respectively. After the maximum accumulation, the ethanol was quickly consumed decreasing nearly to 0 $g_{ethanol}/L$ at 48 h. The opposite pattern was observed for the W medium, where ethanol concentration continued increasing.

Similar to biomass production, the Lphe consumption was affected by the dissolved oxygen and the media composition. For example, under aerobic conditions, the consumption of Lphe was complete for WY and W media. This consumption can occur as follows: lactose is oxidized to pyruvate which is further oxidized by the Krebs cycle to produce α-ketoglutarate which enters the Ehrlich pathway where the Lphe is transformed to form phenylpyruvate. Then, it is decarboxylated producing phenylacetaldehyde, and reduced on 2PE, ending the reaction. This last step involves the use of a reductase or an alcohol dehydrogenase to catalyze the aldehyde reduction coupled with the oxidation of NAD(P)H to $NAD(P)^+$. Hence, the production of 2PE is an energy consumption step without being essential for cell growth. 2PE is a secondary metabolite, which accumulates at the end of the exponential growth phase; for this reason, in the present study, it appeared after 24 h. Since aerobic metabolism produces more energetic

molecules than the anaerobic one [15,36], aerobic consumption rate of Lphe was faster than the anaerobic one.

The low 2PE yield for the LM and WY assays shown in Table 2 can be attributed mainly to the presence in the culture broth of amino acids other than Lphe. These amino acids will also be transformed through the Ehrlich pathway into their respective fusel alcohols. For example, the isopentanol which is produced from leucine was accumulated in higher concentration than 2PE under aerobic conditions, from 120.0±13.5 to 200.1±23.8 mg/L (data not shown), which correspond to a mass from 3.6±0.4 to 6.0±0.7 $mg_{isopentanol}$, respectively. Additionally, under aerobic conditions the 2PE can be transesterified into 2-phenylethylacetate, decreasing its concentration in the media. The ester is formed in the presence of Acetyl CoA and an alcohol dehydrogenase which uses 2PE as a precursor [15,40]. Wittman et al. (2002) [15] studied the 2PE and 2-phenylethylacetate production during the fermentation of glucose (77 g/L) and of Lphe (7 g/L) using KM, with resulting yields of 0.65 g_{2PE}/g_{Lphe} and 0.08 $g_{2\text{-phenylethylacetate}}/g_{Lphe}$ [15].

In the present study, the WY-aerobic presented the highest content of 2PE. This medium contained 45 g/L of lactose and it was supplemented with an external nitrogen source. The combination of both lactose content and high nitrogen contributed to obtaining the highest production of 2PE (0.86 mg_{2PE}/L*h).

Lactose concentration was monitored for this WY medium only (data not shown), and at 24 h it was nearly 0 g/L. Then, KM should begin to consume ethanol as a carbon source, and as shown in Figure 1A this time corresponding to the end of KM exponential growth phase. This observation confirms that 2PE is associated with the stationary phase, where secondary metabolites are produced. Wittman et. al. (2002) [15] studied glucose fermentation using KM. They observed that KM produced ethanol, glycerol and pyruvate. When glucose was depleted at 28 h of fermentation, ethanol, glycerol and pyruvate were used as a carbon source [15].

Assuming that ethanol was consumed because lactose was depleted, the continued accumulation of ethanol for W may indicate that the lactose was not depleted during the 96 h of fermentation.

Lphe consumption and alcohols production of 2PE and ethanol by *Debaryomyces hansenii*

Figure 3 shows the 2PE and ethanol production by DH during the aerobic and anaerobic assays. For the aerobic assays of W and WY and W-anaerobic, the Lphe was totally consumed at 96 h and the faster consumption (5.73 $mg_{Lphe}/L*h$) was observed for WY-aerobic. For the LM-aerobic and LM-anaerobic and WY-anaerobic, the initial concentration of Lphe (492.3±1.3 mg_{Lphe}/L) decreased by 28, 21.3 and 33.9% respectively. The highest 2PE concentration (40.1±6.17 mg/L) occurred at 96 h of WY-aerobic (Figure 3). For the ethanol concentration, it was lower than 300 mg/L for all assays except for LM-anaerobic and WY-aerobic, which presented 1880.89±0.12 and 1700.38±0.23 mg/L at 48 h respectively.

Figures 2 and 3 show that the metabolism of DH behaved differently from KM for the same fermentation conditions. The Lphe was consumed similarly by both yeasts, but the consumption rate was slower for DH. Apparently, in this yeast the lactose carbon was mainly derived to biomass synthesis (yield of 0.73 $g_{biomass}/g_{lactose}$), instead of ethanol (0.04 $g_{ethanol}/g_{lactose}$), which was observed at a lower concentration than in KM assays. As mentioned before, the 2PE is a secondary metabolite, and it could be detected after 24 h or 48 h of fermentation. Its accumulation was more pronounced when ethanol was consumed, maybe because the availability of lactose decreased, which could be associated with inducing 2PE formation.

The statistical analysis (two-way ANOVA, $\alpha = 0.95$, indicated in Table 5) confirmed that the KM strain presented the highest 2PE production when grown in WY under aerobic conditions.

For this reason, WY-aerobic was selected to perform the next step of the co-culture of KM and DH.

3.3 Whey fermentation under co-culture mode

Growth performance and ecological behavior

Figure 4 shows the biomass concentration (dry basis) of the co-cultures assays as a function of time under the WY-aerobic. The growth pattern was similar for the five ratios studied, with exponential growth phase up to 72 h. The 1KM:1DH ratio presented the highest biomass concentration of 25.25±0.75 g/L.

Table 3 shows the μ, cell density, Lphe consumption rate, 2PE productivity and 2PE and ethanol yield under monoculture and co-culture modes. The μ was calculated by cellular counting, and the μ of co-cultures was always lower than in monocultures, except for 1KM:1DH, where it was higher. The yeast colonies were easy to differentiate. Figure 5 shows the normalized concentration of KM and DH as $\log_{10}$ of cell density at any time/cell density at an initial time for monoculture and co-culture. In general, KM showed higher growth rate than DH, even for 1KM:5DH, where DH was inoculated with a concentration 5 times higher than KM. For 1KM:5DH, the final cell density of DH was higher than KM, but KM increased its initial cell density ($1.50\pm0.04 \times 10^7$ CFU/mL) up to 1.63 times, whereas, DH increased its initial cell density ($6.00\pm0.05 \times 10^7$ CFU/mL) by only 1.28 times. Moreover, when the inoculum of KM was 5 times higher than DH (5KM:1DH), the KM yeast dominated from the beginning of the fermentation, and no colonies of DH were observed on the plates.

These results suggest that the interactions between the two yeasts are a competitive relationship. Several reports indicate the production of mycocins by both yeasts. Mycocins are extracellular proteins that can inhibit the β-glucan synthesis, a key compound in the structure

of the cell wall. Also, mycocins interfere with the synthesis of genetic material and consequently with cell division, producing the known killer phenomenon [41,42]. Banjara et al. (2016) [41] observed the production of mycocins by DH strains which were able to inhibit *Candida albicans* and *C. tropicalis* yeasts, especially under pH conditions from 4.5 to 5.5 and temperatures from 25 °C to 30 °C [41]. In addition, Chen et al. (2015) [43] studied the production of mycocins by KM at pH 2 and 8, and the inhibitory effect on *Escherichia coli* was mainly observed in a temperature range from 25 °C to 45 °C [43]. This suggests that KM and DH could also produce mycocins as a survival mechanism and predominate in the environment.

