

Received:
28 December 2018
Revised:
15 February 2019
Accepted:
18 February 2019

Cite as: Magdalini Soupioni,
Sofia Maragkou,
Alexandra Psarologou,
Athanasios A. Koutinas.
Revealing new promoters in
whey fermentation leads to a
new research concept.
Heliyon 5 (2019) e01262.
doi: [10.1016/j.heliyon.2019.e01262](https://doi.org/10.1016/j.heliyon.2019.e01262)



Revealing new promoters in whey fermentation leads to a new research concept

Magdalini Soupioni*, Sofia Maragkou, Alexandra Psarologou, Athanasios A. Koutinas

Food Biotechnology Group, Department of Chemistry, University of Patras, 26504 Patras, Greece

* Corresponding author.

E-mail address: m.soupioni@chemistry.upatras.gr (M. Soupioni).

Abstract

The current investigation reveals new findings concerning the substantial promotional activity of orange juice, orange peel and molasses on whey fermentation using single cell culture of kefir microflora. Specifically, the addition in whey of only 1 % v/v orange juice or 1% v/v molasses - 4% w/v orange peel reduced the fermentation time to 10 from 70 hours. But the lowest fermentation time (9 h) was observed when a composite biocatalyst consisted of cells entrapped with orange peel in corn starch gel was used, where also the highest ^{14}C -labeled lactose uptake rate by kefir was recorded. Bio-ethanol concentrations were increased using all the aforementioned promoters too and volatile byproducts such as esters were raised during the whey-orange juice mixtures fermentations, while terpenes by composite biocatalyst. The findings could be the base for new research projects for the rapid production of novel food stuffs and chemicals using whey as raw material.

Keywords: Food technology, Biotechnology

1. Introduction

Whey, the main side stream of the dairy industry, has a considerable nutritional value and potential for its upgrading by bioconversion, while its high organic load (4.8–5.3% w/v lactose) makes it a highly polluting waste. In order to effectively

manipulate the huge world whey capacity, the production of a variety of bio-products is necessary (Kourkoutas et al., 2002; Panesar et al., 2007; Bekatorou et al., 2006). In the frame of this aim the mixed natural culture of kefir, has been found suitable for simultaneous alcoholic and lactic acid fermentations of whey for the production of ethanol (Athanasiadis et al., 2002), lactic acid (Elezi et al., 2003), baker's yeast (Harta et al., 2004), single cell protein (SCP) as livestock feed (Plessas et al., 2008; Aggelopoulos et al., 2013), and cheese ripening (Dimitrellou et al., 2009; Papavasiliou et al., 2008). Also, it has been found that the use of whey in mixtures with low-cost raw materials and the application of a continuous process promote effectively its fermentation by kefir, leading to the potential production of novel alcoholic drinks (Reddy et al., 1987; Kourkoutas et al., 2002; Athanasiadis et al., 2002; Paraskevopoulou et al., 2003; Athanasiadis et al., 2004). Similarly, proviotic beverages were previously prepared by blending whey and orange or pineapple juice in different ratios using *Lactobacillus acidophilus* and/or *Bifidobacterium bifidum* for mixtures fermentation (Shukla et al., 2013; Shukla and Kushwaha, 2017). At the same time there is serious reducing of various agro-industrial wastes polluting the environment. Generally, the improvement of whey lactose fermentation rate is necessary for the easy and fast production of various bio-products through biotechnological means due to whey stuck fermentations using free cells. As the whey fermentation rate is affected by lactose uptake rate (LUR) by kefir, the ^{14}C -labeled lactose was earlier used to study the kefir fermentation ability through lactose uptake rate (Golfinopoulos et al., 2009; Golfinopoulos et al., 2011; Golfinopoulos et al., 2012; Soupioni et al., 2013). Furthermore, the promotion of single cell protein (SCP) production by kefir was revealed using orange juice containing orange pulp (Plessas et al., 2008) and orange peels were used for immobilization of *Saccharomyces cerevisiae* cells for successful alcoholic fermentation (Plessas et al., 2007). Moreover, as was recently reported an enhanced green production of xanthan gum was achieved by utilizing orange peels (Mohsina et al., 2018). Agro-industrial citrus side streams include (i) the discarded surplus of citrus fruits, (ii) the liquid effluent of citrus juices industry and (iii) its citrus peel. Reviewing evidences of the aforementioned citrus fragments promotional effect, the aim of the present investigation is to examine the effect of the aforementioned citrus side streams on the promotion of batch whey fermentation by kefir free cells, in order to increase the capacity of whey exploitation and to create a new research concept.

