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Discarded Oranges and Brewer's Spent Grains as Promoting Ingredients for Microbial Growth by Submerged and Solid State Fermentation of Agro-industrial Waste Mixtures

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Abstract The exploitation of various agro-industrial wastes for microbial cell mass production of *Kluyveromyces marxianus*, kefir, and *Saccharomyces cerevisiae* is reported in the present investigation. Specifically, the promotional effect of whole orange pulp on cell growth in mixtures consisting of cheese whey, molasses, and potato pulp in submerged fermentation processes was examined. A 2- to 3-fold increase of cell mass was observed in the presence of orange pulp. Likewise, the promotional effect of brewer's spent grains on cell growth in solid state fermentation of mixtures of whey, molasses, potato pulp, malt spent rootlets, and orange pulp was examined. The cell mass was increased by 3-fold for *K. marxianus* and 2-fold for *S. cerevisiae* in the presence of these substrates, proving their suitability for single-cell protein production without the need for extra nutrients. Cell growth kinetics were also studied by measurements of cell counts at various time intervals at different concentrations of added orange pulp. The protein content of the fermented substrates was increased substantially, indicating potential use of mixed agro-industrial wastes of negligible cost, as protein-enriched livestock feed, achieving at the same time creation of added value and waste minimization.

Keywords Agro-industrial wastes · Discarded oranges · Brewer's spent grains · Cell growth · Single-cell protein

Introduction

Agro-industrial wastes are usually discarded in the environment causing serious pollution problems. Wastes especially from the food industry contain considerable amounts of fermentable

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and nonfermentable sugars that could be utilized using microorganisms to produce value-added products. For example, residues from the citrus and other fruit industries have been used as animal feed [1], for oxidative and hydrolytic enzyme production [2], cellulases [3], enzymatic extract production [4], as well as growth media for starter cultures such as kefir for bread making and alcoholic and dairy fermentations [5, 6]. However, bulk quantities of oranges are discarded each year creating serious environmental problems, while big quantities are sold to citrus juice industries at very low prices. The total production of citrus fruits in Greece is only about 1,000,000 tonnes annually, 30 % of which is processed for juice production. By the disposal of oranges and citrus wastes in landfills, the pollution occasionally reaches levels so high that they can be visually detected in the aqueous horizon [7]. Therefore, handling of citrus processing wastes is correlated with a number of economic and environmental problems, such as high organic loads, lack of available disposal areas, and increase of transportation costs. However, many researchers have pointed out the nutritional composition and utilization potential of discarded citrus and citrus wastes for added-value products such as single-cell protein (SCP) [1, 5, 6, 8]. For example, the growth rate of kefir culture was increased when orange pulp was suspended to the growth medium, and increased ethanol production was also observed [6].

Brewers' spent grains (BSG) are available at low or no cost throughout the year and are produced in large quantities by both large and small breweries. The chemical composition of BSG varies according to barley variety, harvest time, and malting and mashing conditions, and includes fiber, protein, cellulose, lignin, starch, pentosans, β-glucans, lipids, and phenolic compounds [9–11]. BSG are usually discarded or used as animal feed after drying. Yeast cells immobilized on BSG have been used successfully in various brewing and winemaking processes [12, 13]. Also, biocatalysts produced by cell immobilization on BSG or delignified BSG were found suitable for alcoholic fermentation of molasses, exhibiting advantages such as ease of handling, low production and process costs, good operational stability, and low by-product formation [11]. On the other hand, their use for SCP is limited. BSG treated with *Aspergillus* sp. were proposed as protein-enriched animal feeds or as nutritious supplements in yeast growth media containing mixtures of molasses and orange pulp as main carbon sources, resulting in significantly improved biomass yields [14].

Apart from discarded oranges and BSG, various other agro-industrial wastes such as whey, molasses, potato residues, malt spent rootlets (MSR), etc., are available as raw materials for animal feed and SCP production to improve the nutritional composition of synthetic growth media or to prepare media exclusively by these wastes [15–18]. The aim of this work was to study the promotional effect of whole discarded orange fruit and BSG on cell growth of various yeast species using substrates consisting of mixtures of the above agro-industrial wastes in submerged (SmF) and solid state fermentation (SSF) processes.