2PE and ethanol production under co-culture mode.

Figure 6 shows the production of 2PE and ethanol by the five co-culture ratios. For all assays, Lphe was depleted and the Lphe consumption rate for 5KM:1DH was the fastest (10.38 $mg_{Lphe}/L*h$), followed by 1KM:2DH (9.58 $mg_{Lphe}/L*h$).

2PE production was higher for co-cultures than for monocultures. The highest productivity was observed for 5KM:1DH (1.93±0.02 $mg_{2PE}/L*h$), which corresponded to the highest mass of 2PE accumulated in the culture broth of 5.57±0.07 mg_{2PE} (corresponding to a concentration of 185.72±2.28 mg/L). Figure 5A shows that at 48 h KM and DH yeasts in co-culture mode produced a 2PE mass of 2.39±0.07 mg_{2PE} or higher, in comparison to the monoculture mode using KM, which took 96 h to produce 2.46±0.70 mg_{2PE} (Figure 2).

For all co-culture assays, an ethanol accumulation of 396.5±17.9 to 458.1±11.1 $mg_{ethanol}$ (corresponding to a concentration of 13217.7±599.9 to 15276±367.9 mg/L) was observed at 24 h, except for 1KM:1DH, where an ethanol mass of 226.8±14.7 mg was observed at 48 h (concentration of 7560±490 mg/L). The highest ethanol mass (458.1±11.1 mg) was observed for 5KM:1DH, corresponding to a yield of 0.34 $g_{ethanol}/g_{lactose}$. Moreover, for all co-culture

assays, the lactose was depleted at 24 h, except for 1KM:1DH, where it occurred at 48 h (data not shown).

The use of co-cultures increased the Lphe consumption rate. Both yeasts consumed nutrients, such as lactose and organic nitrogen, and KM had an advantage over DH because of its faster growth (defined by μ) in comparison to DH during the co-cultures assays. Also, KM has higher lactose oxidation capacity than DH, allowing KM to produce more energy to transform Lphe into 2PE. Rodrigues et al. (2016) [44] studied a co-culture using *Sacharomyces cerevisae* and *Kluyveromyces lactis* for a whey and carob sugars mix fermentation. They observed a faster and complete depletion of sugars when they used the co-culture instead of monoculture fermentation [44].

Since 2PE is a secondary metabolite induced by the presence of Lphe, the presence of Lphe in the culture broth is necessary. When this amino acid is consumed, its amine is incorporated into the essential metabolic pathways of yeasts, such as for protein formation, biomass synthesis or energy production (Krebs cycle). In addition, in co-culture mode both yeasts compete for lactose consumption, and this could quickly energize the cells. All this led to higher 2PE production than the sum of individual production with KM and DH in monoculture mode.

The statistical analysis (ANCOVA of one factor, $\alpha = 0.95$, indicated in Table 6) showed that the yeasts growing under co-culture mode and WY-aerobic were the best conditions to produce 2PE, especially the 5KM:1DH. According to the statistical analysis, the cumulative production of 2PE was a function of biomass content. In contrast, ethanol production was not affected by either biomass or cell density. Table 4 shows the studies where whey was fermented by yeast with the aim to produce 2PE. It is expressed by the 2PE yield obtained in other studies in comparison with the present study. The present study can be a competitive process since the

whey was not supplemented directly with Lphe and both yeasts produced 2PE. Additionally, the use of the co-culture mode improved the transformation of Lphe into 2PE in comparison with the monocultures used in this study and in Leclerq-Perlat et al. (2004).

Almost all co-culture assays accumulated ethanol faster than the monoculture ones. This could be attributed to the faster lactose consumption resulting from the competition to consume the substrate, leading to a faster growth rate (Table 3). Although ethanol production was faster, the ethanol yield was in the range of 0.29 to 0.34 $g_{ethanol}/g_{lactose}$ for co-cultures, similar to the ethanol yield of KM alone (0.36 $g_{ethanol}/g_{lactose}$), except for 1KM:1DH, which had a yield of 0.17 $g_{ethanol}/g_{lactose}$. This behavior has been observed previously, showing that the ethanol yield varies slightly when single yeasts are grown under co-culture mode. Rodrigues et al. (2016) [44] observed a ethanol yield of 0.40 $g_{ethanol}/g_{sugars}$ for *S. cerevisae* and *K. lactis* under both monoculture and co-culture conditions [44]. Once lactose is consumed, the yeasts will use other carbon sources present in the culture broth, such as ethanol and glycerol. Both yeasts will interact to consume these molecules and survive. For example, KM released glycerol which could be consumed by DH. Then, DH produced and released, ethanol, which could be used by KM as an alternative carbon source. This could cause a decrease in ethanol in the culture media after 24 h, and even its complete depletion. The ethanol consumption can occur by alcohol dehydrogenase which reduces a NAD^+ enzyme to NADH, where the ethanol is transformed into an aldehyde. This reaction releases energy which is used for cell preservation and biomass synthesis [36,39]. For this reason, the use of co-cultures could improve the lactose and Lphe consumption, and their transformation into 2PE.

4. Conclusions

Whey fermentation using yeasts for the production of compounds with aromatic and flavoring properties was studied. The production of Maillard reaction products (MRP) was observed in sterilized media, probably due to the thermal reactions. Their relative abundance varied based

on the content of oxygen, lactose and amino acids on the medium. The MRP identified were 5-hydroxymethylfurfural (5Hfurfural), pyranosyl type compounds and acetic acid. The content of the MRP was modified by yeast fermentation. For example, 5Hfurfural was consumed. The enriched fermentation broth matrix produced aromatic and flavoring compounds, such as organic acids, dihydroxyacetone, isopentanol and 2-phenylethanol which were produced and excreted by both yeasts. The biomass synthesis and metabolite production were affected by the media composition, oxygen availability, yeast strain and culture mode. Thus, in the present study, the aerobic fermentation of whey powder enriched with yeast extract and peptone (as organic nitrogen sources) using *Kluyveromyces marxianus* and *Debaryomyces hansenii* at a mixture ratio of 5:1 resulted in the best conditions to produce 2PE. Under these operating conditions, the 2PE yield was 0.16±0.03 g_{2PE}/g_{Lphe}, corresponding to a productivity of 1.93±0.02 $mg_{2PE}/L*h$, which is competitive compared with studies reported in the literature using pure glucose as a substrate. The present study shows that the valorization of whey into value-added biomolecules has great potential.

As future work, it is considered the 2PE production optimization in terms of lactose and Lphe initial concentrations as well as the extraction and purification of 2PE. The distillation under vacuum conditions or the liquid-liquid extraction are downstream methods that have been studied to extract and purify the 2PE in the culture broth. The 2PE is currently used as fragrance, flavoring or antiseptic compound. The detailed information of the downstream processes and market opportunities can be found in Valdez-Castillo et al. (2020) [8] and Qian et al. (2019) [45].