2. Materials and methods

2.1. Microorganism and cell growth

In the present study, kefir bought from the local market (Athens, Greece) and usually used to produce kefir drink was employed. Kefir growth and biomass production

were done at 30 °C in a synthetic medium, according to a previous work (Golfiopoulou et al., 2009). Pressed wet cells (15–20 g) were prepared at the late exponential phase, separated by centrifugation and used directly for fermentation experiments.

2.2. Materials and media

Liquid cheese whey obtained from the regional dairy industry “Agricultural Cooperative Union of Kalavryta” (Kalavryta, Greece) was used. Normally, it was remained after the production of feta cheese, mainly contained about (% w/w) 5 lactose, 0.8 proteins and its pH value was 6.5. The used molasses was the by-product of sugar industry “Hellenic Sugar Industry” and was obtained from “Spiliopoulos Distillery S.A” (Patras, Greece). It consists of (% w/w) water (17–25), sucrose (30–40), glucose (4–9), fructose (5–12), polysaccharides-dextrin, pentosans, polyuronic acids (2–5), and inorganic salts. Oranges were bought from a local super-market (Patras, Greece) in order orange juice and orange peel (white mesocarp) to be used. The white corn starch used for starch gel preparation was manufactured by Hellenic Giotis Co, Greece, containing 85.6 % w/w carbohydrates. All media were sterilized by autoclaving at 120 °C for about 15 min prior to their use for whey fermentation.

2.3. Materials used as promoters in the whey fermentation

Molasses (M) in its original form without any chemical treatment was added in whey until the desirable concentration to be achieved. The oranges were squeezed and the resulted juice (OJ) with pulp was added into whey. The orange peel (OP), white mesocarp) was obtained by removal of the orange–yellow exocarp, cut into small pieces with diameter of 1–1.5 cm, and dried at 120 °C for 2 h before adding into whey.

2.4. Biocatalyst preparation

For biocatalyst preparation 8 g of corn starch were added in 100 mL of deionized water at 90 °C with agitation till gelatination. Then 5 g of dried orange peel (OP) and 6 g of kefir cells were added into approximately 108 g of corn starch gel (CSG) at 40 °C. The resulted CSG-OP biocatalyst was remained at 4 °C for 24 h to become more stable.

2.5. Fermentations

In order to study the effect of temperature and pH on modified whey fermentation rate and lactose uptake rate by kefir, many batch fermentation experiments were carried out in lab-scale of 500-mL Erlenmeyer flask without agitation or air supply. The

molasses, orange juices or orange peel-molasses were added separately in 250 mL of pasteurized whey with liquid kefir biomass of 2.4 % w/v and fermentations were carried out (i) at various pH values 4.5, 5.5 and 7 at 30 °C and (ii) at various temperatures of 10, 20, and 30 °C under 5.5 pH value. Specifically, for the fermentation experiments with molasses or discarded orange juices various proportions of these promoters, namely 1, 2, 5 or 10 % v/v, were added in whey at pH 5.5 and 30 °C. Fermentation experiments were carried out in whey mixtures with 1 % v/v molasses and 10 g of dried orange peel too. Also, an amount of about 119 g of biocatalyst was added separately in plain whey and fermentations were carried out without agitation or air supply. In all cases trial pH value was achieved by the addition of tartaric acid (7 % w/v), as the original whey pH value was 6.5. During whey fermentations the pH value was maintained stable to the selected trial pH, by the addition of 6 M NaOH solution. A small quantity of ^{14}C -labeled lactose [D-glucose-1- ^{14}C], (ARC 0466 lactose, and 0.1 mCi mL $^{-1}$) was added at the beginning of fermentations at 30 °C and pH 5.5, in order to determine the lactose uptake rate by kefir cells. At various time intervals samples of the fermented liquids were collected and stored at -20 °C until further analysis. For statistical reasons all fermentations were carried out in triplicates.

2.6. Determination of ^{14}C -Labeled lactose

The ^{14}C -labeled lactose consumed by kefir cells was determined by liquid scintillation according to a previous work (Golfnopoulos et al., 2012) and expressed as counts per min (cpm) of labelled lactose per g of kefir biomass per h (cpm g $_{\text{biomass}}$ $^{-1}$ h $^{-1}$).

