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Refining Citrus Wastes: From Discarded Oranges to Efficient Brewing Biocatalyst, Aromatic Beer, and Alternative Yeast Extract Production

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Abstract: Agro-industrial wastes can be valorized as biorefinery raw materials through innovative, environmentally friendly bioprocessing for added value products. In this study, a process for citrus waste valorization within the biorefinery concept is proposed, including the development of an effective biocatalyst, based on immobilized cells, for aromatic beer production, and an alternative yeast extract (AYE) production in the same unit. Specifically, orange pulp from discarded oranges was applied as an immobilization carrier of the alcohol-resistant and cryotolerant yeast strain *S. cerevisiae* AXAZ-1. The yeast culture was produced by minor nutrient supplementation using diluted molasses as substrate. An effective Citrus Waste Brewing Biocatalyst (CWBB) was produced and applied for beer fermentation. The aroma-related compounds in beer produced with free yeast cells or the CWBB were evaluated by solid-phase micro-extraction (SPME) gas chromatography–mass spectrometry (GC–MS). The analysis showed that the beers produced by the CWBB had a more complex volatile profile compared with beer fermented by the free cells. More specifically, the CWBB enhanced the formation of esters and terpenes by 5- and 27-fold, respectively. In the frame of the proposed multiprocessing biorefinery concept, the spent CWBB, after it has completed its cycle of brewing batches, was used as substrate for AYE production through autolysis. The produced AYE significantly affected the yeast growth when compared to commercial yeast extract (CYE). More specifically, it promoted the biomass productivity and biomass yield factor by 60–150% and 110–170%, respectively. Thus, AYE could be successfully used for industrial cell growth as an efficient and cheaper substitute of CYE. Within a circular economy framework, the present study highlights the potential use of citrus waste to produce aromatic beer combined with AYE production as an alternative way to valorize these wastes.

Keywords: biorefinery; citrus wastes; valorization; immobilized biocatalyst; *S. cerevisiae*; aromatic beer; yeast extract.

Citation: Ganatsios, V.; Terpou, A.; Bekatorou, A.; Plessas, S.; Koutinas, A.A. Refining Citrus Wastes: From Discarded Oranges to Efficient Brewing Biocatalyst, Aromatic Beer, and Alternative Yeast Extract Production. *Beverages* **2021**, *7*, 16. https://doi.org/10.3390/beverages7020016

Academic Editors: Luis F. Guido and Pavel Dostálek

Received: 18 February 2021
Accepted: 29 March 2021
Published: 31 March 2021

1. Introduction

The emerging interest in food wastes, side-streams, and by-products valorization is due to the fact that 1/3 of food production is wasted globally, accounting for about 1.3 billion tons/year of food waste [1]. Meanwhile, global food production is estimated to rise up to 60% by 2050, generating a proportionate increase in food waste [2]. With regard to the environmental issues related to food waste, the United Nations implemented

Sustainable Development Goals (SDGs), targeting to reduce at least 50% of global food loss and waste by 2030 [3]. These environmental issues and the growing global food production, combined with the increasing demand for food additives and high-quality foods, have fostered research efforts to develop innovative biorefinery technologies based on renewable raw materials, such as agro-industrial wastes (AIWs) [1,4,5]. To succeed in developing economically viable systems, a variety of AIWs have been proposed as biorefinery substrates and converted into valuable marketable products, including chemicals, added-value novel foods and food ingredients, and biofuels [5–8].

Citrus and especially the orange juice industry is one of the largest fruit-processing sectors worldwide, experiencing a steady growth in the past decade mainly due to the dramatic increase in consumer demand. In 2014 alone, the world orange production reached 72.9 million tons, rising up to 78.6 million tons by 2019, with approx. 8.6% of the global production obtained from Mediterranean countries [9]. The citrus waste industry includes discarded fruits and liquid or solid wastes of juice production. The main waste of juice processing is orange pulp and peels (30–50% *w/w* of the fruit) and its disposal represents a growing problem, since the plant material is usually susceptible to microbial spoilage, limiting any further exploitation. An additional issue that increases the amount of waste is the landfill disposal of fruit surpluses.

Several biotechnological strategies have been proposed to valorize the bulk of the disposed citrus wastes, including enzymatic and alkali treatment for animal feed production, use as organic fertilizers, production of biofuels, recovery and evaluation of hydrolytic enzymes, and isolation and characterization of added-value by-products, such as polyphenols and other antioxidant constituents, vitamins, or essential oils [4,10–12]. Despite these efforts, large quantities of citrus wastes are still disposed, creating considerable environmental problems, since many of the proposed processes can be quite complex and expensive to introduce in the industrial sector.

Fermentation technologies based on immobilized cell biocatalysts on solid AIWs have been proposed as efficient and low cost methods for brewing, winemaking, and dairy fermentations, targeting improved bioprocessing [7,13–16]. Within another concept, yeast biomass (*Saccharomyces cerevisiae*) grown on AIWs was successfully applied as feedstock for the production of alternative yeast extract (AYE) [17]. Both cell biomasses and immobilized biocatalysts could be used for AYE production after autolytic treatments [18,19]. Therefore, the production of AYE to valorize citrus wastes is of high importance due to its high potential for commercialization. Moreover, the simultaneous production of immobilized biocatalysts for food fermentations, using orange pulp as the support material, may provide another path for citrus waste exploitation.