Acknowledgements

Financial support from Fonds de recherche du Québec – Nature et technologies (FRQNT) (No. 87352) and Fonds de recherche du Québec – Nature et technologies (FRQNT) - Bourses de

2ème et 3ème cycles pour stage en Centre collégial de transfert de technologies (No. 279900). NSERC Discovery Grants, James and Joanne Love Chair in Environmental Engineering at York University and Institut National de la Recherche Scientifique-Eau Terre Environnement are acknowledged. One author, Vinayak Laxman Pachapur, is thankful to the "MITACS Post-Doctoral scholarship program" for financial assistance, with CRIQ as an industrial partner.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors are responsible for the content and writing of the paper.

REFERENCES

1. Goverment of Canada. Canadian Dairy Industry at a Glance - Canadian Dairy Information Centre (CDIC). *Canadadian Dairy Information Centre* (2019). Available at: http://www.dairyinfo.gc.ca/index_e.php?s1=cdi-ilc&s2=aag-ail. (Accessed: 6th April 2019)
2. Smithers, G. W. Whey-ing up the options - Yesterday , today and tomorrow. *Int. Dairy J.* **48,** 2–14 (2015).
3. Panesar, P. S. & Kennedy, J. F. Biotechnological approaches for the value addition of whey. *Crit. Rev. Biotechnol.* **32,** 327–348 (2012).
4. Anand, S., Khanal, S. N. & Marella, C. Whey and Whey Products. in *Milk and Dairy Products in Human Nutrition: Production, Composition and Health* (eds. Park, Y. W. & Haenlein, G. F. W.) 477 (John Wiley & Sons, Ltd, 2013). doi:10.1002/9781118534168
5. FAO. *Overview of global dairy market developments in 2018. Dairy Market Review* (2019).
6. Index mundi. Dairy, Cheese Production by Country in 1000 MT - Country Rankings. (2020). Available at: https://www.indexmundi.com/agriculture/?commodity=cheese&graph=production. (Accessed: 10th March 2020)
7. Slavov, A. K. General characteristics and treatment possibilities of dairy wastewater - a review. *Food Technol. Biotechnol.* **55,** 14–28 (2017).
8. Valdez Castillo, M. *et al.* Yeast-driven whey biorefining to produce value-added aroma, flavor, and antioxidant compounds: technologies, challenges, and alternatives. *Crit. Rev. Biotechnol.* **0,** 1–21 (2020).
9. Lappa, I. K., Papadaki, A. & Kachrimanidou, V. Cheese Whey Processing : Integrated Biorefinery. *Foods* **8,** 1–37 (2019).
10. Conde-Báez, L., López-Molina, A., Gómez-Aldapa, C., Pineda-Muñoz, C. & Conde-Mejía, C. Economic projection of 2-phenylethanol production from whey. *Food*

Bioprod. Process. **115,** 10–16 (2019).
11. Cheirsilp, B., Suksawang, S., Yeesang, J. & Boonsawang, P. Co-production of functional exopolysaccharides and lactic acid by Lactobacillus kefiranofaciens originated from fermented milk, kefir. *J. Food Sci. Technol.* **55,** 331–340 (2018).
12. Mauriello, G. *et al.* Characterization of lactic acid bacteria strains on the basis of neutral volatile compounds produced in whey. *J. Appl. Microbiol.* **90,** 928–942 (2001).
13. El-Holi, M. A. & Al-Delaimy, K. S. Citric acid production from whey with sugars and additives by Aspergillus niger. *African J. Biotechnol.* **2,** 356–359 (2003).
14. Dragone, G., Mussatto, S. I., Oliveira, J. M. & Teixeira, J. A. Characterisation of volatile compounds in an alcoholic beverage produced by whey fermentation. *Food Chem.* **112,** 929–935 (2009).
15. Wittmann, C., Hans, M. & Bluemke, W. Metabolic physiology of aroma-producing Kluyveromyces marxianus. *Wiley Intersci.* **19,** 1351–1363 (2002).
16. Fuzhou Farwell company. Phenethyl Alcohol - Buy Cas No 60-12-8. (2020). Available at: https://www.alibaba.com/product-detail/Farwell-Cas-No-60-12-8_60822357559.html?spm=a2700.7724857.normalList.17.7db571c6ouxg8z&s=p. (Accessed: 26th February 2020)
17. Jiangsu Juming Chemical Process Technology Co., L. Natural 2-phenylethanol 99% - Buy 60-12-8. (2020). Available at: https://www.alibaba.com/product-detail/Flavor-expert-60-12-8-Natural_62236291311.html?spm=a2700.7724857.normalList.38.3424347ayH8EBx. (Accessed: 26th February 2020)
18. Leclercq-Perlat, M. N., Corrieu, G. & Spinneler, H. E. Comparison of Volatile Compounds Produced in Model Cheese Medium Deacidified by Debaryomyces hansenii or Kluyveromyces marxianus. *J. Dairy Sci.* **87,** 1545–1550 (2004).
19. Lawford, H. G. & Rousseau, J. D. Mannose fermentation by an ethanologenic recombinant Escherichia coli. *Biotechnol. Lett.* **15,** 615–620 (1993).
20. Villadsen, J., Nielsen, J. & Lidén, G. *Bioeaction Engineering Principles. Solvent Extraction in Biotechnology* (Springer, 2011). doi:10.1007/978-3-662-03064-6_2
21. Osorio-González, C. S. *et al.* Lipid production in Rhodosporidium toruloides using C-6 and C-5 wood hydrolysate: A comparative study. *Biomass and Bioenergy* **130,** 105355 (2019).
22. Singla, R. K., Dubey, A. K., Ameen, S. M., Montalto, S. & Parisi, S. The Control of Maillard Reaction in Processed Foods. Analytical Testing Methods for the Determination of 5-Hydroxymethylfurfural. in *Analytical Methods for the Assessment of Maillard Reactions in Foods* 15–26 (SpringerBriefs in Molecular Science. Springer, Cham, 2018). doi:10.1007/978-3-319-76923-3_1
23. Huffman, L. M. & Ferreira, L. D. B. Whey - based Ingredients. in *Dairy Ingredients for Food Processing* (eds. Ramesh, C. C. & Arun, K.) 179–198 (John Wiley & Sons, Inc., 2011). doi:10.1139/h11-060
24. Munanairi, A. *et al.* The multiple Maillard reactions of ribose and deoxyribose sugars and sugar phosphates. *Carbohydr. Res.* **342,** 2575–2592 (2007).
25. Christensen, A. D., Kádár, Z., Oleskowicz-Popiel, P. & Thomsen, M. H. Production of bioethanol from organic whey using Kluyveromyces marxianus. *J. Ind. Microbiol. Biotechnol.* **38,** 283–289 (2011).
26. Flores-Cosio, G., Arellano-Plaza, M., Gschaedler, A. & Amaya-Delgado, L. Physiological response to furan derivatives stress by kluyveromyces marxianus SLP1 in ethanol production. *Rev. Mex. Ing. Quim.* **17,** 189–202 (2018).
27. Duarte, L. C., Carvalherio, F., Neves, I. & Gírio, F. M. Effects of Aliphatic Acids , Furfural , and Phenolic Compounds on Debaryomyces hansenii CCMI 941. *Appl.*