2.7. Statistical methods

During each of whey fermentation, the standard deviations (SD) of the recorded values for residual sugar and ethanol concentrations as well as lactose uptake rates by kefir were calculated and the proper graphs were done using the software Origin 9. The statistical treatment of the data, including summary statistics and one-way analysis of variance (ANOVA) was performed using the Computer software, Statistical Package for Social Sciences (SPSS Inc., Chicago, IL) version 21.0 for Windows. Significant differences between means were identified by multiple range tests (considered significant for $P < 0.05$).

2.8. Analyses

The ^{14}C -labelled lactose was measured on a PACARD-3255 liquid scintillation counter, interfaced to an APPLE-2 personal computer for data evaluation (Golfnopoulos et al., 2011).

Fermentation kinetics was monitored measuring Baume density ($^{\circ}\text{Be}$) and residual sugar in the fermentation broth. Residual sugar in the whey samples was determined on a Shimadzu LC-9A HPLC system (Tsaousi et al., 2008). Ethanol was determined by gas chromatography using a Shimadzu GC-8A system, with a Teknokroma HayeSep Q 80/100 mesh column, a FID detector, and a C-R6A Chromatopack integrator. It was used as carrier gas at 20 mL min^{-1} . The injection port and detector temperatures were 200 and $220 \text{ }^{\circ}\text{C}$, respectively. The column temperature was programmed at $80\text{--}180 \text{ }^{\circ}\text{C}$ ($16 \text{ }^{\circ}\text{C min}^{-1}$). Samples of $1 \mu\text{L}$ were injected directly into the column. Determinations were done by means of standard curves, where 1-Butanol was used as internal standard at a concentration of 0.5 \% v/v (Kopsahelis et al., 2009).

In Table 1 the lactose conversion to ethanol calculated by the following equation is presented:

$$(\text{Initial sugar conc.} - \text{Residual sugar conc.})/\text{Initial sugar conc.} \times 100$$

The volatile byproducts produced during whey, whey-orange juice and whey-molasses mixtures fermentations by kefir at $30 \text{ }^{\circ}\text{C}$ and $\text{pH } 5.5$ were analyzed either by gas chromatography (GC) or by means of gas chromatography-mass spectroscopy (GC/MS SPME 17A SHIMADZU) as described in a previous study (Kandyliis et al., 2008). Specifically, volatiles such acetaldehyde, ethyl acetate, 1-propanol, isobutyl alcohol, and amyl alcohols were determined by GC as mentioned above. For the GC/MS analysis, the volatile compounds were isolated by the head space solid phase micro-extraction (SPME) method and their identification was effected by comparing (i) the linear retention indices based on the even n -alkanes (C10-C24) with those of standard compounds and by the literature retention indices and (ii) MS data with those of standard compounds and by MS data obtained from

Table 1. Kinetic parameters and ethanol production using kefir free cells during whey fermentation in presence of promoters in comparison to whey fermented by kefir entrapped with orange peel in corn starch (CSG-OP), at $30 \text{ }^{\circ}\text{C}$ and $\text{pH } 5.5$.

| Promoter concentration (v/v) | Initial sugar (g/100 mL) | Fermentation time (h) | Ethanol concentration (g/100 mL) | Residual sugar (g/100 mL) | Ethanol productivity [g/(100 mL h)] | Conversion (%) |
|------------------------------|--------------------------|-----------------------|----------------------------------|---------------------------|-------------------------------------|----------------|
| 1% Molasses | $4.50 \pm 0.1^*$ | 22 | $2.79 \pm 0.1^*$ | $1.10 \pm 0.0^*$ | $0.12 \pm 0.0^*$ | 75.56 |
| 1% Orange Juice | 4.50 ± 0.0 | 10 | 1.90 ± 0.2 | 1.70 ± 0.0 | 0.19 ± 0.0 | 62.22 |
| 1% Molasses +4% orange peel | 4.90 ± 0.1 | 9 | 2.80 ± 0.1 | 1.40 ± 0.2 | 0.28 ± 0.0 | 71.43 |
| Whey | 5.03 ± 0.1 | 70 | 1.34 ± 0.3 | 0.24 ± 0.1 | 0.02 ± 0.0 | 95.23 |
| CSG-OP | 4.83 ± 0.1 | 10 | 3.68 ± 0.2 | 1.62 ± 0.1 | 0.22 ± 0.1 | 66.46 |

*Average (N = 3) concentrations \pm SD.