Based on the above, the aim of this work was to develop a citrus waste biorefinery to produce an efficient brewing biocatalyst and an AYE. The fermentation efficiency of the immobilized biocatalyst in beer production and the efficiency of the AYE in microbial biomass production were evaluated. Therefore, the presented work includes (i) the production of a Citrus Waste Brewing Biocatalyst (CWBB), with demonstration of its fermentation efficiency and its effect on the aroma profile of the produced beer; and (ii) the production of an AYE after autolysis of the spent CWBB after it has completed its brewing purpose, and a study of its effect on cell growth when compared with commercial yeast extract (CYE).

2. Materials and Methods

2.1. Yeast Strain

The yeast *Saccharomyces cerevisiae* AXAZ-1 used in the study is an alcohol-resistant and psychrotolerant strain, isolated from a local vineyard (Achaia, Greece) [20]. During the study, the yeast strain was maintained on YPDA medium (regenerated every 2 months), which consisted of 40 g/L glucose, 10 g/L yeast extract, 10 g/L peptone, and 25 g/L agar. Yeast biomass was produced by growth in a liquid culture medium consisting of 4 g yeast extract/L, 1 g $(NH_4)_2SO_4$/L, 1 g KH_2PO_4/L, 5 g $MgSO_4 \cdot 7H_2O$/L, and 40 g glucose monohydrate/L. The culture was incubated under aerobic conditions at 30 °C

and harvested by centrifugation at 4000 rpm for 10 min at 25 °C (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at the end of the exponential phase. All media were sterilized (135 °C, 1.5 atm, 15 min) prior to use.

2.2. Orange Pulp Suspension

Mature oranges (*Citrus sinensis*) of the Washington Navel variety were supplied by a local producer (Achaia, Greece). The oranges were initially washed by water to remove dirt and other foreign material. Afterwards, the external yellow exocarp parts of the skins were removed manually, and the remaining body was blended for 10 min to produce the pulp suspension [21]. The orange pulp was separated from the orange juice by filtration and washed with 100 mL of sterile water per 100 g of orange pulp to remove the juice residues. It was then drained and sterilized for 15 min at 120 °C and 1.5 atm. The collected juice was refrigerated to avoid spoilage until further use.

2.3. Immobilized Biocatalyst Preparation

The harvested yeast biomass (16 g of wet pressed *S. cerevisiae*) was suspended in 800 mL of liquid mixture of diluted molasses (Spiliopoulos Co. distillery, Patras, Greece) and orange juice to prepare a broth of 40 g/L initial sugar concentration. Molasses are proposed for sustainable production of yeast biomass from AIWs or by-products, within the general biorefinery concept. Then 200 g of sterilized orange pulp were added, and the mixture was left to ferment for about 6 h until all the sugar was utilized (final Baume hydrometer density of 0–0.5 °Be). The liquid was then decanted, and the produced biocatalyst was washed twice with 400 mL of sterile glucose medium (40 g/L) to remove any free cells [14]. The viable cell population of the immobilized biocatalyst (CWBB) was subsequently tested by analysis of yeast viability on YPDA media. In brief, 10 g of CWBB were blended with 90 mL of sterile $\frac{1}{4}$ Ringers solution in a sterile food grade plastic bag and mixed in a stomacher blender (Bagmixer 400, Model VW, Interscience, Saint Nom, France). The suspension was then subjected to serial decimal dilutions in $\frac{1}{4}$ strength Ringer's solution [22]. Viable counts of yeast cells were determined in triplicate by pour plating 0.1 mL on YPDA media. The viable immobilized yeast cells of the CWBB were found to be ~8.5 log CFUs/g of wet weight of orange pulp.

2.4. Beer Production

The brewing experiments were performed using lager wort supplied by the Athenian Brewery S.A. (Patras, Greece), but without hops addition [14]. Amounts of the immobilized biocatalyst (CWBB, 10 g) and 300 mL of wort (initial density 8.3 °Be) were introduced into sterilized glass cylindrical fermenters of 1 L total volume. Repeated anaerobic batch fermentations were carried out at 15 and 10 °C using the same CWBB in each batch. The CWBB was washed with 100 mL of fresh wort before the next fermentation batch. The same process was carried out for comparison reasons using free yeast cells (8.5 log CFUs/mL). When the fermentable sugar was exhausted, the biocatalysts (free yeast cells or CWBB) were removed from the products [23]. Samples of the freshly produced beers (green beer) were collected at the end of each batch fermentation and analyzed for ethanol, residual sugar, and volatile compounds.

2.4.1. Ethanol and Residual Sugar

The ethanol concentration was determined by both means of gas chromatography (GC), on a Shimadzu GC-8A system, and high-performance liquid chromatography (HPLC), on a Shimadzu HPLC system (Kyoto, Japan). The GC chromatograph was equipped with a Teknokroma HAYE SEP Q 80/100 column (Teknokroma, Barcelona, Spain), a C-R6A Chromatopack integrator (Shimadzu, Kyoto, Japan) and a flame ionization detector (FID). The carrier gas was He with a flow rate of 20 mL/min. The injection port and the detector temperatures were both set at 210 °C and the column oven temperature was set at 130 °C. The sample injection volume was 2 μL. Determinations were done by means of standard

curves. Ethanol productivity was calculated as mL of ethanol/L liquid volume (% v/v). Different dilutions of pure ethanol (Fisher Scientific, Loughborough, UK) were used in each chromatographic system as the internal standard (IS).