Biochem. Biotechnol. **121,** 413–425 (2005).
28. Saunders, L. P., Bowman, M. J., Mertens, J. A., Da Silva, N. A. & Hector, R. E. Triacetic acid lactone production in industrial Saccharomyces yeast strains. *J. Ind. Microbiol. Biotechnol.* **42,** 711–721 (2015).
29. Talapatra, S. K., Talapatra, B. & Nicolaou, K. C. Polyketide pathway. Biosynthesis of diverse classes of aromatic compounds. in *Chemistry of Plant Natural Products: Stereochemistry, Conformation, Synthesis, Biology, and Medicine* 1–1180 (2015). doi:10.1007/978-3-642-45410-3
30. Hirst, M. B. & Richter, C. L. Review of aroma formation through metabolic pathways of Saccharomyces cerevisiae in beverage fermentations. *Am. J. Enol. Vitic.* **67,** 361–370 (2016).
31. Chaturvedi, S., Bhattacharya, A. & Khare, S. K. Trends in Oil Production from Oleaginous Yeast Using Biomass: Biotechnological Potential and Constraints. *Appl. Biochem. Microbiol.* **54,** 361–369 (2018).
32. Saini, P., Beniwal, A. & Vij, S. Physiological response of Kluyveromyces marxianus during oxidative and osmotic stress. *Process Biochem.* **56,** 21–29 (2017).
33. Beniwal, A., Saini, P., Kokkiligadda, A. & Vij, S. Physiological growth and galactose utilization by dairy yeast Kluyveromyces marxianus in mixed sugars and whey during fermentation. *3 Biotech* **7,** 1–13 (2017).
34. Adler, L., Blomberg, A. & Nilsson, A. Glycerol metabolism and osmoregulation in the salt-tolerant yeast Debaryomyces hansenii. *J. Bacteriol.* **162,** 300–306 (1985).
35. Gori, K. *et al.* Proteomic changes in Debaryomyces hansenii upon exposure to NaCl stress. *FEMS Yeast Res.* **7,** 293–303 (2007).
36. Hazelwood, L. A., Daran, J. M., Van Maris, A. J. A., Pronk, J. T. & Dickinson, J. R. The Ehrlich pathway for fusel alcohol production: A century of research on Saccharomyces cerevisiae metabolism (Applied and Environmental Microbiology (2008) 74, 8, (2259-2266)). *Appl. Environ. Microbiol.* **74,** 3920 (2008).
37. Neves, M. L., Oliveira, R. P. & Lucas, C. M. Metabolic flux response to salt-induced stress in the halotolerant yeast Debaryomyces hansenii. *Microbiology* **143,** 1133–1139 (1997).
38. Breuer, U. & Harms, H. Debaryomyces hansenii — an extremophilic yeast with biotechnological potential. *Wiley Intersci.* **23,** 415–437 (2006).
39. Rittman, B. E. & McCarty, P. L. *Environmental Biotechnology: Principles and applications*. *Biotechnology* (McGraw-Hill Education, 2001). doi:10.1016/B978-0-12-385015-7.00012-0
40. Etschmann, M. M. W., Sell, D. & Schrader, J. Production of 2-phenylethanol and 2-phenylethylacetate from L-phenylalanine by coupling whole-cell biocatalysis with organophilic pervaporation. *Biotechnol. Bioeng.* **92,** 624–634 (2005).
41. Banjara, N., Nickerson, K. W., Suhr, M. J. & Hallen-Adams, H. E. Killer toxin from several food-derived Debaryomyces hansenii strains effective against pathogenic Candida yeasts. *Int. J. Food Microbiol.* **222,** 23–29 (2016).
42. Nascimento, B. L. *et al.* Yeast Mycocins: a great potential for application in health. *FEMS Yeast Res.* **20,** 2005–2008 (2020).
43. Chen, Y., Aorigele, C., Wang, C., Simujide, H. & Yang, S. Screening and Extracting Mycocin Secreted by Yeast Isolated from Koumiss and Their Antibacterial Effect. *J. Food Nutr. Res.* **3,** 52–56 (2015).
44. Rodrigues, B. *et al.* Growth kinetics and physiological behavior of co-cultures of Saccharomyces cerevisiae and Kluyveromyces lactis, fermenting carob sugars extracted with whey. *Enzyme Microb. Technol.* **92,** 41–48 (2016).
45. Qian, X. *et al.* Current status and perspectives of 2-phenylethanol production through

biological processes. *Crit. Rev. Biotechnol.* **39,** 235–248 (2019).

Table 1. Compounds with aroma and flavor properties identified in controls (sterilized and incubated) and metabolites found in culture broth under aerobic and anaerobic conditions at 72 h of fermentation

Compounds	Relative area (counts*min) x $1x10^7$																	
	Aerobic									Anaerobic								
	Lactose medium			Cheese whey medium			Cheese whey with yeast extract and peptone medium			Lactose medium			Cheese whey medium			Cheese whey with yeast extract and peptone medium		
	CT	KM	DH	CT	KM	DH	CT	KM	DH	CT	KM	DH	CT	KM	DH	CT	KM	DH
Maillard reaction products																		
Acetic acid	0.26	0.18	0.18	3.2	0.26	6.77	3.69	1.90	-	0.20	1.36	2.31	0.21	0.96	7.11	1.95	0.41	4.47
Maltol	-	-	-	0.22	0.11	1.36	5.41	-	-	-	-	-	0.15	0.05	9.75	2.73	-	5.86
3D6Mpyranone	2.47	1.35	3.40	9.47	0.52	10.60	11.60	0.60	0.98	0.32	1.47	3.32	1.99	0.21	9.53	9.90	0.57	11.70
5Hfurfural	19.60	-	-	39.50	5.24	1.42	4.92	-	-	105	-	-	16.70	1.25	1.65	4.41	-	4.18
4HDfuranone	0.21	-	-	-	-	-	0.59	-	-	-	-	-	-	-	-	0.30	-	-
2Hfuranone	0.87	-	-	4.91	0.15	4.91	3.93	-	0.14	1.20	-	-	-	0.76	-	1.71	4.16	1.09
1-hydroxy-2-propanone	-	-	-	-	-	-	0.46	-	-	-	-	-	-	-	-	0.16	-	0.56
Metabolites																		
Propanoic acid	-	0.69	0.33	-	0.76	-	-	4.55	0.19	-	-	-	-	-	-	-	-	-
Butanoic acid	-	0.14	-	-	-	0.85	-	-	-	-	-	-	-	-	1.09	-	0.34	-
Glycerol	-	-	-	-	12.20	-	-	10.90	-	-	-	-	-	27.70	-	-	21.20	-
Dihydroxyacetone	-	-	-	-	-	0.57	-	-	0.71	-	-	-	-	-	0.93	-	-	0.62
Methionol	-	-	-	-	-	-	-	0.19	-	-	-	-	-	-	-	-	-	-
Isopentanol	-	0.63	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.22	-
2-phenylethanol	-	2.10	0.52	-	1.21		-	5.91	1.11	-	0.39	0.10	-	0.98	-	-	0.72	0.12

CT = control; KM = *Kluyveromyces marxianus*; DH = *Debaryomyces hansenii*; 3D6Mpyranone = 3,5-dihydroxy-6-methyl-2,3-dihydropyran-4-one; 5Hfurfural = 5-hydroxymethylfurfural; 2Hfuranone = 2(3H)-Furanone,dihydro-4-hydroxy; 4HDfuranone = 4-Hydroxy-2,5-dimethyl-3(2H)-furanone

Table 2 Overall performance of yeasts during the LM, W and WY media fermentations assays using the monocultures.