Materials and Methods

Materials

Cheese whey was obtained from the Agricultural Cooperatives Union of Kalavryta (Achaia Province, Greece). Molasses was supplied by the BG Spiliopoulos SA alcohol distillery (Patras, Greece). BSG and MSR were supplied by the Athenian Brewery SA (member of the Heineken NV group, Patras, Greece). The pH of the fermentation media was controlled using a 2 M NaOH solution (Fixanal Fluka Analytical). For the determination of total

(Kjeldhal's) nitrogen, K_2SO_4 (Fluka), $CuSO_4 \cdot 5H_2O$ (Merck), 0.1 M NaOH (Fixanal Fluka Analytical), and NaOH pellets (Chem-Lab) were also used.

Microorganisms

The kefir culture was isolated from commercially available kefir grains (Meliton SA, Thessaloniki, Greece). It was grown at 30 °C in a synthetic medium consisting of 20 g/L lactose (Fluka, Buchs, Switzerland), 20 g/L glucose (Fluka, Buchs, Switzerland), 4 g/L yeast extract (Fluka), 1 g/L $(NH_4)_2SO_4$ (Fluka), 1 g/L KH_2PO_4 (Fluka), and 10.2 g/L $MgSO_4 \cdot 7H_2O$ (Fluka). The pH of the medium was adjusted to 5.5 prior to inoculation. The psychrotolerant and alcohol-resistant yeast strain *Saccharomyces cerevisiae* AXAZ-1, isolated from the Greek agricultural area [19], was grown at 30 °C in a medium consisting of 40 g/L glucose, 4 g/L yeast extract, 1 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, and 5 g/L $MgSO_4 \cdot 7H_2O$. The thermotolerant yeast *Kluyveromyces marxianus* IMB3 was obtained from the University of Ulster [20], and it was grown in the same media as *S. cerevisiae* AXAZ-1. All media were sterilized by autoclaving at 130 °C and 1.5 atm for 15 min prior to use.

Preparation of Substrates

The external (yellow exocarp) parts of orange skins were removed, and then the whole remaining fruit was blended for 10 min. The blended product, hereinafter referred to as "orange pulp" (including the juice), was diluted with water at a ratio of 1:1 (by weight). Potato pulp was prepared in the same manner and was diluted with water at a ratio of 1:2 (by weight). Molasses was diluted with water to a density of 4°Be. Cheese whey, BSG, and MSR were used without any pretreatment.

SmF of Liquid Waste Mixtures in the Presence of Orange Pulp

Growth of kefir, *K. marxianus* IMB3, and *S. cerevisiae* AXAZ-1 was carried out in 300 ml Erlenmeyer flasks by a SmF process of different mixtures of liquid agro-industrial wastes, as shown in Table 2. The total volume of the substrates was fixed to 200 ml with water. Cheese whey was the main substrate in the case of *K. marxianus* and kefir which contains lactose-fermenting species. The initial pH of the substrates was adjusted to 5.5 in the case of kefir and *S. cerevisiae* and to 7 for *K. marxianus* using a 2 M NaOH solution. The substrates were then sterilized by autoclaving for 15 min at 120 °C. After cooling, the substrates were separately inoculated with 1 g of harvested culture of each microorganism, and growth was carried out with air supply at 500 ml/min (pressure, 6,900 Pa), for 4 days at 30 °C. The protein content before and after the SmF treatment of the substrates that yielded the optimum results for cell mass yield was analyzed by determination of total nitrogen (Table 3).

SSF of Solid and Liquid Waste Mixtures in the Presence of BSG

Growth of kefir, *K. marxianus* IMB3, and *S. cerevisiae* AXAZ-1 was also carried out by SSF of the above liquid wastes including solid brewery wastes, such as BSG and MSR. After preparation of the substrate mixtures as shown in Table 4 and pH adjustment to 5.5, they were sterilized by autoclaving, and then equal amounts were spread to petri dishes. The substrates were inoculated with 1 g of harvested culture and were incubated for 4 days at

30 °C for cell growth. The amounts of BSG and MSR added were chosen so as to have 70–80 % *w*/*w* moisture content of the mixed substrates.