Sugar was determined by HPLC, on a Shimadzu HPLC system consisting of an SCR–101N stainless-steel column, an LC–9 A pump, a CTO–10 A oven (60 °C), and an RID–6 A refractive index detector. Three times distilled and filtered water was used as mobile phase (0.8 mL/min) and 1-butanol of 0.1% v/v (Merck, Darmstadt, Germany) was used as the IS. Beer samples were filtered on 0.2 µm micro-filters, prior to injection.

2.4.2. SPME GC/MS Analysis

The volatile compounds of the beer produced by the CWBB were determined by means of gas chromatography–mass spectroscopy (GC–MS), as described in previous studies [24,25]. The volatiles were isolated by the headspace solid-phase micro-extraction (SPME) technique. The conditions of the headspace-SPME sampling were as follows: 10 mL of the liquid sample, 3 g NaCl, and the IS (4-methyl-2-pentanol) were transferred into a 20-mL glass vial and sealed with a screwcap with PTFE-lined silicone septum. The vials were placed in a water bath at 60 °C for 5 min and magnetically stirred at 250 rpm; then the fiber was exposed to the headspace for 45 min at the same temperature. The fiber applied for the absorption of volatiles was 2 cm long and was coated with a 50/30 mm divinylbenzene/carboxen on poly-dimethyl-siloxane bonded to a flexible fused silica core (Supelco, Bellefonte, PA, USA). The length of the fiber in the headspace was kept constant. Desorption of volatiles took place in the injector of the gas chromatograph in splitless mode at 240 °C for 3 min.

GC/MS analysis was performed on a Shimadzu GC-17A gas chromatograph coupled to a Shimadzu MS QP5050 mass spectrometer. Helium was used as the carrier gas (1.8 mL/min). The mass spectrometer was operated in a scan range of 45–400 m/z. A capillary column of 0.32 mm i.d. and 0.25 µm film thickness (Supelco COWax-10 60 m, Sigma-Aldrich, Darmstadt, Germany) was used for the separation of volatile compounds. The oven temperature was programmed at initially 35 °C for 6 min, and then raised to 60, 200, and 250 °C at a rate of 2, 5, and 25 °C per min, respectively, and finally it was maintained at 250 °C for 6 min. The injector and interface temperatures were both set at 240 °C.

Identification of volatile compounds was done by comparing (i) the Kováts' retention indices based on the even n-alkanes (C10–C24) with those found in the literature; and (ii) MS data obtained from the NIST107, NIST21, and SZTERP libraries. Semi-quantitative determinations were performed by dividing the peak area of a compound with the peak area of the IS and multiplying the result with the concentration of the IS (1.62 mg/L).

2.5. Autolysis of the Spent Brewing Biocatalyst and AYE Production

Autolysis of the CWBB (300 g), after its use in the successive beer fermentation batches, was performed by suspension in 0.8 L of distilled water, as described in [18], and adjustment of the pH to 6.0 using 2 N NaOH or 2 N HCl solutions. Autolysis was carried out at controlled temperatures of 45, 50, 55, and 60 °C in a water bath. After autolysis, samples were harvested, pasteurized at 80 °C for 30 min, and then cooled down to room temperature (~25 °C). Then the samples were centrifuged at 11,000 rpm for 10 min at 4 °C and the supernatant was decanted [25]. The biocatalyst was frozen to −45 °C with a cooling rate of 3 °C/min. The frozen samples were subsequently freeze-dried for 24 h at 5×10^{-3} mbar and at −45 °C in a Freeze Drying System (Freezone 4.5, Labconco, Kansas City, MO, USA) [25]. The AYE samples were analyzed for minerals (Ca, Mg, Fe and Cu) after ashing at 550 °C and diluted with concentrated H_2SO_4, on an AA-6500 Series Atomic Absorption Spectrometer (Shimadzu, Japan).

2.6. Effect of the AYE on Yeast Cell Growth

Yeast growth experiments were carried out in triplicate in 50 mL conical flasks, where 0.1 g of the psychrotolerant yeast strain *S. cerevisiae* AXAZ-1 were introduced as inoculum. The cultivation broth contained 1 g $(NH_4)_2SO_4$/L (Chem-Lab, Zedelgem, Belgium), 1 g KH_2PO_4/L (Chem-Lab, Belgium), 5 g $MgSO_4 \cdot 7H_2O$/L (Chem-Lab, Belgium), 25 g glucose monohydrate/L (Fisher Scientific, UK), and various concentrations (0.3–0.5%) of yeast extract, either CYE (Fisher Scientific, UK) or AYE [26]. Sterilized washing water (from the orange pulp preparation stage) with a 15.3 g/L initial sugar content was used to adjust the final volume of the cultivation broths to 50 mL. The flasks were incubated for 48 h at 30 °C with an agitation speed of 200 rpm. The produced yeast biomass was centrifuged after incubation, dried, and weighed [24]. At various time intervals, the optical density of the cultivation broths was measured at 440 nm on a UV–VIS spectrophotometer (JASCO V-630) to observe the kinetics of cell mass production.