Wiley and NIST libraries. Semiquantitative analysis was performed by dividing the peak area of a compound with the peak area of the internal standard and multiplying the result with the concentration of the internal standard (1.62 mg/L) (Kandyliis et al., 2008).

2.9. Electron microscopy

Scanning electron microscope (SEM) micrographs of the orange peels before and after whey mixtures fermentations were obtained, in order to evaluate the adhesion of kefir cells on them, during bioprocess. Therefore, pieces of orange peels before and after fermentation, as well as pieces of the prepared CSG-OP biocatalyst dried for 2 hours at 120 °C. All samples were coated with gold in a Balzers SCD 004 Sputter Coater for 10 min and examined in a JEOL model JSM-6300 scanning electron microscope.

3. Results and discussion

The main aim of this investigation is a contribution on the exploitation of discarded oranges. It is satisfied by the study of the effect of oranges components such as peel and orange juice on the rate of whey fermentation of which stuck fermentations usually are observed. Therefore, this investigation focuses on the development of a new promoter to avoid stuck whey alcoholic fermentations using free cells. Stuck whey fermentations promoted in the past by immobilized cells on delignified cellulosic materials (Kourkoutas et al., 2002). The use of oranges components as promoter of whey fermentation creates a new situation, due to that they are food ingredients and therefore could better manage various new food stuffs produced from whey, in comparison with the employment of delignified cellulosic materials as promoter. In the frame of that goal, the effect of orange juice and peel separately are examined. In order to better optimize the promotional effect, the effect of orange peel/starch gel composite biocatalyst (CSG-OP), that of molasses and the mixture of orange peel-molasses were studied too. Molasses was examined here, because in the frame of this work was discovered promotion of whey fermentation using free kefir cells in presence of molasses. Therefore, fermentations of whey were carried out using CSG-OP, 1% v/v orange juice, 1% v/v molasses and orange peel-1% v/v molasses, in comparison with whey fermentations without each of them. The idea adopted, taking into account the promotion of SCP production using orange juice containing orange pulp (Plessas et al., 2007). The natural culture of kefir consisting mainly of lactic bacteria (83–90 %) and yeasts (10–17 %) was considered to be the advantageous culture for the whey mixtures fermentations. Kefir free cells entrapped with orange peel in corn starch gel were used for comparison reasons in fermentations. This composite biocatalyst examined in this investigation for its promotional effect due to that it can be used to obtain two bioprocesses in the same batch of a bioreactor

(Servetas et al., 2013). In addition, ^{14}C -labeled lactose was employed in order to determine lactose uptake rate by kefir and thus to evaluate the fermentative ability of this culture during the present bioprocesses.

3.1. Effect of citrus agro-industrial waste components on whey fermentation related with lactose uptake rate by kefir

Fig. 1a and b show the effect of citrus agro-industrial wastes on the promotion of fermentation rate and ethanol production rate, in comparison with the effect of 1% v/v molasses during whey fermentation at 30 °C and 5.5 pH value. The results show that the addition of 1% v/v molasses gave a substantial increase of fermentation rate showing promotional effect on process as the whey fermentation time was significantly lower ($P < 0.05$) than that in plain whey. Likewise, the addition of 1% v/v orange juice promoted further the process, as now the whey fermentation time was significantly lower ($P < 0.05$) than that in both previous cases. Furthermore, the addition of orange peel in whey containing 1% v/v molasses promoted more the fermentation compared of that containing only 1% v/v molasses but the fermentation time was not significantly lower ($P > 0.05$) than that of 1% v/v orange juice addition. This obvious enhancing of microorganisms fermenting capacity can be attributed to the nutrients contained in the orange juice and molasses. In order to examine a biocatalyst based on orange peel the composite biocatalyst consisted of kefir cells entrapped with orange peel in corn starch gel was used in whey fermentations. This biocatalyst resulted to about the same fermentation rate and fermentation time with that in presence of orange juice. The observed lowest fermentations times by the addition of 1% molasses with 10 g of orange peel into whey, as well as by using the biocatalyst, can be explained by the immobilization of kefir cells on solid orange peel during fermentation. The adhesion of kefir cells on orange peel was obvious at electronic micrographs in Fig. 2, where the porous surface of orange peel is presented (Fig. 2a) and the characteristic shapes of bacteria ranging from spheres to rods are clearly shown on it (Fig. 2b). Also in Fig. 2c is shown how cells are distributed in the whole mass of composite biocatalyst and cells immobilized on the surface of entrapped orange peel. Therefore, cells are contained in starch gel layer and on the surface of orange peel. The kefir immobilization on suitable substrates strongly advantages the whey fermentation kinetics, as was already reported (Soupioni et al., 2013) and is also confirmed in the present work. Fig. 1b, in which the ethanol formation rate is indicated, shows that the composite biocatalyst CSG-OP gives the highest ethanol concentration, while the fermentation of whey without promoters leads to significantly lower ($P < 0.05$) ethanol concentration (Table 1), even-though the fermentation proceeds at lower final $^{\circ}\text{Be}$ density (Fig. 1b). It is obvious that there is a complete identification of the lactose uptake rate (Fig. 3) with the whey fermentation rate (Fig. 1a), when a small proportion of various agro-industrial raw materials were separately added in whey. Specifically, CSG-OP