Medium Fermentation	Initial lactose concentration (g/L)	Lphe concentration (mg/L)			Growth rate - μ (h^{-1})		Lphe consumption rate ($mg_{Lphe}/L*h$)		Maximun yield production (g_{2PE}/g_{Lphe})		Maximun productivity ($mg_{2PE}/L*h$)	
		Initial	At 72 h for KM	At 72 h for DH	KM	DH	KM	DH	KM	DH	KM	DH
LM-aerobic	20	491	237.50±43.1	350.90±15.50	0.06	0.09	5.41±0.71	3.54±0.26	0.20±0.01	0.11±0.01	0.52 ± 0.05	0.16±0.01
LM-anaerobic	20	491	316.70±27.30	386.40±1.20	0.08	0.07	3.35±0.11	2.26±0.25	0.05±0.01	0.01±0.00	0.09 ± 0.05	0.02±0.00
W-aerobic	45	6.7	0	0	0.06	0.09	0.21±0.00	0.21±0.00	0.76±0.01	0.38±0.03	0.05± 0.00	0.03±0.01
W-anaerobic	45	6.7	0	0	0.08	0.11	0.21±0.00	0.21±0.00	0.55±0.1	0.30±0.07	0.04±0.01	0.02±0.00
WY-aerobic	45	494	0	132.40±75.81	0.13	0.16	7.04±0.00	5.27±1.16	0.16±0.02	0.11±0.04	0.86±0.18	0.42±0.06
WY-anaerobic	45	494	352.90±5.71	410.60±6.62	0.10	0.11	1.20±0.04	1.58±0.62	0.11±0.01	0.04±0.01	0.16±0.01	0.06±0.01

LM = Lactose medium; W = cheese whey medium; WY = cheese whey medium enriched with yeast extract and peptone

Table 3. Overall performance of yeasts during the fermentations assays for WY media using the monocultures and co-cultures.

Parameter	Culture						
	KM	**DH**	**1KM:5DH**	**1KM:2DH**	**1KM:1DH**	**2KM:1DH**	**5KM:1DH**
Growth rate - μ (h^{-1})	0.13	0.16	KM=0.01 DH=0.06	KM=0.14 DH=0.06	KM=0.21 DH=0.13	KM=0.08 DH=0.12	KM=0.10 DH=0.00
CD ($1x10^7 CFU_{KM}/mL)_{0h}$	1.60±0.10	NA	1.50±0.04	1.20±0.10	1.20±0.00	2.41±0.20	3.90±0.20
CD ($1x10^7 CFU_{KM}/mL)_{8h}$	11.80±1.20	NA	20.00±0.10	31.00±3.00	10.00±0.13	5.00±0.01	41.00±2.30
CD ($1x10^7 CFU_{KM}/mL)_{24h}$	111.00±3.00	NA	30.00±0.00	49.50±3.00	221±0.1	27.00±6.00	45.00±5.00
CD ($1x10^7 CFU_{KM}/mL)_{48h}$	880.00±70.00	NA	65.00±5.00	49.20±3.00	83.00±2.00	116.00±6.00	920.00±0.50
CD ($1x10^7 CFU_{KM}/mL)_{96h}$	56.50	NA	94.00±6.00	43.50±4.5	72.5±0.1	11.90±0.50	61.00±13.00
CD ($1x10^7 CFU_{DH}/mL)_{0h}$	NA	1.40±0.10	6.00±0.05	1.97±0.10	1.51±0.00	1.51±0.00	1.60±0.01
CD ($1x10^7 CFU_{DH}/mL)_{8h}$	NA	20.00±4.20	21.00±2.00	11.00±2.00	35.00±0.13	7.00±0.01	0.10±0.01
CD ($1x10^7 CFU_{DH}/mL)_{24h}$	NA	95.00±3.00	35.00±5.00	15.00±0.00	49.50±2.50	32.00±1.00	0.00±0.00
CD ($1x10^7 CFU_{DH}/mL)_{48h}$	NA	265.00±6.50	115.00±5.00	13.00±3.00	20.00±2.00	23.00±3.00	0.00±0.00
CD ($1x10^7 CFU_{DH}/mL)_{96h}$	NA	99.00±8.00	0.00±0.00	6.50±0.50	2.00±0.01	1.00±0.50	0.00±0.00
L-phenylalanie consumption rate ($mg_{Lphe}/L*h$)	7.04±0.00	5.27±1.16	4.54±0.00	9.58±0.00	9.54±0.00	9.48±0.00	10.38±0.00
2-phenylethanol productivity ($mg_{2PE}/L*h$)	0.86±0.18	0.42±0.06	1.46±0.22	1.63±0.03	1.30±0.10	1.41±0.18	1.93±0.02
2-phenylethanol yield production (g_{2PE}/g_{Lphe})	0.16±0.02	0.11±0.04	0.27±0.03	0.31±0.08	0.26±0.02	0.28±0.04	0.38±0.00
Ethanol productivity ($mg_{ethanol}/L*h$)	0.69±0.04	0.04±0.00	0.56±0.01	0.62±0.01	0.16±0.01	0.55±0.02	0.64±0.02
Ethanol yield production ($g_{ethanol}/g_{lactose})_{24h}$	0.36±0.00	0.04±0.00*	0.30±0.00	0.33±0.00	0.17±0.00*	0.29±0.00	0.34±0.00

CD = Cell density; NA= Not applicable.
*The value was obtained at 48 h of fermentation

Table 4 Fermentation of whey using yeasts to produce 2-phenylethanol

Culture media	Microorganisms	T (°C)	pH	Time (h)	Mode operation	Yield (g_{2PE}/g_{Lphe})	References
Whey + $(NH_4)_2SO_4$ + Lphe	*Kluyveromyces marxianus*	30	4.8	96	Batch	0.75	Conde-Báez et al., 2019;
Whey +Beet sucrose +$(NH_4)_2SO_4$ +Lphe	*Saccharomyces cerevisae*	30	5.4	72	Batch	0.65	Chreptowicz et al., 2018;
Whey	*Debaryomyces hansenii*	25	4.8	96	Batch	0.06	Leclerq-Perlat et al., 2004;
Whey	*Kluyveromyces marxianus*	30	4	Not reported	Continuos	Not reported	Dragone et al., 2009
Whey + Yeast extract and Peptone	*Kluyveromyces marxianus*	25	4.5	96	Batch/aerobic Batch/anaerobic	0.16 0.11	This study
Whey + Yeast extract and Peptone	*Debaryomyces hansenii*	25	4.5	96	Batch	0.11 0.04	This study
Whey + Yeast extract and Peptone	Co-culture of *Kluyveromyces marxianus* and *Debaryomyces hansenii*	25	4.5	96	Batch	0.32	This study

