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Biomass and Biopolymer Production using Vegetable Wastes as Cheap Substrates for Extremophiles

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Waste biomass is continuously generated in huge amounts by cultivation, harvest, selection and industrial transformation of fruits, vegetables and crops that are exploited for production of foods, chemicals, fertilizers and bioenergy. The management and the proper disposal of such biomass (whose levels in Italy reach about 22,000,000 t/y) is a worldwide problem both in environmental and in economical terms. On this basis we investigated a possible strategy of valorization of these residues, with particular regard to some agro-industry wastes and crop residues. Agro-industrial wastes included tomato and lemon processing wastes, fennel and carrot selection residues that are produced by some of the most important activities of Italian food industry sector. Crop residues selected for this study included giant roots and cardoon residues, that are two lignocellulosic cultures that in recent year attracted attention in relation to their potential use in bioenergy (bioethanol, biodiesel) production. All the wastes were tested as growth media in batch fermentation (BF) and dialysis fermentation (DF) modes for the production of biotechnologically useful microorganisms, namely two thermophilic species and two halophilic species. All the wastes afforded appreciable microbial biomass production and moreover they positively affected also production of enzymes and biopolymers by the studied extremophiles. The results obtained encourage to exploit such wastes as zero cost fermentation media for biotechnologically useful microorganisms, thus also providing an alternative and less environmental impacting method for vegetable wastes management.

1. Introduction

Vegetable waste biomass is produced in huge amount by agro-food industries, forestry crop and agricultural crop production. Vegetables' cultivation, harvest, selection and industrial transformation for production of foods, chemical, fertilizers et cetera generate different kinds of residues. All these processes result in the production of residual material that is represented by starting material that has been discarded (because rotten or with bad spots not noticed in the field, or that wasn't been selected for the packing lines and not shipped to the consumer) or by the by-products of industrial processing. Proper disposal of such waste biomass, usually classified as "special wastes", is crucial for the reduction of environmental pollution but on the other hand it constitutes an economical issue for industry or farming, both with regard to their transportation and treatment. On the other hand, such residual biomass still contain significant amounts of value added products of different chemical type including polysaccharides, polyphenols, carotenoids, et cetera that according to the "biorefinery" concept could be recovered and then re-used in other production chains. Waste biomass enter into the biorefinery to be converted by means of chemical, physical and biotechnological techniques into a wide spectrum of products including platform chemicals, power and renewable energy. In this context, we pointed out our attention to vegetable waste biomass such food processing wastes and crop residues since agro-industrial, based on processing and preservation of fruits and vegetables for food production, play a key role in Italian economy. Nevertheless, every year they produce a significant amount of waste biomass that account for nearly 22,000,000 t (about from 5 till to 50% of input materials) depending on the processing and on the feedstock types, and that