2.7. Calculation of the Activity Energy E_a

The activation energies E_a (kJ/mol) of the fermentation systems (free and immobilized cells at 10 and 15 °C) were calculated based on an Arrhenius-type equation:

$$ln(dP/dt) = ln(AX) - E_a/RT$$

where P is the ethanol concentration (g/L), t is the fermentation time (h), T is the absolute temperature (K), A is the Arrhenius pre-exponential factor of the fermentation (1/h), R is the ideal gas constant (kJ/K·mol), and X is the cell mass concentration (g/L) [27].

2.8. Statistical Analysis

The results for initial sugar concentration, residual sugar, E_a, concentration of volatile compounds, mineral contents, cell mass concentration, productivity, and yield are presented as the mean values of three repetitions plus standard deviations. The significance of the differences between the means of various groups was checked by one-way analysis of variance (ANOVA) at the 5% level of significance. p-values below 0.05 were considered significant. Fermentation experiments were carried out in duplicate.

2.9. Biorefinery Process Design

The proposed biorefinery concept for the valorization of citrus industry wastes through food production (beer and AYE) is presented in Figure 1. Discarded oranges and citrus juice industry wastes could be separated into pulp and juice in the same tank. The juice can be used as the cultivation broth for growth of brewer's yeast (e.g., *S. cerevisiae*) with addition of diluted molasses. The produced yeast will be then immobilized on the pulp and the prepared CWBB can be used in the same plant or in the fermentation industry as a biocatalyst for brewing, wine making, and distillates production. Likewise, the spent CWBB, after it has completed its cycle as a brewing biocatalyst, can be used as food or feed material, enriched in protein and vitamins, within the concept of rational exploitation of resources and organic load reduction. In the proposed process, CWBB is used for repeated wort fermentation batches and after it is no longer operationally stable, it is treated for AYE production, which can be used as a food additive (e.g., as a flavor enhancer) or as a microbiology nutrient (e.g., for the growth of yeast in the same industrial plant or in the biotechnological industry in general).

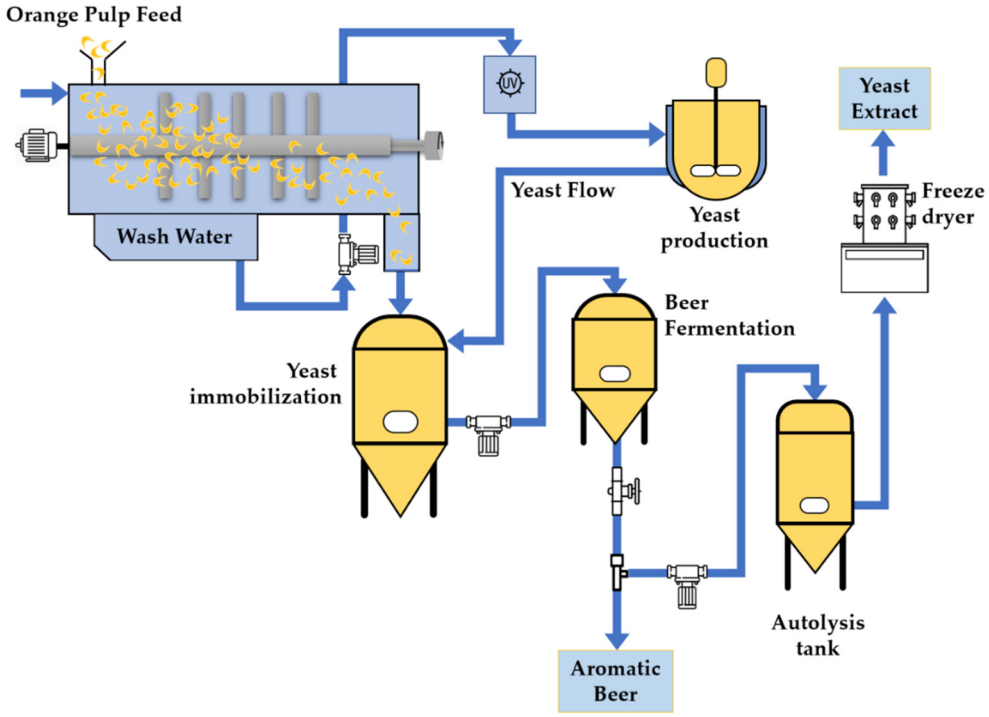

Figure 1. Refining concept for orange pulp valorization.

3. Results and Discussion

The aim of this investigation was to propose alternative ways for the exploitation of citrus industry wastes (whole discarded fruit, liquid, and solid residues), within the biorefinery concept. Specifically, the work involved (i) the production of an immobilized cell biocatalyst (CWBB) and the evaluation of its fermentation efficiency for good quality beer production (as evaluated by composition and aroma analysis); and (ii) the production of a new type of yeast extract (AYE) after autolysis of the spent CWBB and evaluation of its effect on yeast cell growth as compared with commercial extracts (CYE).

