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AGROINDUSTRIAL WASTES AS UNCONVENTIONAL SUBSTRATES FOR GROWING OF *BACILLUS* STRAINS AND PRODUCTION OF BIOSURFACTANT

The study was aimed at the development of economical methods for biosurfactant production by the use of unconventional substrates. The research investigated the potential of utilising agroindustrial wastes to replace synthetic media for cultivation of *Bacillus* strains and biosurfactant production. In total, 21 of the waste products from dairy, sugar, fatty, and fruit and vegetable processing industries, breweries, distillery were examined. Three bacterial strains were identified by 16S rRNA gene sequencing: *Bacillus subtilis* (I′-1a), *Bacillus* sp. (T-1), *Bacillus* sp. (T′-1). Biosurfactant production was examined in an indirect way by measuring of surface tension (ST), blood agar lysis, oil spreading and drop collapsing tests. The best unconventional substrates for bacteria growing and biosurfactant production at 30 °C under aerobic conditions were molasses, brewery effluents, and fruit and vegetable decoction from the processing factory.

1. INTRODUCTION

Currently, the main drawback for widespread use of biosurfactants is disadvantageous economics of their production. Although biosurfactants have numerous advantages (being non-toxic, nonhazardous, biodegradable, environmentally friendly, selective, effective under extreme conditions, with wide potential in industrial applications, and unique surface-active properties), their production costs higher than those of synthetic surfactants is a major drawback. Biosurfactants could potentially replace synthetic surfactants if costs of their production were lowered substantially. Achieving

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this goal requires finding alternative inexpensive substrates and highly efficient microorganisms for biosurfactant production. The choice of inexpensive raw materials is important to the overall economics of the process because they account for 50% of the final product cost. The best way to reduce substrate cost for biotechnology at present is to use wastes with the right balance of carbohydrates and lipids to support optimum bacterial growth and biosurfactants production, and which are either free or carry a cost credit for environmental benefit [1]. As is known, millions tons of hazardous and non-hazardous wastes are generated each year throughout the world. There is a great need for better management of these wastes via the concept: reduce, reuse, and recycle [1]. So far, several renewable substrates include various agricultural and industrial by-products and waste materials have been intensively studied for microorganism cultivation and biosurfactant production at a laboratory scale, for example: olive oil mill effluent [2], waste frying oil [3], oil refinery wastes [4], soapstock [5, 6], molasses [7, 8], whey [7, 9, 10], starch wastes [10–12], cassava flour processing effluent [13] and distillery waste [9].

In the present research, the potential of agroindustrial wastes has been investigated to replace synthetic media for supporting the growth of the *Bacillus* strains and biosurfactant synthesis.

2. MATERIALS AND METHODS

Isolation, identification and characterization of bacterial isolates. Bacterial strains (T-1, T′-1 and I′-1a) used in this study were isolated from sludge of a 100 year old oil refinery in Czechowice-Dziedzice (Poland) as described by Berry et al. [14] and Płaza et al. [15, 16]. The aged sludge was acidic (pH 2), highly contaminated with polycyclic aromatic hydrocarbons. Bacterial isolates were identified based on the 16S rRNA gene sequence analysis. A direct-colony, polymerase chain reaction (PCR) was exploited to amplify the 16S rRNA gene in a 30-cycle PCR using universal primers 27F and 1492R. The PCR conditions used were: initial denaturation at 95 °C for 8 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and elongation at 72 °C for 1 min, followed by elongation at 72 °C for 10 min. The amplified PCR products were purified using the Qiagen-PCR purification kit as per the manufacturer's instructions. The purified PCR products were sequenced from both ends at the DNA Sequencing Core facility of the University of Michigan at Ann Arbor. The 16S rRNA gene sequences were analysed at the Ribosomal Database Project (RDP) II (http//:rdp.cme.msu.edu). The top 10 most homologous sequences were aligned using the CLUSTALW program v1.83 at the European Bioinformatics site (www.ebi.ac.uk/clustalw). The similarity matrix was prepared using the DNAdist program in the PHYLIP package with Jukes–Cantor corrections. Isolates were identified

as the genus/species to which they showed highest 16S rRNA gene sequence similarity in the RDP database.