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have to be disposed. We investigated food residual biomasses including residues from tomato and lemon after canning and liquor production, respectively, and residues from carrot and fennel after selection for wholesale market: such residues were selected since they are among the most abundant Italian agricultural productions. Indeed, nearly 6,500,000 t/y (data from ISTAT, National Institute of Statistics of Italy, year 2010) of tomato are produced for canning in Italy and about 117,000-149,500 t are discarded as wastes constituted by peels and seeds; lemons production, constituted by about 570,000 t/y (data from ISTAT, year 2010) of fruits that are mainly used for production of juices and liquors, results in production of wastes till 60% of starting material; Italian production of fennels comprises nearly 85% of world total production, and their processing for wholesale market results in the production of higher amounts of wastes; since 40% of the vegetable is discarded; finally, more than 630,000 t/y (data from ISTAT, year 2010) of carrots are harvested and processed and about the 40% of them are discarded during packing or after juice production. All these waste biomasses are rich of polysaccharides, fibers and other valuable molecules that could be used as starting materials in other production chains. Waste biomass crops we choose comprised residues from cultivation and harvesting of giant reed (*Arundo donax* L.) and cardoon (*Cynara cardunculus*), two crops that are object of growing interest as bioenergy producing crops with minimum or no energy inputs. The growing interest for giant reed (*A. donax* L.) is justified by the high tolerance of this lignocellulosic crop to a wide range of environmental stresses that make possible its cultivation on marginal degraded or contaminated lands thus reducing competition with food crops which generally require a better quality arable land (Di Nasso et al. 2013). The shoots (reeds) of this species, also suggested in projects of phytoremediation of polluted soils, has been studied for its potential exploitation in bioethanol, biodiesel or biopolymer productions. Nevertheless, at the end of its cropping cycle, their rhizomes remain in the soils and they have to be eliminated to afford further agricultural utilizations: such residual part of the plant that is a rich source of polysaccharides, could further be used for other purposes (Di Nasso et al. 2013). The species *C. cardunculus* is a perennial herb with annual growth cycle, that has been traditionally grown for horticultural purposes, nonetheless cardoon biomass can be used for different purposes: animal feed, paper pulp production, bioactive compounds extraction, solid biofuel for direct heating or for electric power generation, biofuels production (Fernandez et al., 2006). In our study, we suggest an alternative re-use strategy to valorize such residues, i.e. as cheap substrate for microbial biomass production. As they are able to grow in both hot and salty environments with great temperature fluctuations, such microorganisms in recent years are the object of interest as source of biotechnologically useful molecules including enzymes, lipids, endo- and exopolymers, that are already used or have been suggested for different industrial applications. Two thermophilic and two halophilic species were chosen for this study. Due to their high carbohydrate content, the selected wastes could be employed as sole carbon source in place of synthetic (and costly) media to produce extremophiles and their related biomolecules such as enzymes and biopolymers.

2. Materials and Methods

Vegetable wastes: Tomato (*Lycopersicon esculentum* variety "Hybrid Rome"), lemon (*Citrum limon*), fennel (*Foeniculum vulgare*) and carrot (*Daucus carota*) were kindly supplied from Fontanella Industry (Sa), Solagri s.c. (Sant' Agnello di Sorrento, Na), Amato V. "Sammy Export" (Pagani, Sa), Tafuri Brothers for Polli Industry (Battipaglia, Sa), respectively. Giant reed roots (*Arundo donax*) and cardoon (*Cynara cardunculus*) were harvested at the experimental farm of the University "Federico II" and kindly supplied by prof. M. Fagnano. Every waste was dried under vacuum, minced to comparable particle size and then used for bacterial growth.

Bacterial strains and culture conditions: *Haloterrigena hispanica* (type strain FP1^T; DSM 18328^T; EMBL number AM285297) (Romano et al., 2007), *Geobacillus thermoleovorans* subsp. *stromboliensis*, (type strain Pizzo^T; DSM 15392^T, DSMZ, Braunschweig, Germany; EMBL number AJ704828) (Romano et al., 2008), *Halobacillus alkaliphilus* (type strain FP5^T; DSM 18525^T; EMBL number AM 295006) (Romano et al., 2005), *Geobacillus thermantarcticus*, initially named *Bacillus thermantarcticus* (Coorevits et al., 2012; Lama et al., 2004) were grown on their complex and minimal media with selected sugars as previously described (Di Donato et al., 2011). For growth on waste as sole carbon source, 200 mL of each bacteria pre-cultures, prepared as above mentioned, were transferred into the bioreactor filled with 1L minimal media containing 10 g L^{-1} waste for batch cultivation (BF); for dialysis cultivation (DF), they were transferred in 875 mL minimal media in a bioreactor including a dialysis tube (MWCO 12,000 – 14,000 Da) containing 10 g L^{-1} waste in 125 mL of the corresponding minimal medium. Microbial biomass analysis: after 5, 4, 3 and 1 days of incubation for strains FP1, FP5, Pizzo and M1, respectively, dialysis tube was removed and cells were harvested by centrifugation at 18,500 *g* × 20 min, washed with 50 mM Na-phosphate pH 7.0, centrifuged and freeze-dryed. Gravimetrical determinations of dry cell weight were run in triplicate. Control experiments were carried out by measuring the dry cell weight after growth of the four bacteria in the respective complex media.