3.1. Activation Energy and Fermentation Kinetics

Brewing was performed at 15 °C and 10 °C using the psychrotolerant and alcohol-resistant yeast strain *S. cerevisiae* AXAZ-1, either free or immobilized on orange pulp waste (CWBB). The yeast cells were successively immobilized on the pulp, most likely through hydrogen bonding on the pulp surface (as it consists of cellulose and pectin polysaccharides) as well as by natural entrapment in the pulp pores [28–30]. In both cases (free and immobilized cells), the temperature was decreased from 15 to 10 °C for adaptation of the yeast culture to the lower temperature. As expected, the temperature significantly affected the fermentation time (Table 1) for both free cells (136 h at 15 °C; 260 h at 10 °C) and the CWBB biocatalyst (124 h at 15 °C; 248 h at 10 °C), as also reported in previous studies [13,31]. The reduced fermentation activity at a lower fermentation temperature is associated with cell wall permeability and fluidity changes [30,32]. However, the CWBB fermentation kinetics were slightly improved (5–20% lower fermentation time) when compared with the free cells (Table 1), most likely due to the protective effect of cell immobilization against cold-induced stress [30].

Table 1. Process parameters of the fermentation of wort by free cells and the Citrus Waste Brewing Biocatalyst (CWBB).

Biocatalyst	Batch	Temp. (°C)	Initial Sugar (g/L)	Fermentation Time (h)	Residual Sugar (g/L)	Alcohol Conc. (% *v*/*v*)	E_a (kJ/K·mol)
Free cells	1	15	141.0 ± 0.4 ^{a}	134	0.4 ± 0.2 ^{c}	5.3 ± 0.5 ^{a}	89.8 ± 2.9 ^{b}
	2	15	139.3 ± 0.2 ^{b}	135	0.4 ± 0.1 ^{c}	5.3 ± 0.3 ^{a}	
	3	15	141.2 ± 0.3 ^{a}	136	0.2 ± 0.1 ^{c}	5.4 ± 0.4 ^{a}	
	4	10	141.4 ± 0.1 ^{a}	260	0.8 ± 0.1 ^{b}	5.1 ± 0.3 ^{a}	
	5	10	141.1 ± 0.2 ^{a}	248	0.9 ± 0.1 ^{b}	4.9 ± 0.2 ^{a}	
	6	10	141.3 ± 0.2 ^{a}	240	1.1 ± 0.2 ^{b}	4.8 ± 0.3 ^{a}	
CWBB	1	15	141.1 ± 0.3 ^{a}	124	1.1 ± 0.1 ^{b}	4.8 ± 0.2 ^{a}	56.6 ± 4.1 ^{a}
	2	15	141.2 ± 0.2 ^{a}	120	1.3 ± 0.1 ^{b}	4.6 ± 0.5 ^{a}	
	3	15	141.2 ± 0.3 ^{a}	120	0.9 ± 0.2 ^{b}	4.9 ± 0.3 ^{a}	
	4	10	141.4 ± 0.5 ^{a}	248	2.2 ± 0.1 ^{a}	4.4 ± 0.7 ^{a}	
	5	10	141.2 ± 0.1 ^{a}	240	1.8 ± 0.2 ^{a}	4.5 ± 0.5 ^{a}	
	6	10	141.3 ± 0.3 ^{a}	200	1.4 ± 0.2 ^{b}	4.6 ± 0.6 ^{a}	

Different superscript letters in a column indicate statistically significant differences among the biocatalysts (ANOVA, Tukey's HSD, $p < 0.05$).

The repeated batch fermentation kinetics and the calculated activation energy (E_a) indicate a higher fermentation efficiency of the CWBB in comparison with the same amount of free yeast cells (Table 1).

Specifically, the E_a in the case of immobilized cells was reduced by 36.8%. A similar reduction was also observed in previous studies, highlighting the ability of natural cellulosic immobilization carriers to act as protective matrices for yeast cells, as well as to serve as "sugar pumps" attracting the fermentable sugar molecules (possibly by hydrogen bonding), making them more accessible to the immobilized cells [28]. The reduction of E_a in the case of the CWBB is significant since components such as the essential oil deriving from the immobilization carrier (orange pulp) could have an inhibitory effect on cell activity and viability [33]. Various other factors, such as a high sugar content, can influence the E_a of alcoholic fermentation and the quality of the final product [34]. In this study, the presence of antimicrobial compounds, such as limonene deriving from the immobilization carrier, did not have a significant effect on the fermentation kinetics. On the contrary, the E_a of the fermentation with the CWBB was 56.6 kJ/K·mol, while that of the free cell culture was higher (89.8 kJ/K·mol), which may also be attributed to the presence of nutrients (N-sources, minerals) in the orange pulp that enhanced the fermentation capacity of the yeast cells [35]. Finally, repeated batch fermentation experiments at different low temperatures proved the operational stability of the CWBB, while the beer alcohol content remained at acceptable levels (4.4–4.9% *v*/*v*).