Table 5 Two-Way ANOVA table for the fermentation assays using *Kluyveromyces marxianus* and *Debaryomyces hansenii* under monoculture mode. Dependent variable: 2-phenylethanol concentration

Source of variation		*Pr(>|t|)*	Residual standard error	Degrees of freedom	Multiple R-squared	Adjusted R-squared	p-value
Medium and fermentation conditions	*Yeast strain*						
LM-Aerobic	KM	$2.25x10^{-5}$*	9.216	96	0.79	0.74	$2.2 x10^{-16}$
	DH	0.089					
LM-Anaerobic	KM	0.676					
	DH	0.891					
W-aerobic	KM	0.784					
	DH	0.938					
W-anaerobic	KM	0.682					
	DH	0.936					
WY-aerobic	KM	0.001*					
	DH	$3.33 x10^{-12}$*					
WY-anaerobic	KM	0.209					
	DH	0.979					

"*" Significant at 5% level ($P \leq 0.05$). Pr(>|t|); proportion of the *t* distribution at that degree of freedom which is greater than the absolute value of *t* statistic. KM; *Kluyveromyces marxianus*, DH; *Debaryomyces hansenii*

Table 6 ANCOVA table for the fermentation assays using *Kluyveromyces marxianus* and *Debaryomyces hansenii* under co-culture mode. Dependent variable: 2-phenylethanol concentration

Source of variation		*Pr(>|t|)*	Residual standard error	Degrees of freedom	Multiple R-squared	Adjusted R-squared	p-value
Medium and fermentation conditions	*Yeast strain*						
WY-aerobic	KM	2.74x10-4*	19.42	102	0.879	0.84	2.2×10^{-16}
	DH	0.125					
	1KM:5DH	1.15x10-5*					
	1KM:2DH	2.85x10-7*					
	1KM:1DH	4.58x10-6*					
	2KM:1DH	1.43x10-7*					
	5KM:1DH	7.78x10-10*					

* Significant at 5% level ($P \leq 0.05$). Pr(>|t|); proportion of the *t* distribution at that degree of freedom which is greater than the absolute value of *t* statistic. KM; *Kluyveromyces marxianus*, DH; *Debaryomyces hansenii*

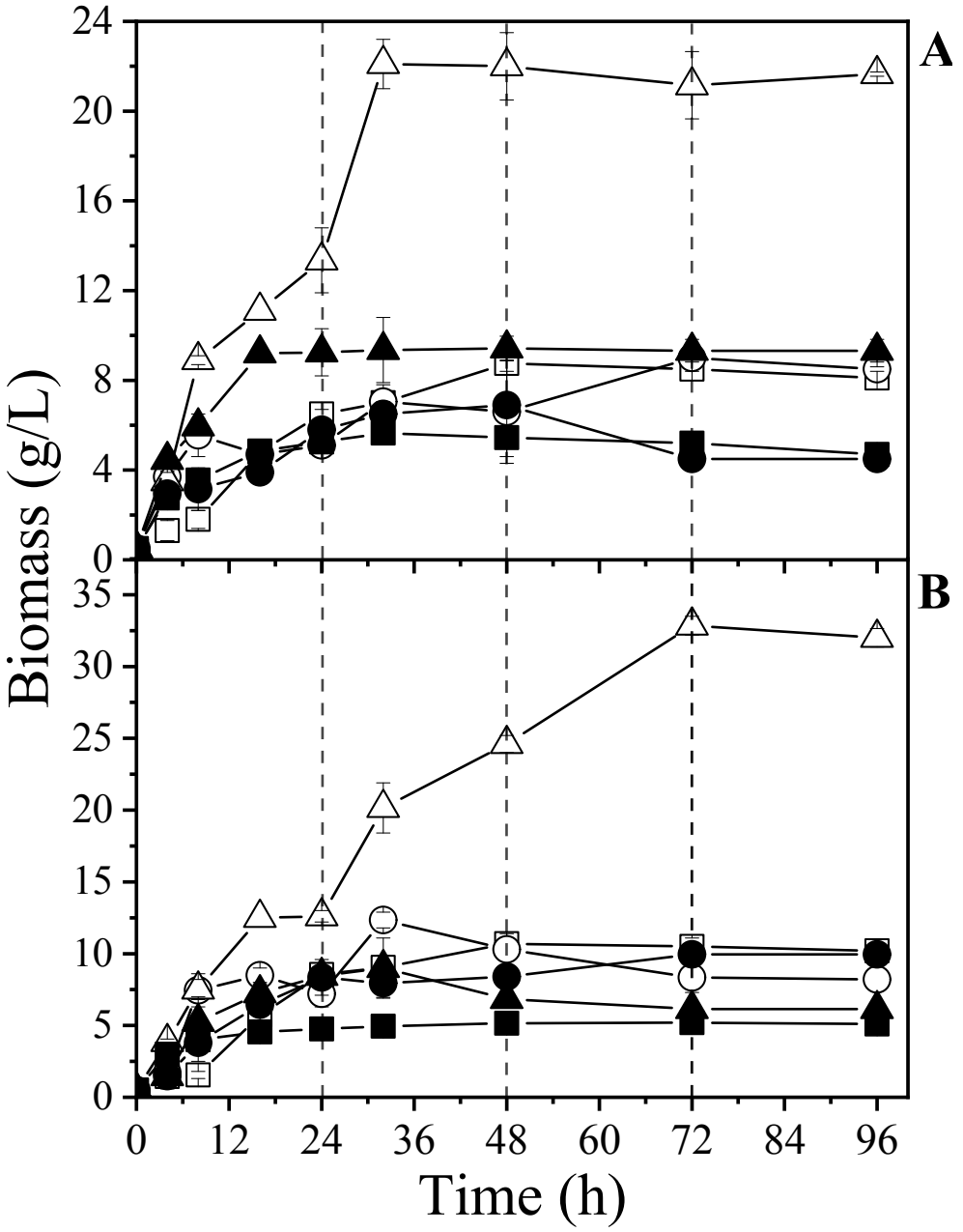

Figure 1. Biomass production in culture broth of Lactose medium (LM), cheese whey medium (W); cheese whey supplemented with yeast extract and peptone (WY) using *Kluyveromyces marxianus* (A) and *Debaryomyces hansenii* (B) yeasts. Open symbols indicate the aerobic □-LM, ○-W and Δ-WY media fermentations. Solid symbols indicate the anaerobic ■-LM, ●-W and ▲- WY media fermentations.