Statistical analysis: significance of results was assessed by *t* test using SYSTAT 7.0 software. Total dry cell weight obtained for all vegetable media was compared with complex media and sugar media results.

Carbohydrate and reducing sugars determination: Polysaccharides were extracted and their total carbohydrate content was determined as previously described (Tommonaro et al., 2008). The reducing sugars concentration was determined by means of the 3,5-dinitrosalicylic acid (DNS) method using appropriate sugar as standard (Miller, 1959).

Poly-hydroxybutyric acid (PHB) production: PHB production by strain FP1, its isolation and structural determination were performed as previously described (Di Donato et al., 2011).

Enzyme activities production: Intracellular alpha-glucosidase (E.C. 3.2.1.20) from strain FP5 was recovered after cell sonication (Di Donato et al., 2011). Extracellular alpha-amylase (E.C. 3.2.1.1) from strain Pizzo was recovered by ammonium sulphate (80%) precipitation, and partially purified by dialysis (MWCO 12,000-14,000 Da) against 50 mM sodium acetate buffer pH 5.6. Alpha-amylase activity was determined as previously described (Finore et al., 2011). Extracellular xylanase (E.C. 3.2.1.8) from strain M1 was determined in the extracellular milieu without any fractionation step (Lama et al., 2004).

3. Results and Discussion

The ability of vegetable wastes to support microbial growth as sole carbon sources was tested in two different fermentation processes: batch fermentation (BF) and dialysis fermentation (DF); in the latter, the powdered residues were placed in a dialysis tube inside the bioreactor. Control experiments were carried out in complex media (CM) and in defined media (DM) supplemented with sugars and other nutrients as trace elements, vitamins and salts. Bacterial biomass production was assessed gravimetrically by measuring dry cell weights: the obtained results are showed in figures 1-4. The tomato and carrot media well sustained growth of *H. hispanica* strain FP1 (FP1) both in BF and DF fermentation processes, affording higher amounts with respect to minimal media supplemented with galactose or trehalose as sole carbon sources (figure 1). Carrot medium also resulted the best one in promoting cell growth in the case of *H. alkaliphilus* strain FP5 (FP5) whose dry cell yield was comparable with that obtained on tomato medium; both waste media gave higher cell amounts compared with DM with ribose and cellobiose as sole carbon sources (figure 2). The thermophilic *G. thermoleovorans* strain Pizzo (Pizzo) growth was higher in the case of reed roots medium in BF conditions, and when using tomato and lemon wastes as sole carbon sources; reed residues provided cell yields that were comparable to CM and higher than all tested sugars (figure 3). The thermophilic *G. thermantarcticus* strain M1 (M1) was tested on cardoon medium that afforded higher cell production than CM and DM supplemented with glucose as sole carbon source (figure 4).

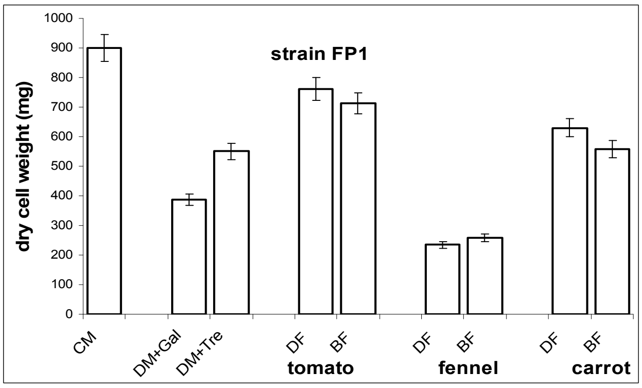

Figure 1: strain FP1 dry cell weight on CM, on DM with sugars, on tomato, fennel and carrot waste media.