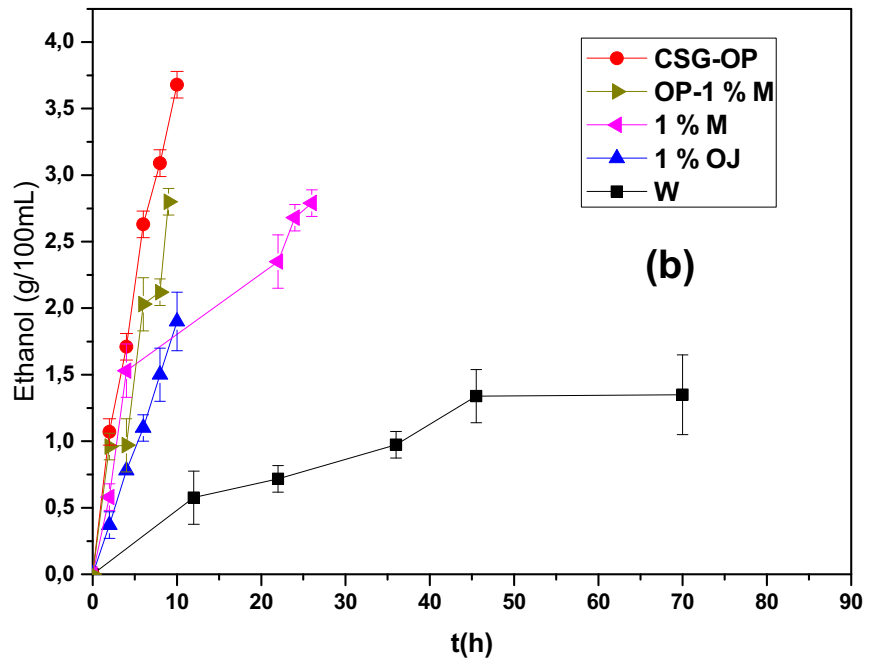
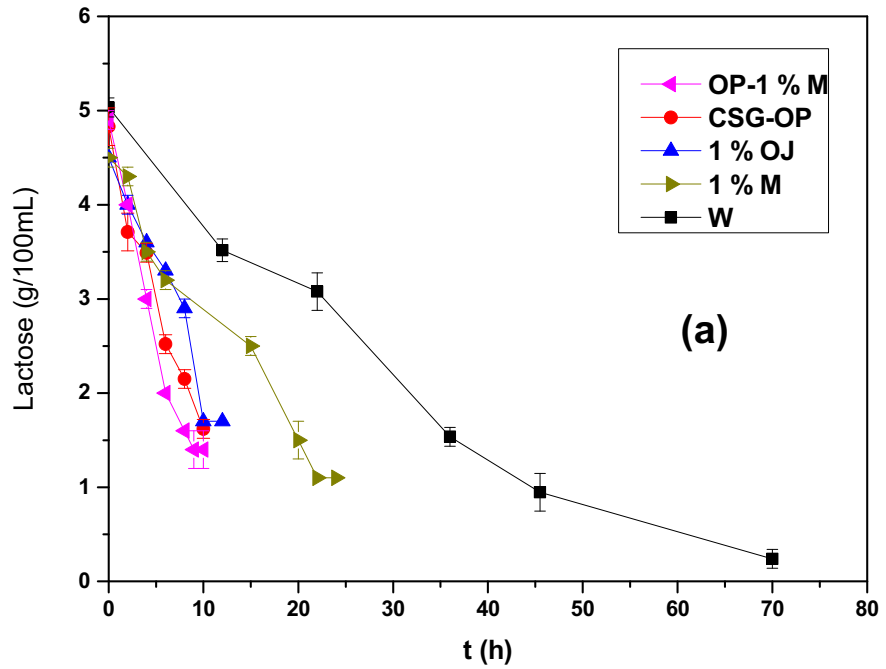