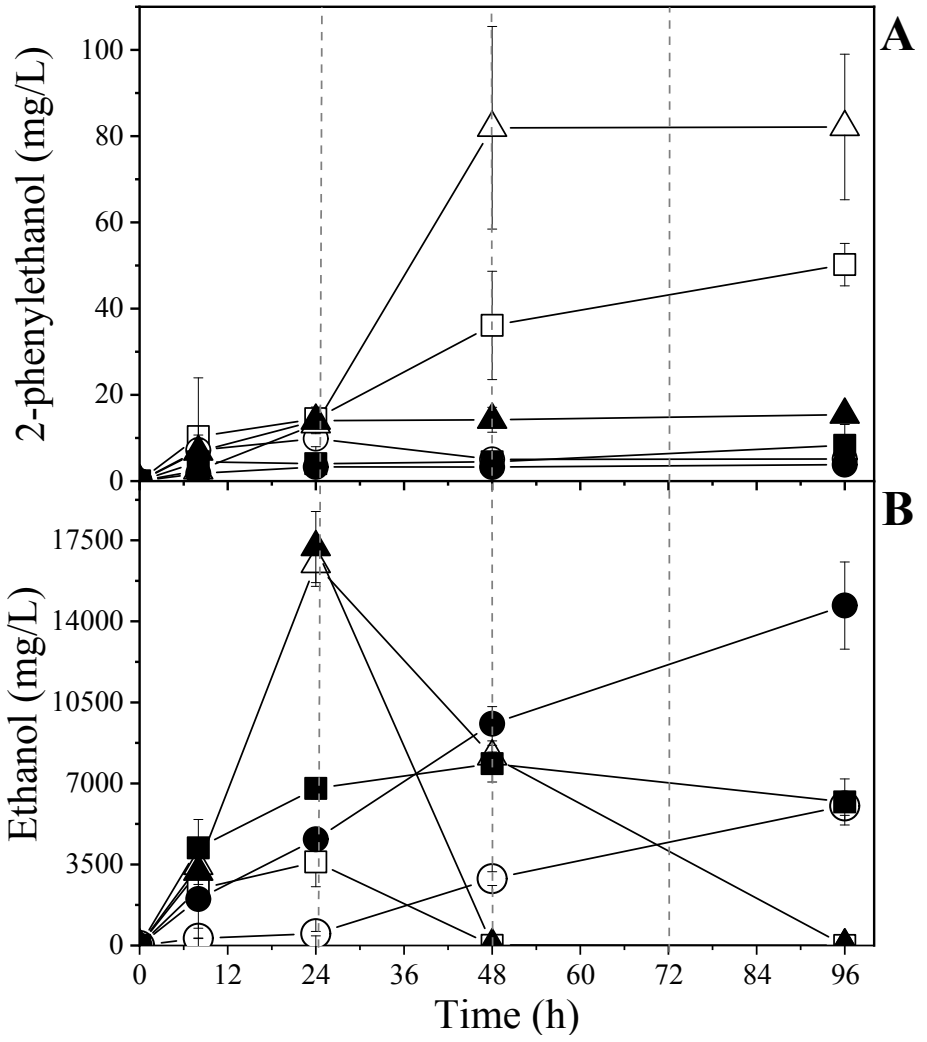

Figure 2. Production of alcohols by *Kluyveromyces marxianus* monocultures during the fermentations of Lactose medium (LM), cheese whey medium (W); cheese whey supplemented with yeast extract and peptone (WY); open symbols indicate the aerobic □-LM, ○-W and ∆-WY media fermentations. Solid symbols indicate the anaerobic ■-LM, ●-W and▲- WY media fermentations

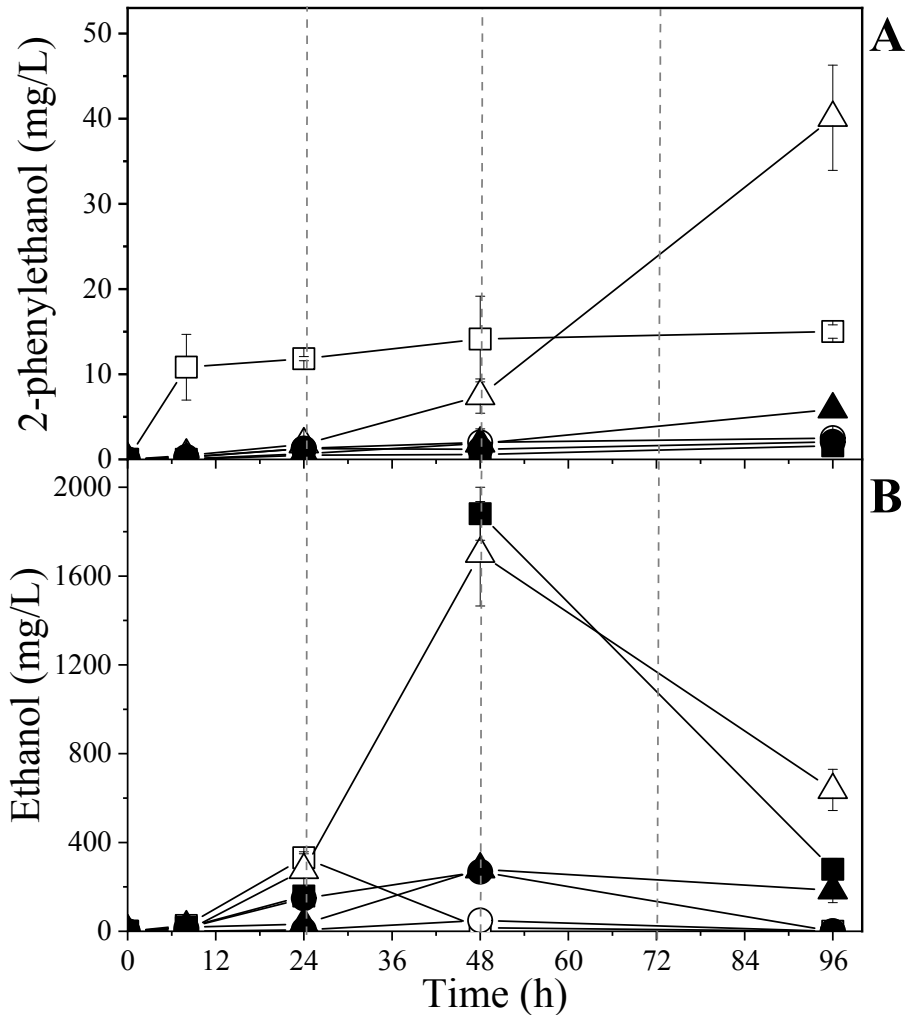

Figure 3. Production of alcohols by *Debaryomyces hansenii* monocultures during the fermentations of Lactose medium (LM), cheese whey medium (W); cheese whey supplemented with yeast extract and peptone (WY); open symbols indicate the aerobic □-LM, ○-W and △-WY media fermentations. Solid symbols indicate the anaerobic ■-LM, ●-W and▲- WY media fermentations