Proximate Analysis of the Substrates

Moisture content (percent *w*/*w*) was determined by weighing before and after heating at 80 °C overnight. Ash (percent *w*/*w*) was determined by burning 1–2 g of the samples in porcelain crucibles on a burner flame for 15 min, followed by heating in a furnace at 550 °C for at least 3 h. For HPLC analysis of fermentable sugars (sucrose, fructose, lactose, glucose, galactose) and ethanol, 1 g of sample was suspended to 5 ml deionised water and was shaken on a vortex for 60 s. The supernatant was then filtered by 0.45 μm membrane filters. In the case of cheese whey, analysis was performed after filtration and dilution with water to 20 % *v*/*v*. HPLC analysis was performed as described below. For fat analysis, 5 g of predried sample was extracted with diethyl ether in a Soxhlet apparatus for about 2.5 h. The extract was then condensed in a rotary evaporator, dried at 105 °C for 0.5 h, and weighed. In the case of cheese whey, the determination of fat was carried out by the Gerber method, which involves separation of fat by treatment with sulfuric acid and separation by centrifugation. Total nitrogen expressed as grams of crude protein per 100 g sample on dry weight basis (grams/100 g dw) was determined in raw materials before and after treatment using Kjeldahl's procedure (AOAC official method 991.20). The determination of phenolic compounds was carried out according to Singleton and Rossi [21], which involved alkaline hydrolysis, extraction of phenolic compounds with diethyl ether/ethyl acetate (1:1), separation of the aqueous and organic phases, and determination of total phenolics as milligrams of gallic acid/gram of dry sample with the Folin–Ciocalteau reaction. The determination of the antioxidant capacity was performed according to Brand Williams et al. [21] as free radical-scavenging activity (percent reduction of the 2,2-diphenyl-1-picrylhydrazyl radical). The determination of chemical oxygen demand (COD) was carried out by both colorimetric and photometric tests according to standard methods (APHA, 1995). The results are presented in Table 1 as means of at least four repetitions.

Residual Sugar, Cell Growth Kinetics, and Cell Mass Yields

Residual sugar was determined on a Shimadzu LC-9A HPLC system. A Shim-Pack (SCR-101N) column, a refractive index detector, three times distilled water as mobile phase (0.8 ml/min), and 1-butanol (0.5 % *v*/*v*) as internal standard were used. The column temperature was 60 °C, sample dilution was 1 % *v*/*v*, and injection volume was 40 μL. The determination of residual sugar in the SSF substrates was done in the supernatant of a 20 % *v*/*v* suspension prepared by mixing thoroughly with water and leaving to stand for 12 h.

Cell growth kinetics was determined by microscopy cell counts at various time intervals using a glass hemocytometer slide. Specifically, 1 ml of the substrate was transferred to a test tube that contained 9 ml of Ringer's solution. After shaking the tube on a Vortex apparatus, 1 ml of that solution was plated on the hemocytometer, and then five different squares of the hemocytometer grid were chosen for the counting. The results were expressed as cells per milliliter of sample.

The specific growth rate was calculated by the equation $N=N_0\times\exp[\mu\times(t-t_L)]$, $t>t_L$, where N_0 is the initial cell count, N is the cell count at time t, and t_L is the time of lag phase.

The cell mass yield was calculated as grams of cell mass/gram of consumed sugar (g/g_s) by the equation cell mass yield=(final cell mass−initial cell mass)/(initial sugar−final sugar)×100. Cell mass in the liquid substrates before and after treatment was determined

Table 1 Proximate composition of BSG, MSR, molasses, cheese whey, and orange and potato pulps

	MSR	BSG	Whey	Molasses	Orange pulp	Potato pulp
Moisture (% *w*/*w*)	12.9±1.4	73.8±4.1	93.5±0.4	22.8±1.0	82.9±1.4	82.8
Solids (% *w*/*w*)	87.1±1.4	26.2±4.1	6.5±0.4	77.2±1.0	17.1±1.4	17.2
pH	5.9[a]	5.0[a]	4.2	4.7	4.1	5.5
Ash	6.78±0.31[c]	4.16±0.22[c]	1.9±0.5[c]	7.5±0.5[e]	0.36[e]	1.04[d]
Fat	4.44±1.03[c]	6.19±2.14[c]	3.8±1.7[c]	0.0[e]	0.8[c]	0.58[c]
Crude protein	31.12±0.13[c]	24.0±2.8[c]	14.2±2.5[c]	7.3±2.3[e]	2.85[c]	2.47[d]
Fermentable sugar[b]	0[c]	2.57[c]	5.18±0.48[d]	42.5±6.1[e]	8.31[e]	0.88[d]
COD (mg/L)	112±2[f]	55±8[f]	52,558±5,165	>80,000	103±4[f]	101±2[f]
Total phenolics$_{aq}$ (mg/g DM)	4.22±2.38	9.65±3.90	na	7.72±1.50	15.78±2.16	6.02±1.03
Total phenolics$_{org}$ (mg/g DM)	7.65±3.10	6.50±2.10	na	1.76±0.08	4.98±1.04	2.74±0.65
Total antioxidant capacity$_{org}$ (IC_{50} mg/ml)	4.30±1.5	1.63±0.44	4.35±0.40	4.00±1.02	2.03±0.78	3.79±0.90