3.2. Aromatic Attributes in Beer Produced with the CWBB

Beer is one of the most popular alcoholic beverages consumed worldwide, available in many different types and with complex aroma profiles. The volatile compounds produced during beer fermentation (congeners) and those deriving from the raw material have a major impact on the quality of the final product [23]. In this study, the effect of the CWBB biocatalyst on the aroma of the produced beer was evaluated by headspace SPME GC–MS analysis and compared with the aroma of beer produced by free cells. The detected compounds are presented in Figure 2. In general, the novel beer produced with the CWBB had an enhanced total volatile content compared to the beer produced with the free yeast cells. More specifically, the headspace of the novel beer (CWBB) contained volatile organic acids (522.3 mg/L), alcohols (223.5 mg/L), terpenes (9.72 mg/L), carbonyl compounds (21.4 mg/L), and esters (84.3 mg/L). In total, 33 different volatile compounds were detected in beer fermented with the immobilized yeast cells, and 23 were found in the beer produced with free cells (Table 2).

Therefore, the GC–MS analysis showed that wort fermentation with the CWBB can enhance the volatile profile of the products with desirable (formed by yeast or extracted) compounds, such as the fruity esters (ethyl 2-hydroxyhexanoate and ethyl-butanoate), providing extra sweet and fruity odors to the final product.

The application of CWBB in wort fermentation significantly increased the limonene and geraniol terpenes in the produced beer, obviously due to the presence of the orange pulp [36]. Among the terpenes, limonene is known to provide a citrus aroma in alcoholic beverages [37]. GC–MS analysis also showed that the use of CWBB led to the presence of terpenes in the final product, such as β-myrcene, β-linalool, and β-terpineol, which can also provide unique aroma notes to beer products, such as floral, woody, balsamic, or spicy notes [31,37]. The formation of terpene volatiles is initiated from isopentenyl and dimethylallyl diphosphates, generating monoterpenes during alcoholic fermentation [31], and only selective *S. cerevisiae* strains are known to promote the *de novo* synthesis of monoterpenes [38]. As a result, the novel beer (made by CWBB) is enhanced in terpene compounds, more possibly due to extraction from the immobilization carrier (orange pulp).

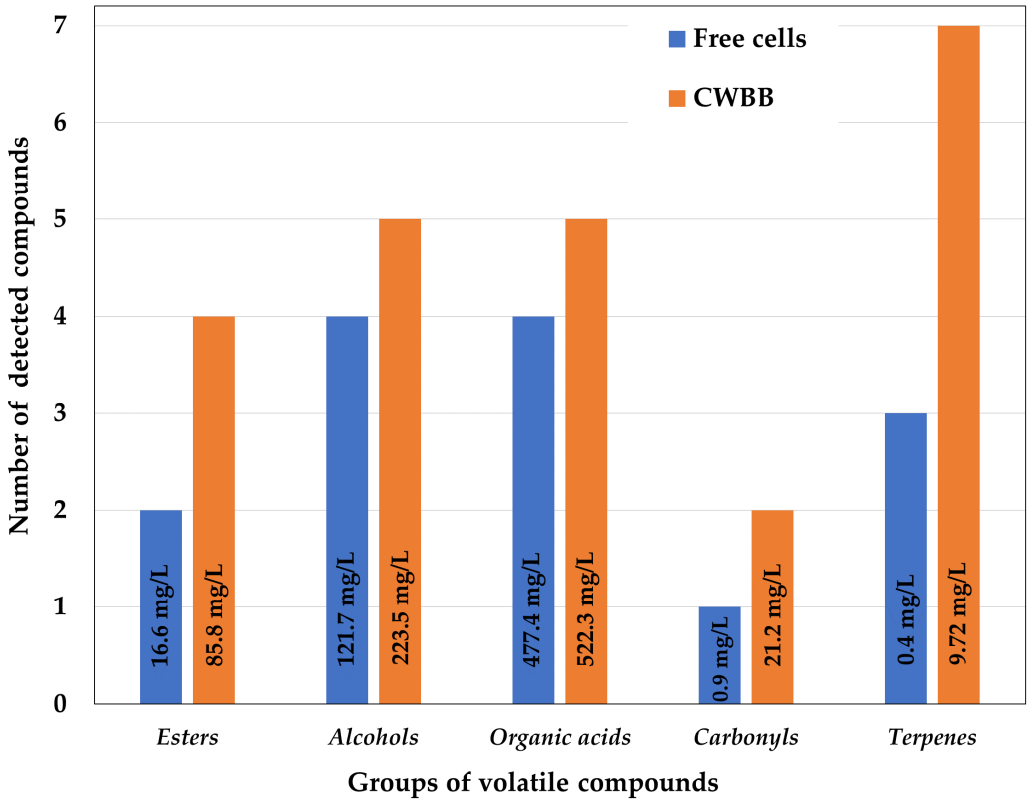

Figure 2. Number and content of volatile compounds (mg/L) detected in beer produced at 10 °C with free yeast cells or with the CWBB biocatalyst.

Table 2. Volatile compounds detected in the beers brewed at 10 °C using the CWBB or free yeast cells.