Isolates were maintained on agar slants (SMA–Standard Methods Agar, bioMerieux) containing 8 g of peptone, 2.5 g of yeast extract, 1 g of glucose and 15 g of agar per 1 dm^3 of distilled water at 4 °C.

Isolates growth on solid media and in liquid cultures. In the experiment, the following solid and liquid wastes were used: whey, diary wastewaters, brewery effluents, brewery spent grain, sugar wastewaters, beet pulp, molasses, soapstock, oil slime, acidic wastewaters from fatty factories, fusel, slop, potato decoction, apple and citrus pomaces, fruit and vegetable decoction from the processing factory. The wastes were collected and stored at –18 °C until needed.

Whey was boiled for 10 min, cooled to 4 °C, and solid particles were removed by filtration through the cotton. The operation was repeated. Spent grain, apple and citrus pomaces (100 g) were mixed with 1 dm^3 of distilled water and incubated at 4 °C by 24 h. After sedimentation, solid particles were removed by filtration through the cotton. Beet pulp (100 g) was mixed with 1 dm^3 of distilled water, boiled by 30 min, cooled and then filtered through cotton. Molasses (100 g) was diluted in 1 dm^3 of distilled water. Potato peels were collected and washed with tap water followed by distilled water. Then blenching operation was carried out by immersing the peels in boiling water for 20 min. After sedimentation, the potato substrate was filtered through cotton. All the wastewaters were filtered through cotton to remove solid particles. Concentrations of the waste products prepared in this way and other liquid wastes were established as 100%.

20 g/dm^3 of agar were then added to solid and liquid samples of wastes, the mixtures were sterilized in an autoclave under 1 atm at 121 °C for 15 min and poured into plates. The concentrations (in vol. %) of the media used were: 100, 50, 25 and 10. The minimum medium (MM) of the following composition (g/dm^3): KNO_3 – 3.0, Na_2HPO_4 – 2.2, KH_2PO_4 – 1.4, NaCl – 0.1, $MgSO_4 \cdot 7H_2O$ – 0.6, $CaCl_2 \cdot 6H_2O$ – 0.04, $FeSO_4 \cdot 7H_2O$ – 0.02 was used to dilute the wastes. 24 hour slant cultures of isolates to be tested were spread on the medium with a microbiological loop. The plates were incubated for 72 h at 30 °C. The growth was examined after 24, 48 and 72 h. As the control, the bacterial growth on minimum medium (MM) was evaluated. All growth studies were done in triplicate.

The bacterial suspensions, obtained from a nutrient agar slant incubated for 24 h at 30 °C, in the liquid Standard Methods medium of the following composition (g/dm^3): peptone – 8, yeast extract – 2.5, glucose – 1 was adjusted to $OD_{600\ nm}$ 0.65 (ca. 10^7–10^8 CFU/cm^3). Then 3 cm^3 of the bacterial suspensions were inoculated in 300 cm^3 Erlenmeyer flasks containing 150 cm^3 of sterilized organic wastes as nutrient sources. The cultures were grown aerobically at 30 °C for 96 h with constant shaking (110 rpm). Growth curves were obtained by monitoring the optical density at 600 nm on an

UV/VIS spectrophotometer (Varian). Three independent experiments were conducted for each bacterial strain and for each chosen organic waste product.

Determination of biosurfactant production. Hemolytic activity was carried out as described by Carrilo et al. [17] with the minor modifications. Isolated strains were screened on blood agar plates containing 5 vol. % of blood and incubated at 30 °C for 96 h. Hemolytic activity was detected as the presence of a clear zone around a colony.

The drop collapse technique was carried out in polystyrene lid of a 96 microwell plate (Biolog, Harward, CA, USA) as described by Jain et al. [18] and Bodour and Maier [19]. 100 µl bacterial cultures were added to wells of a 96 well microtiter plate lid, than 2 µl of crude oil were added to the surface of the cultures.

Oil spread technique was carried out according to Morikova et al. [20] and Youssef et al. [21]. 50 cm^3 of distilled water were added to Petri dishes followed by addition of 100 µl of crude oil to the surface of the water. Then, 10 µl of the bacterial cultures were put on a crude oil surface. The diameter *d* of the clear zone on the oil surface was observed.