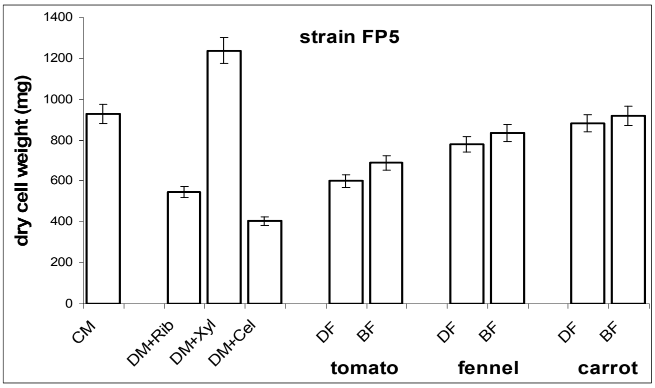

Figure 2: strain FP5 dry cell weight on CM, on DM with sugars, on tomato, fennel and carrot waste media.

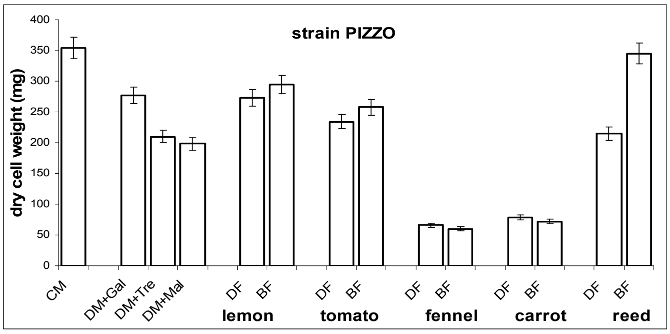

Figure 3: strain FP5 dry cell weight on CM, on DM with sugars, on lemon, tomato, fennel, carrot, reed waste media.

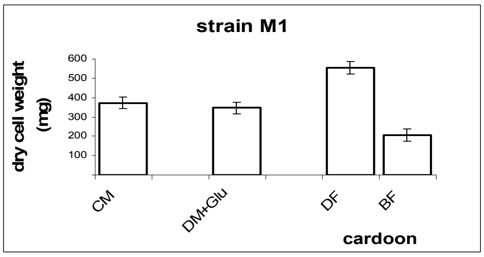

Figure 4: strain M1 dry cell weight on CM, on DM with sugars, on cardoon waste medium.

In order to verify if the tested bacteria actually and to which extent used such residues as sole carbon sources for their growth, the variation of reducing sugars released in the growth medium and of wastes' polysaccharides content were quantified. All these analysis were performed in after DF conditions that allowed a better recovery of wastes after cell growth and also a more reliable sugar content determination. This assay was performed in comparison with control experiments, in which vegetable wastes were treated in the same conditions used for microbial growth (temperature, time, agitation, etc) but in the absence of extremophilic strains. The results obtained are means of three independent experiments and are reported in table 1. Polysaccharide content in wastes was determined by a recently developed method for quantifying the alkali soluble polysaccharides in vegetable matrix (Tommonaro et al., 2008). Consumption of polysaccharides ranged from 10% to 30% of starting content for reed roots, cardoon, fennel, tomato and lemon, while it was higher for FP1 growth on carrot in which the 75% of polysaccharide content was depleted. All the tested vegetable wastes released in the growth media different amounts of reducing sugars, ranging from 26 mg/mL to 1380 mg/mL as showed in table 1. The highest decrease in released sugars amount was found for the food residues: about 93% of sugars released by carrot was used during FP1 growth, 81% of soluble sugars from fennel were used for FP5 growth, and 74% and 76% of tomato and lemon soluble sugars, respectively, were used for Pizzo growth (Di Donato et al., 2011). A minor decrease was observed in the case of crops residues; indeed, in the case of reed, the sugar depletion was about 25%, while for cardoon there was no appreciable depletion in released sugar content (table 1).