Volatile Compounds	KI	KI_{ref}	Free Cells (mg/L)	CWBB (mg/L)
Organic acids				
Hexanoic acid	1895	1926	19.0 ± 2.1 [a]	10.8 ± 3.0 [b]
Octanoic acid	2044	2111	318.4 ± 12.5 [a]	351.3 ± 9.1 [b]
Decanoic acid	2289	2336	130.2 ± 7.5 [a]	129.2 ± 8.3 [a]
Undecanoic acid	2301	2266	9.8 ± 1.01 [b]	25.3 ± 4.7 [a]
Undecylenic acid	2320	2390	n.d.	5.7 ± 0.9
Alcohols				
2-Methyl-1-butanol	1190	1213	85.9 ± 8.3 [b]	167.9 ± 11.1 [a]
1-Hexanol	1319	1354	1.8 ± 0.5 [a]	0.2 ± 0.1 [b]
1-Octanol	1507	1563	9.9 ± 1.3 [a]	3.1 ± 0.7 [a]
2-Furanmethanol	1627	1680	n.d.	4.1 ± 0.8
Phenyl ethyl alcohol	1847	1933	24.16 ± 4.1 [b]	48.2 ± 5.9 [a]
Terpenes				
β-Myrcene	1182	1176	n.d.	2.3 ± 0.4
Limonene	1225	1212	0.2 ± 0.1 [b]	2.7 ± 0.6 [a]
γ-Terpinene	1244	1265	n.d.	0.3 ± 0.1
β-Linalool	1522	1556	n.d.	2.3 ± 0.4
α-Terpineol	1652	1688	Tr	0.6 ± 0.1
β-Terpineol	1723	1711	n.d.	0.1 ± 0.05
Geranial	1860	1862	0.2 ± 0.1 [b]	1.4 ± 0.5 [a]
Esters				
2,3-Butanedione	988	973	0.2 ± 0.1 [a]	0.3 ± 0.1 [a]
Ethyl acetate	885	889	16.4 ± 3.1 [b]	84.3 ± 6.9 [a]
Ethyl butanoate	1022	1040	n.d.	1.2 ± 0.1
Ethyl 2-hydroxyhexanoate	1372	1386	n.d.	0.04 ± 0.02
Carbonyl compounds				
Nonanal	1281	1406	n.d.	12.4 ± 0.9 [a]
Decanal	1417	1507	0.9 ± 0.1 [b]	8.8 ± 1.4 [a]

Different superscript letters in a row indicate statistically significant differences among the biocatalysts (ANOVA, Tukey's HSD, $p < 0.05$); n.d.: not detected; Tr: traces < 0.03 mg/L.

3.3. Efficient AYE Production from Spent CWBB

The yeast extract and single-cell protein industries are highly interested in replacing cultivation media with alternative, cheaper materials [39]. Yeast extract is commonly produced via *S. cerevisiae* autolysis at high temperatures (45–55 °C). Previous studies revealed that AIWs are potential raw materials for yeast extract production [18] as a nutrient that can lead to lower process costs. A parameter that affects production cost is the fermentation rate, in relation with productivity and yield [40]. In this work, the spent CWBB after the brewing experiments was used for AYE production through autolysis.

Comparison of the mineral analysis results for AYE and CYE revealed that AYE contained higher amounts of minerals, while copper was only detected in the case of AYE (Table 3). Copper is an essential trace element for most living organisms as it is involved in oxygen transport and many enzymatic redox reactions that are important for the maintenance of life. The presence of significant amounts of copper in yeast fermentation can inhibit yeast activity and can even cease the fermentation. On the other hand, yeast strains can reduce the copper levels during fermentation [41]. Copper was detected in small amounts (2.3 mg/100 g of yeast extract) only in AYE, an amount that does not pose any threat to fermentation systems—on the contrary, it can enhance yeast viability [41,42]. This was verified during the study of yeast biomass production (*S. cerevisiae*) in media containing either AYE or CYE (Table 4). It was observed that AYE can lead to a significant increase in biomass yield, in the range 110–170%, compared with CYE. In addition, the use

of AYE enhanced the biomass productivity by 60–150% compared to CYE. The significantly enhanced mineral content of AYE (Table 3), as well as other yeast or orange pulp nutrients present in CWBB, is the most possible explanation for this outcome. The mineral composition of molasses and orange pulp used in this study, as reported by [43], is also presented in Table 3. Further details on the composition of molasses and orange pulp can be found in other studies carried out in parallel with this study [43–45].

Table 3. Mineral content of the alternative yeast extract (AYE) in comparison with commercial yeast extract (CYE).

Minerals	Content (mg/100 g)			
	AYE	CYE	Orange Pulp/Water (1:1) [43]	Molasses (Undiluted) [43]
Calcium	221.0 ± 8.0 ^{a}	120.0 ± 4.1 ^{b}	28.5	163.6
Magnesium	185.0 ± 3.7 ^{b}	200.0 ± 4.0 ^{a}	0.02	1420.0
Iron	0.6 ± 0.1 ^{b}	5.0 ± 0.9 ^{a}	n.d.	7.1
Copper	2.31 ± 0.15	n.d.	0.32	0.60

Different superscript letters in a row indicate statistically significant differences among the strains (ANOVA, Tukey's HSD, $p < 0.05$); n.d.: not detected.

Table 4. Biomass productivity in fermentation using the *S. cerevisiae* AXAZ-1 strain after the addition of alternative yeast extract (AYE) and commercial yeast extract (CYE) in various concentrations.