DM dry matter, *aq* aqueous extract, *org* organic extract, *na* not available

[a] In aqueous extract

[b] Total amount of sucrose, glucose, galactose, fructose, and galactose, if present

[c] Percent *w*/*w* DM

[d] Percent *w*/*v*

[e] Percent *w*/*w* wet

[f] Aqueous extract

by centrifugation at 5,000 rpm for 10 min. The harvested cultures retained about 70 % moisture; therefore, the cell mass yield was expressed in wet weight basis. In the SSF substrates the cell mass was determined gravimetrically, by weighing the substrates before and after treatment. All experiments were carried out in triplicate, and the mean values plus standard deviations are presented.

Results and Discussion

Rationale of the Investigation

This investigation was done in the frame of efforts worldwide to minimize and exploit agro-industrial wastes, thus producing added value. To contribute to these efforts, the promotional effect of discarded whole oranges and solid brewery wastes on the growth of various microorganisms in SmF and SSF processes (SSF) of mixed agro-industrial wastes without addition of synthetic nutrients was evaluated. The thermotolerant lactose-fermenting yeast *K. marxianus* IMB3 and the mixed dairy culture kefir (with yeasts as the dominant species due to the aerobic type method used for inocula preparation) have been previously studied for the exploitation of cheese whey [15, 20, 22]. The strain *S. cerevisiae* AXAZ-1 is a psychrotolerant and alcohol-resistant yeast extensively studied for alcoholic fermentation processes, such as wine making, brewing, distillates, and ethanol production [23]. The basic

ingredients of the substrates used were common food industry wastes that have been widely used for microbial mass production as well as for ethanol and other chemicals production: cheese whey, the major liquid waste of the dairy industry, molasses from the cane or beet sugar industries, and potato pulp from the potato processing industries or restaurant wastes. Table 1 shows the results of the proximate analysis of the agro-industrial wastes used in this study [18]. The sugar, protein, fiber, and mineral content of these samples, as well as the presence of minor compounds such as vitamins and trace elements, make them suitable for microbial treatment. Therefore, biotechnological utilization can convert these materials from potential pollutants to added-value products.

Whole discarded oranges and solid brewery wastes have been mainly evaluated as raw materials for recovery of various products such as pectin, phenolics, bioactive compounds, fiber, biofuels, etc. [24, 25] rather than SCP. Therefore, this investigation is an optimization study on cell growth of various food-related microorganisms in mixed substrates of the above agro-industrial wastes in SmF or SSF processes, focusing on the effect of the presence of orange pulp or BSG in the mixtures. The treated materials could potentially be used as protein-enriched livestock feed.

Effect of Orange Pulp on Cell Growth by SmF

Table 2 shows cell mass production yields of *K. marxianus*, kefir, and *S. cerevisiae* during SmF of various mixtures of cheese whey, molasses, potato pulp, and whole orange pulp. In the case of *K. marxianus*, increased contents of whole orange pulp resulted to a substantial increase (about 3-fold) of cell mass yield, although the main carbon sources (whey and molasses) were reduced in the substrate mixture. The same was observed in the case of kefir culture. Regarding *S. cerevisiae*, experiments 1 and 2 (Table 2) showed that the increase of orange pulp increased cell mass yield by about 2-fold when the carbon source was only molasses. Furthermore, the addition of potato pulp and whey reduced the cell mass yield when the orange pulp content was reduced. These results show that the increase of the content of orange pulp in the substrate mixtures increased the cell mass production yields for all the tested microorganisms, although the carbon sources were increased. The best yield

Table 2 Effect of orange pulp on *K. marxianus*, kefir, and *S. cerevisiae* growth by SmF of mixtures of various agro-industrial wastes