Table 1: Carbohydrates depletion of vegetable media during fermentation

waste	**Strain**	**Polysaccharides (mg/g dry waste)**		**Reducing sugars (µg/g dry waste)**	
		Control	**After Growth**		
Reed	Pizzo	170.1 ± 3.4	153 ± 2.9	230 ± 16	175 ± 13
Cardoon	M1	166.3 ± 2.9	150 ± 2.5	139 ± 11	157 ± 15
Carrot	FP1	58.1 ± 2.2	14.5 ± 0.5	1380 ± 59	93.1 ± 4.5
Fennel	FP5	27.6 ± 1.2	18.6 ± 0.9	294 ± 14	57.3 ± 2.2
Tomato	Pizzo	32.3 ± 0.9	26.5 ± 1.2	26 ± 10	6.8 ± 0.3
Lemon	Pizzo	29.6 ± 1.3	25.1 ± 1.1	1030 ± 50	247 ± 11

Finally, besides assessing the suitability of vegetable waste as fermentation media for bacterial biomass production, also their effectiveness in promoting, the production of extremophile biomolecules of potential biotechnological interest was investigated. Attention was pointed out to the production of both endo- and exo-biomolecules that are usually produced by the above mentioned bacterial strains. In particular, strain FP1 has been reported (Romano et al., 2007) to produce poly-hydroxybutyrate (PHB) a biodegradable endopolymer that can find several applications for packaging, medicine and agriculture, and whose industrial production costs strongly depend on the bacterial growth medium. Carrot media provided a comparable amount of PHB (1.25 ± 0.05 mg/g dry cell) with respect to the growth carried out on CM (1.35 ± 0.06 mg/g dry cell): the identity of isolated polymer as poly(4-hydroxybutyrate) (P4HB) was confirmed by ^{1}H-NMR analysis (Di Donato et al., 2011). The halophilic bacterium FP5 exhibited an endo-cellular alpha-glucosidase activity, whose production was promoted by fennel waste medium, that allowed to recover an enzyme specific activity corresponding to nearly 74% of the value obtained by using complex medium for strain growth, 0.623 ± 0.03 U/mg and 0.837 ± 0.02 U/mg protein, respectively (Di Donato et al., 2011). The alpha-glucosidase activity from FP5 is object of interest in relation to its ability to catalyze hydrolysis of glycosydic linkages at alkaline pH (9.0) in the presence of high salt concentrations. The thermophilic bacterial species Pizzo and M1 produce exo-cellular glycoside hydrolases namely amylase and xylanase activities (Romano et al., 2007; Lama et al., 2004). With regard to Pizzo extracellular amylase, the most interesting results were obtained when using lemon and reed wastes as fermentation media: in the first case, the amylase specific activity was about 39% (Di Donato et al., 2011) of the value registered when the growth was carried out on CM, while in the case of reed roots it was higher (110 %) than the growth carried out with standard CM medium. Pizzo amylase is a thermostable enzyme therefore it is suitable for those industrial processes that usually require high temperatures (for bread, sweeteners, and more interestingly for bioethanol production). M1 extracellular xylanase production was assessed on cardoon medium: a promising result was obtained since this waste afforded a higher production of this enzyme, whose specific activity was 160% with respect to that obtained by using CM medium, 1.82 ± 0.09 U/mg and 1.14 ± 0.02 U/mg protein, respectively. Also M1 extracellular xylanase is a thermostable enzyme, having its optimal temperature at 80 °C, and likewise other xylanases it can find application in different fields ranging from food to the recovery of fermentable sugars from hemicelluloses in bioethanol production.

3. **Conclusions**

Industrial processing of vegetables, fruits and crops result every year in the production of significant quantities of residues, whose management represents a great economical and environmental issue. Nevertheless, this complex biomass, according to the biorefinery approach, can be exploited for the production of value added compounds. In the present study, agroindustry wastes were used as sole carbon sources for the production of extremophilic microorganisms of potential biotechnological interest; moreover such wastes promoted also production of enzymes and biopolymers, thus suggesting their potential use as cheap substitute of synthetic fermentation. Up-scale of this laboratory-scale fermentation process could be useful either as lower environmental impacting method of waste handling, either as more sustainable and less expensive method of production of both useful microorganisms and related biomolecules.

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