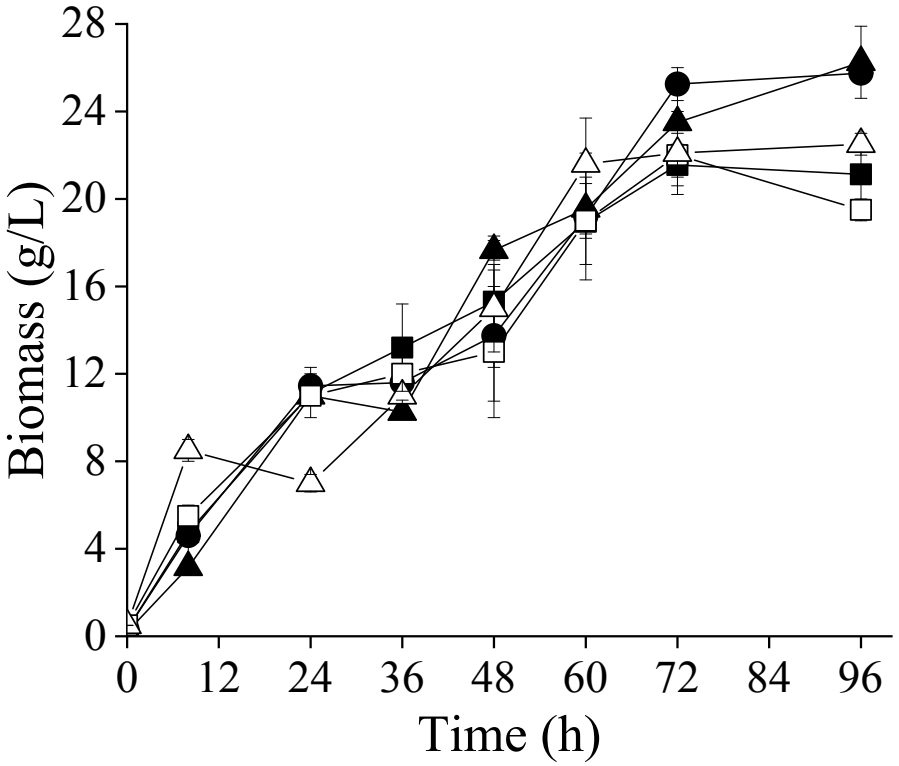

Figure 4. Kinetics of biomass production of co-cultures for the cheese whey supplemented with yeast extract and peptone medium (WY) fermentation under aerobic conditions. Symbols indicate the performance of □-1KM:5DH, ■-1KM:2DH, ●-1KM:1DH, ▲-2KM:1DH, and ∆-5KM:1DH

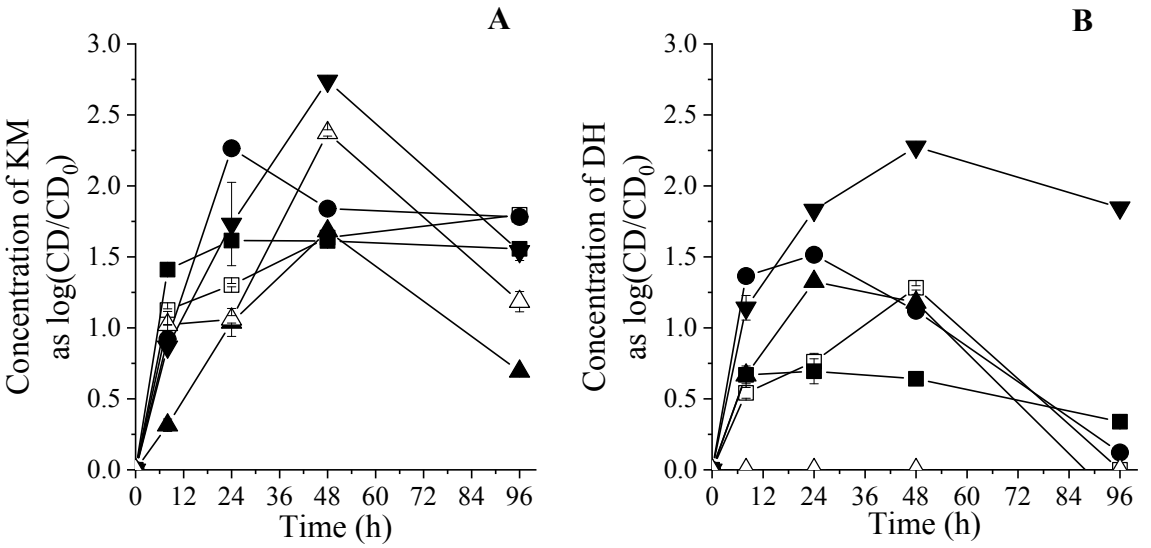

Figure 5. Growth of (A) *Kluyveromyces marxianus* and (B) *Debaryomyces hansenii* as monoculture and co-culture mode for cheese whey supplemented with yeast extract and peptone (WY) medium-aerobic. Symbols indicate the performance of ▼-KM or DH monoculture, ▽-DH monoculture, □– 1KM:5DH, ■–1KM:2DH, ●– 1KM:1DH, ▲–2KM:1DH, ∆–5KM:1DH.

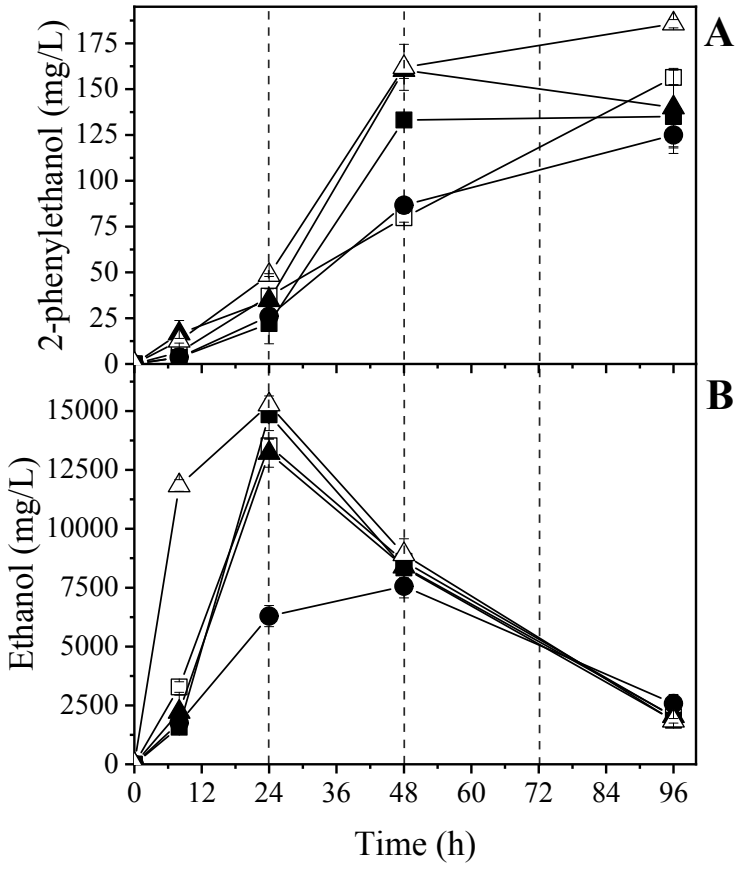

Figure 6. Production of alcohols during the aerobic cheese whey supplemented with yeast extract and peptone (WY) medium fermentation using the co-cultures. Symbols indicate the performance of □– 1KM:5DH, ■–1KM:2DH, ●–1KM:1DH, ▲–2KM:1DH, ∆–5KM:1DH.