Fig. 1. (a) Fermentation kinetics and (b) Ethanol formation during fermentation by kefir free cells using whey (W), molasses (M) 1 %, orange juice (OJ) 1 %, orange peel with molasses (OP-1 % M) and kefir entrapped with orange peel in corn starch (CSG-OP), at 30 °C and pH value 5.5.

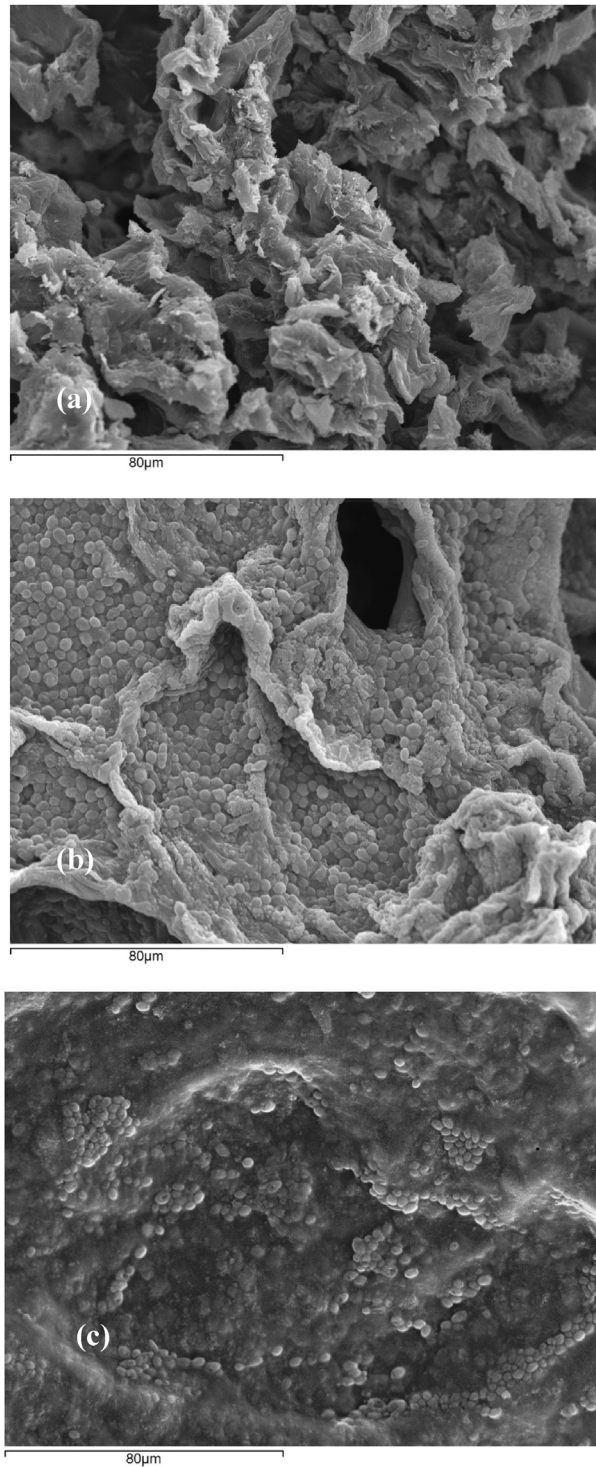


Fig. 2. Micrographs of a scanning electron microscope showing (a) Orange peel (OP), (b) kefir cells immobilized on OP and (c) kefir cells entrapped with orange peel in corn starch gel, after fermentation of whey mixture in presence of them.