Surface tension (ST) was determined with a Kruss processor tensiometer (model K12 Kruss, Germany) by the plate method. Before measuring, the bacterial cultures were centrifuged at 10 000*g* for 20 min and the supernatant was used for the surface activity measurements. To increase the accuracy, an average of three independent experiments was used for the study. Surface tension of water was taken 71.79 ± 0.3 mN/m.

All the assays were performed in triplicate with distilled water as a control.

3. RESULTS AND DISCUSSION

The isolates were screened and selected for further studies as described by Płaza et al. [15]. Three bacteria (T-1, T′-1 and I′-1a), halothermotolerant Gram-positive spore forming species were chosen for identification. The 16S rRNA gene sequences showed that the isolates were *Bacillus* spp.; I′-1a was identified as *B. subtilis*, but T-1 and T′-1 were identified as *Bacillus* sp. The 16S rRNA gene sequencing could not clearly assign isolates T-1 and T′-1 to any species of *Bacillus* as both isolates showed higher than 99% similarity to two distinct species (*B. subtilis* and *B. licheniformis* for T-1 and *B. subtilis* and *B. amyloliquefaciens* for T′-1). The morphological and biochemical characteristics of the three isolates were presented by Płaza et al. [22].

The results of growing *Bacillus* strains on solid media are presented in Table 1 (concentrations of all wastes was 100%). *Bacillus* strains grew very well on the following solid media: two brewery effluents, beet pulp, molasses, slop and fruit and vegetable decoction. Two from three brewery wastewaters tested appeared to be good candidates to replace conventional media, probably due to right balance of carbohydrates and lipids to support optimum growth of the strains.

Table 1

Growth of *Bacillus* strains on solid media composed of various waste products[a]

Waste products	*Bacillus* strains		
	T-1	T′-1	I′-1a
1. Whey 1	–	–	–
2. Dairy wastewater	–	++	+
3. Whey 2	–	–	–
4. Brewery effluent 1	++	+++	+++
5. Brewery effluent 2	+	+	+
6. Brewery effluent 3	+++	+++	+++
7. Brewery spent grain	++	+	+
8. Sugar wastewaters	++	+	–
9. Beet pulp	+++	++++	+++
10. Molasses	++++	++++	++++
11. Soapstock 1	–	–	–
12. Soapstock 2	–	–	–
13. Oil slime	–	–	–
14. Acidic fatty wastewater 1	–	–	–
15. Acidic fatty wastewater 2	–	–	–
16. Fusel	–	–	–
17. Slop[b]	+++	+++	+++
18. Potato decoction	++	++	++
19. Apple pomace	+	+	+
20. Citrus pomace	+	+	+
21. Fruit- and-vegetable decoction	+++	+++	+++

[a]++++ very good growth, +++ good growth, ++ medium growth, + poor growth, – no growth.

[b]Concentration of slop was 50 vol. %.

Results presented in Fig. 1 show that only 4 wastes (brewery effluents, molasses and fruit and vegetable decoction) from all tested ones were good substrates for growth of the isolates at 30 °C in liquid media under aerobic conditions. Molasses and fruit and vegetable decoction were found to be the best substrates for growing of *Bacillus* strains. Both waste products had high values of COD and BOD, and nutritional components of both wastes were efficiently utilized for biomass built-up.

In this work, the protocol proposed by Youssef et al. [21] was applied to screen production of biosurfactants by *Bacillus* strains growing at 30 °C in brewery effluents, molasses and fruit and vegetable decoction. In the first method used to screen biosurfactant producing isolates blood agar lysis was used. From all of *Bacillus* strains growing in brewery effluents, molasses and fruit and vegetable decoction, hemolytic activity was observed (Table 2). Youssef et al. [21] reported 13.5% of the hemolytic strains to lower the surface tension to the values below 40 mN/m. Various compounds are

produced by microorganisms which can cause lysis of red blood cells however they do not necessarily have to be surface active molecules. For this reason, many authors suggested that this method should be supported by other techniques based on surface activity measurements [8, 19].