Microorganism		Substrate composition				Initial sugar (g)	Residual sugar (g)	Cell mass yield (g/g_s)
		Whey (ml)	Molasses (ml)	Potato (ml)	Orange (ml)			
K. marxianus	1	30	10	10	100	21.30±0.34	1.56±0.11	0.87±0.04
	2	100	10	10	30	20.78±0.85	0.18±0.07	0.35±0.06
	3	30	100	10	10	18.55±0.92	0.10±0.04	0.27±0.06
Kefir	1	100	10	10	30	15.24±0.46	0.43±0.11	0.48±0.12
	2	100	30	10	10	30.24±1.24	1.01±0.21	0.41±0.08
S. cerevisiae	1	0	50	0	100	25.44±2.12	2.99±0.45	0.80±0.12
	2	0	100	0	50	27.77±2.43	5.50±1.01	0.64±0.04
	3	0	100	10	40	35.60±2.33	1.21±0.34	0.53±0.07
	4	10	100	10	30	30.32±1.89	2.43±0.44	0.53±0.05
	5	30	100	10	10	31.43±1.67	9.78±1.57	0.39±0.09

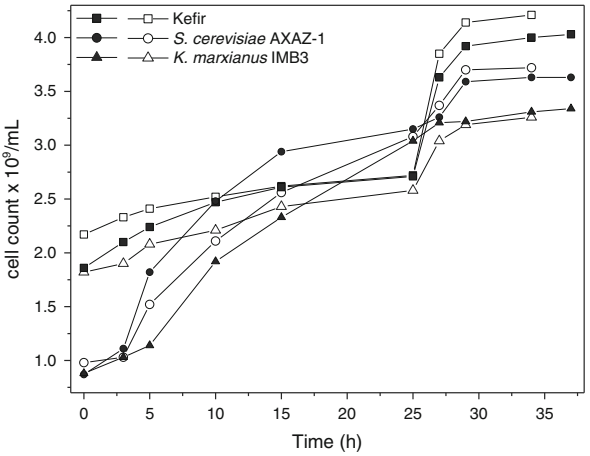

Fig. 1 Cell growth kinetics during SmF at 30 °C of the substrates with the highest content of orange pulp (optimum) (*open symbols*) and those with the lowest content of orange pulp (*closed symbols*)

was obtained in the case *K. marxianus* and the lowest in the case of kefir, probably due to better carbohydrate bioconversion and lactose permease activity expressed by *K. marxianus* [26].

The kinetics of fermentation at the optimum substrate composition indicated that sugar consumption was completed at about 29 h in the presence of orange pulp. Likewise, all microorganisms resulted to about the same sugar conversion. Figure 1 presents cell growth kinetics at two different orange pulp contents used (highest and lowest, respectively), also proving the promotional effect of orange pulp on cell growth. Table 3 presents the crude protein contents of the treated substrates. *K. marxianus* and kefir resulted in the highest protein concentration. Even when the main carbon source (whey, 100 ml) in the case of kefir was higher, *K. marxianus* resulted in about the same protein content at lower carbon source content (whey, 30 ml) due to the higher concentration of orange pulp in the substrate.

The high cell mass yields obtained, which in some cases were noticeably high (e.g., >0.80 g/g_s for *K. marxianus* and *S. cerevisiae* in the optimum substrates) may be due to the presence of orange pulp, which contains complex B vitamins such as thiamine, pyridoxine hydrochloride, calcium D-pantothenate, and nicotinic acid that are components of the coenzyme pyruvate dehydrogenase. This coenzyme contributes to the transformation of pyruvate into acetyl-CoA which then enters the citric acid cycle under aerobic conditions to carry out cell metabolism and growth [27].

Table 3 Protein content before and after the SmF treatment of the optimum substrate mixtures of agro-industrial wastes with *K. marxianus*, kefir, and *S. cerevisiae*

Microorganism	Substrate composition				Crude protein (g/100 g dw)	
	Whey (ml)	Molasses (ml)	Potato (ml)	Orange (ml)	Before growth	After growth
K. marxianus	30	10	10	100	14.13±0.25	30.23±1.38
Kefir	100	10	10	30	12.54±0.57	31.02±1.58
S. cerevisiae	0	50	0	100	10.21±0.59	23.58±0.69

Table 4 Effect of BSG on *K. marxianus*, kefir, and *S. cerevisiae* growth by SSF of mixtures of various agro-industrial wastes