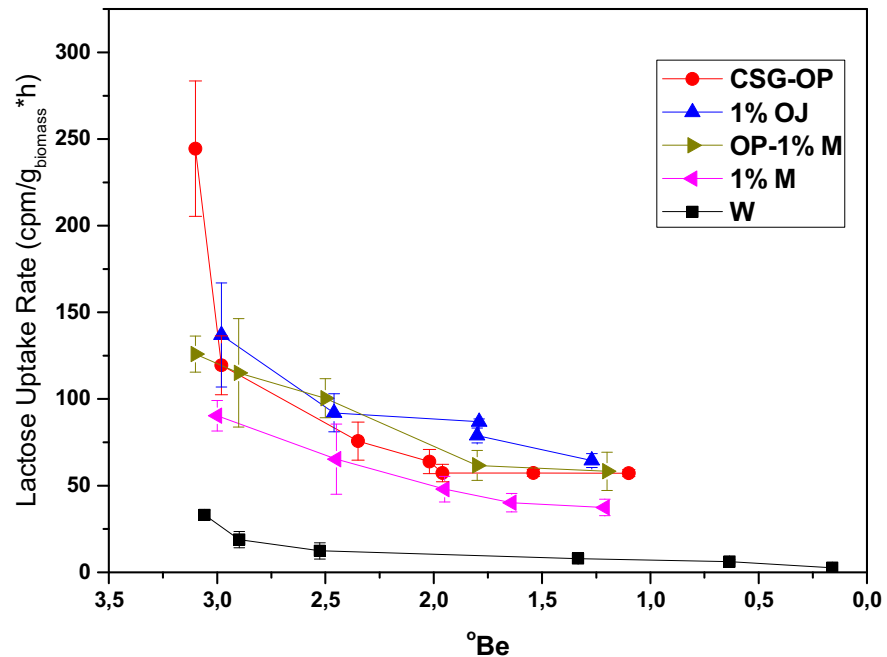


Fig. 3. ^{14}C -lactose uptake rate by kefir free cells using whey (W), molasses (M) 1 %, orange juice (OJ) 1 %, orange peel with molasses (OP-1 % M) and kefir entrapped with orange peel in corn starch (CSG-OP), at 30 °C and pH value 5.5.

resulted to significantly higher ($P < 0.05$) glucose uptake rate and plain whey fermentation gave the significantly lower ones. Also, it is remarkable that the fermentations in whey-molasses, whey-orange juice and whey-molasses-orange peel mixtures stopped at about 1.3 °Be density. This concentration can be attributed to non-fermentable diluted constituents of whey and of added materials and not to residual lactose concentration, which was very low (Table 1). This is in agreement with the results reported before, regarding the whey-molasses mixtures (Athanasiadis et al., 2002). However, the bioprocess becomes much simpler by the addition of 1 % orange juice in whey, in contrast to its improvement by the use of immobilized or entrapped cells or by using kefir granular biomass and raisin extracts (Athanasiadis et al. 2002, 2004).

3.2. Effect of citrus agro-industrial wastes on volatiles production

Due to the fermentation of whey by kefir can be employed for a milk-whey drink production (Golfnopoulos et al., 2009; Athanasiadis et al., 2004) and the new promoters such as orange juice and peel are foods, the main volatiles concentrations were determined in fermented broths and the results are presented in Table 2. From the results, it is clear that the concentrations of acetaldehydes, ethyl-acetate and some alcohols is significantly higher ($P < 0.05$) in the case of adding mainly molasses or orange juice as promoters in whey, than of not using them. Reasonable

Table 2. Volatile by-products (in $\mu\text{g L}^{-1}$) produced during fermentation by kefir free cells of plain whey (W) or whey with orange juice (1% OJ) or whey with molasses (1% M) in comparison to by-products (in mg L^{-1}) of whey fermented by kefir entrapped with orange peel in corn starch (CSG-OP), at 30 °C and pH 5.5.