Yeast Extract Conc. (%)	Initial Biomass (g)	Sugar Conc. (g/L)	Biomass Conc. (g/L)	Biomass Productivity (g/L/h)	Biomass Yield (g/g)
CYE 0.3%	0.1	40	6.72 ± 1.05 ^{b}	0.22 ± 0.04 ^{b}	0.17 ± 0.01 ^{bb}
CYE 0.4%	0.1	40	8.64 ± 1.72 ^{b}	0.57 ± 0.07 ^{b}	0.22 ± 0.01 ^{b}
CYE 0.5%	0.1	40	5.46 ± 0.81 ^{b}	0.19 ± 0.05 ^{b}	0.15 ± 0.05 ^{b}
AYE 0.3%	0.1	40	13.44 ± 2.77 ^{a}	0.55 ± 0.11 ^{a}	0.36 ± 0.01 ^{a}
AYE 0.4%	0.1	40	20.16 ± 2.46 ^{a}	0.88 ± 0.15 ^{a}	0.51 ± 0.05 ^{a}
AYE 0.5%	0.1	40	15.84 ± 1.23 ^{a}	0.41 ± 0.08 ^{a}	0.39 ± 0.04 ^{a}

Different superscript letters in a column, regarding the type of yeast extract at the same concentration (CYE and AYE), indicate statistically significant differences among the strains (ANOVA, Tukey's HSD, $p < 0.05$).

3.4. Discussion

In previous studies, mixed substrates, consisting of AIWs such as brewers' spent grains, spent malt rootlets, cheese whey, molasses, orange, and potato pulps, were used for the production of single-cell protein, edible mushrooms, AYE, enriched livestock feeds, etc. [43–45]. In [43], a mycelium-enriched (*Pleurotus ostreatus*), mixed AIW product was evaluated as a potential raw material for AYE production for food, feed, or microbiology uses. Specifically, the product was autolyzed, freeze-dried, powdered, and analyzed for total ribonucleic acid content, showing the potential for use as a commercial natural food flavor enhancer [43]. This study focused on orange waste utilization, proposing, apart from AYE, the production of an effective brewing biocatalyst (CWBB) and a novel type of beer. The 5-fold increase in esters observed in the beer produced by the CWBB affects the aroma of the product, providing fruity odor notes. Enrichment in the aroma compounds via the application of biocatalysts, immobilized on the natural cellulosic carriers prepared from the AIWs, also has been highlighted in previous studies (wine making and brewing) [14,24,25,30]. In this study, waste orange pulp was proposed as the immobilization carrier, and being rich in cellulose, pectin, and citrus essential oils, it enhanced the volatile content during wort fermentation. As a result, more terpenes were found in beer fermented by the CWBB compared with the beer samples fermented with free yeast cells. Sweet oranges are known to contain about 0.4–0.5% *w*/*w* essential oil, including a wide variety of terpenes, with limonene being their most significant representative [46]. The enhanced terpene profile in beer fermented with CWBB can be attributed to the

extraction of terpenes form orange pulp [46]. Regarding the growth of *S. cerevisiae* using the AYE from CWBB in the growth medium, the presence of orange pulp was also identified as responsible for the promotional effect of AYE [47]. Therefore, the utilization of the discarded oranges to prepare CWBB for use in the production of beer with enriched aroma, and the subsequent production of an effective AYE from the spent CWBB, may facilitate the industrialization of the proposed processes for valorization of citrus wastes within the biorefinery concept. Future studies should focus on technoeconomic evaluations for the complete utilization of discarded oranges (peels, pulp, juice, and solid residues), while process designs should take into account scale-up issues, such as contamination problems, efficient sterilization of the substrates, etc.

4. Conclusions

The proposed CWBB biocatalyst led to a substantial increase in esters and terpenes in beer as compared with free cells. A significant promotion of yeast growth was also observed when the AYE made by autolysis of the spent CWBB was used in a growth medium consisting of spent orange juice and diluted molasses. Therefore, the proposed process, based on bioprocessing of citrus wastes within the biorefinery concept, can result in (i) an effective brewing biocatalyst, prepared by yeast immobilization on orange pulp that advances the aroma of beer. The spent biocatalyst after it has completed its cycle of brewing batches, can be used as (ii) a protein-rich feed or (iii) a food additive, as well as for effective yeast extract production after autolysis, or for (iv) food (flavor enhancer) or (v) microbiology applications (growth medium nutrient). Therefore, the exploitation of citrus wastes as raw materials for beer production, yeast biomass production, and AYE in the same industrial unit, apart from satisfying ethical and environmental issues, can lead to the production of low-cost competitive products and added-value for the involved industries.

Author Contributions: Conceptualization, supervision, resources, A.A.K. and A.B.; methodology, investigation, data curation, V.G.; validation, software, V.G., A.T., S.P.; writing—original draft preparation, review, and editing, A.B., A.T.; funding acquisition, A.B. and V.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research has been co-financed by the European Union (European Social Fund—ESF) and Greek national funds through the Operational Program "Education and Lifelong Learning" of the National Strategic Reference Framework—Research Funding Program: Heracleitus II. Investing in knowledge society through the European Social Fund.

Conflicts of Interest: The authors declare no conflict of interest.

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