Microorganism		Substrate composition						Initial sugar (g)	Residual sugar (g)	Cell mass yield (g/g_s)
		Whey (ml)	Molasses (ml)	Potato (ml)	Orange (ml)	MSR (g)	BSG (g)			
K. marxianus	1	30	10	10	100	0	80	29.60±2.12	10.06±2.35	0.21±0.05
	2	30	10	10	100	20	40	49.53±7.56	0.23±0.10	0.03±0.01
	3	30	10	10	100	40	20	39.53±4.11	0.23±0.09	0.01±0.006
Kefir	1	100	10	10	30	25	60	17.82±1.41	5.63±1.11	0.10±0.03
	2	100	10	10	30	60	30	26.49±2.24	0.20±0.09	0.07±0.03
	3	100	10	10	30	50	0	34.52±3.61	18.68±2.16	0.06±0.02
S. cerevisiae	1	0	50	0	100	0	70	18.22±1.78	8.61±1.38	0.26±0.05
	2	0	50	0	100	20	40	18.67±1.61	8.04±1.33	0.08±0.03
	3	0	50	0	100	30	20	39.17±3.66	10.17±2.41	0.03±0.01

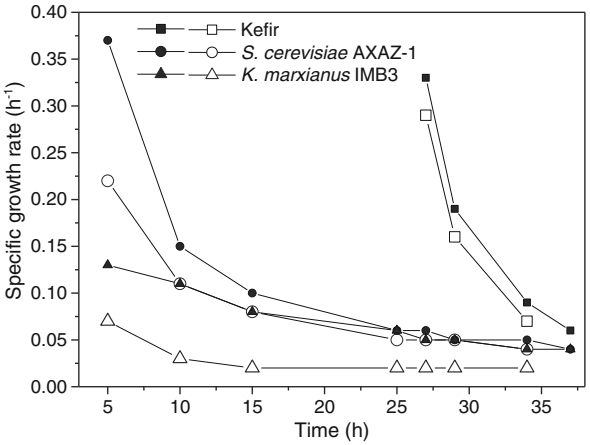

Fig. 2 Specific growth rates during SmF at 30 °C of the substrates with the highest content of orange pulp (optimum) (*open symbols*) and those with the lowest content of orange pulp (*closed symbols*)

Effect of BSG on Cell Growth by SSF

Table 4 shows cell mass production yields during SSF of mixtures of whey, molasses, potato pulp, orange pulp, BSG, and MSR by *K. marxianus*, kefir, and *S. cerevisiae*. In the case of *K. marxianus* and kefir, the increase of the BSG content in the substrate mixture resulted in an increase of cell growth. In the case of *S. cerevisiae*, the results showed that the increase of BSG in the substrate increased cell mass yield when the main carbon source was molasses and orange pulp. It should be noted that in the SSF experiments, the main carbon sources were cheese whey, molasses, and potato pulp, and their amounts were kept constant in the substrate mixtures. MSR were added to keep the moisture levels of the substrates stable. They are a dry fibrous, lignocellulosic raw material that does not contain directly fermentable sugar. The SSF experiments resulted in lower cell mass production yields compared with the SmF treatments which can be attributed to the absence of agitation or air supply which are essential for yeast growth, the lower amounts of fermentable sugar available, and the inability of yeast species to utilize lignocellulosics.

Conclusion

The results of this study show a promotional effect of whole orange pulp and BSG on cell growth of yeast species in mixed food industry wastes without addition of extra synthetic nutrients (e.g., minerals). Orange pulp produced after the recovery of juice as well as BSG has also been previously found suitable to promote cell growth and alcoholic fermentation as yeast immobilization supports [5, 6, 11–13]. In the present study, orange pulp is the blended product of the whole fruit, including juice, after removal of the outer yellow part. Therefore, the effect on cell growth can also be attributed to the nutrients contained in the juice of the fruit. Figures 1 and 2 illustrate that the increased concentration of orange pulp in the substrates resulted in increased cell growth rates of all the tested microorganisms being consistent with the results presented in Table 2. The significance of the investigation is attributed to the possibility of the simultaneous exploitation of various agro-industrial wastes such as discarded oranges, brewery solid wastes, cheese whey, molasses, and potato pulp,

for microbial cell mass production, thus minimizing these wastes and creating added value. The treated materials can be used as protein-enriched livestock feeds to improve meat and milk production. The production cost would be low due to the negligible cost of the raw materials.

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