| Volatile compound | W | 1% OJ | 1% M | CSG-OP |
|----------------------------------|---------------|---------------|-----------------------------------|--------------|
| Esters | | | | |
| Ethyl acetate | 29.2 | 32.57 | 40.8 \pm 4.65 | 15.4 |
| Octanoic acid ethyl ester | 48.5 | 75.8 | ND | 20.4 |
| Decanoic acid ethyl ester | 96.32 | 130.1 | ND | 21.9 |
| Acetic acid, 2-phenylethyl ester | 155.3 | 269.8 | ND | 104.2 |
| Pentanoic acid ethyl ester | 17.5 | 10.6 | ND | 4.5 |
| Total | 346.82 | 518.87 | 40.8 \pm 4.65 | 166.4 |
| Alcohols | | | | |
| Phenylethyl alcohol | 7.69 | 86.2 | ND | 1.4 |
| Propanol-1 | ND | ND | 16.03 | ND |
| Isobutyl alcohol | ND | ND | 28.43 | ND |
| Amyl alcohols | ND | ND | 75.1 | ND |
| Total | 7.69 | 86.2 | 119.56 | 1.4 |
| Ketones | | | | |
| 5-Nonanone | 40.92 | 67.1 | ND | 143.3 |
| Organic acids | | | | |
| Tetradecanoic acid | 19.32 | 602.3 | ND | 20.2 |
| Carbonyls | | | | |
| Acetaldehyde | ND | ND | 17.2 | ND |
| Terpenes | | | | |
| Limonene | ND | ND | ND | 115.3 |

amounts of volatile compounds were produced, especially during whey fermentation by kefir cells entrapped in corn starch gel with orange peel (Tables 1 and 2). Specifically, considerable amounts of acetic acid, 2-phenylethyl ester (104.2 mg L^{-1}) and limonene (115.3 mg L^{-1}) were determined in this case, and they have great odour. Conclusively, orange juice, molasses and biocatalyst increase the main volatiles concentration without deteriorating the organoleptic quality of a novel drink which could be produced. Especially the new prepared CSG-OP biocatalyst could also be used in fermented food applications to improve productivities and quality.

3.3. Scientific and technological consideration of results

Orange juice and peel are promoters of the alcoholic fermentation of whey, increasing substantially the rate of fermentation. Specifically, orange juice and CSG-OP biocatalyst increased about 10 fold the ethanol productivity (Table 1). The use of orange juice in the fermentation of whey with free cells of kefir creates

a simple process in comparison with promotional bioprocess using raisin extracts which needs preparation of kefir granular biomass (Athanasiadis et al., 2004; Koutinas A.A. et al., 2007), or in comparison with cell immobilization on delignified cellulosic materials (Athanasiadis et al., 2003). Furthermore, the promotional effect of orange juice is higher than that of all the aforementioned promoters. The CSG-OP biocatalyst gives the possibility to have promotional effect in the case of entrapped cells and probably in the two products production by two layers fermentation after immobilization of different microorganisms in every one of them (i.e. in SG and OP). Moreover, this investigation reveals the promotional effect of 1 % molasses, which increases 6 fold the fermentation rate in comparison with that without it. It also creates a simple process of low cost using a material as is molasses having low cost. The analysis of volatiles shows creation of esters and terpenes that improve flavor profile of food stuffs which could be produced using whey as raw material. The low concentration of alcohols, organic acids and carbonyls satisfies this possibility (Table 2). The results of glucose uptake rate using labeled ^{14}C glucose are adapted with the promotional effect of fermentation rate.

3.4. Perspectives

Because orange juice and orange peel are foods having important aroma and nutritious compounds, it is great to be added in food bioprocesses. Whey is a raw material for various bioprocesses using in each of them different microorganism to produce food additives and foods. However, orange juice and peel have been proved to have great promotional effect in alcoholic fermentation using kefir. Furthermore, kefir is a mixed culture consisting of various yeasts genera and species and various lactic acid bacteria. Therefore, would be expected similar activity in other bioprocesses using various microorganism and whey for the production of food additives, new food stuffs and chemicals. The last is conceptual and could be subject for several new research projects.

4. Conclusions

This investigation reveals new findings such as orange juice and orange peel are promoters of whey alcoholic fermentation. Likewise, the third finding is that 1 % molasses is also promoter of whey alcoholic fermentation using kefir. The promotional effect of orange juice increases 10 fold ethanol productivity as compared with fermentation without it and the effect of 1 % molasses is 6 fold. The composite biocatalyst CSG-OP is also promoter of whey fermentation using kefir and results to higher bioethanol concentration in comparison with plain whey fermentation and that after addition of 1 % molasses, OP-M and orange juice. Orange juice increases esters while CSG-OP increases terpenes production.

Declarations

Author contribution statement

Magdalini Soupioni: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Sofia Maragkou, Alexandra Psarologou: Performed the experiments.

Athanasios A. Koutinas: Analyzed and interpreted the data; Wrote the paper.

Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

The authors would like to express their thanks to Professor Kanellaki M. for helpful comments on the present manuscript.

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