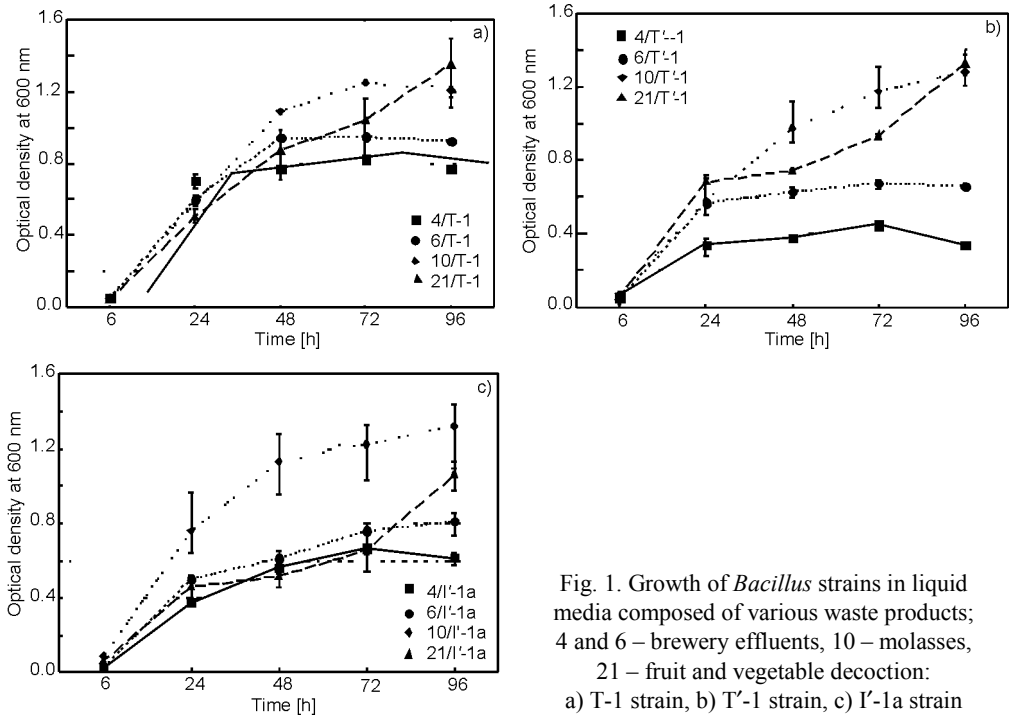

Fig. 1. Growth of *Bacillus* strains in liquid media composed of various waste products; 4 and 6 – brewery effluents, 10 – molasses, 21 – fruit and vegetable decoction: a) T-1 strain, b) T′-1 strain, c) I′-1a strain

The surface active properties of the isolates therefore were tested by the following methods: drop collapse method, oil spreading technique, and finally surface tension measurements. Both, the drop collapse and oil spreading techniques can be used as qualitative and quantitative assays. They have several advantages in requiring small volumes of samples, are rapid and easy to carry out, and do not require specialized equipment. The drop collapse method is not as sensitive as the oil spreading technique in detecting low levels of biosurfactant production [21]. In our investigation for *Bacillus* strains growing in molasses medium both methods gave positive results. In the case of the fruit and vegetable decoction, negative drop collapse technique results and low values of the oil spread technique were observed. For brewery effluents, oil spread method showed positive reaction in the case of three strains (T-1, T′-1, I′-1a) and drop collapse technique only for two strains (T-1 and I′-1a) (Table 2).

Although the use of both drop collapse and oil spreading techniques constitute an easy and quick assay to screen biosurfactant production. However, the surface tension measurements were carried out to confirm the results obtained. In Table 2, the values

of the surface tension are presented. In most of the *Bacillus* strains the surface tension was lower than 40 mN/m. Only one strain (T′-1) growing in brewery effluent showed surface tension over 40 mN/m. *Bacillus* strains decreased the surface tension to 26.7 –28.8 mN/m and to 30.8–36.5 mN/m for molasses and fruit and vegetable decoction, respectively. Results showed that those waste products had similar potential for growing of *Bacillus* strains and biosurfactant production, and can replace synthetic media. The use of economic substrates meets one of the requirements for a competitive process for biosurfactant production. Our early investigation confirmed that three *Bacillus* strains were also able to grow and produce biosurfactant in brewery effluent medium under aerobic and thermophilic conditions during the phase of stationary growth [22].

Table 2

Surface active properties of isolates growing in liquid media composed of various waste products

Waste products	Strains	Surface tension σ [mN/m]	Drop collapse method	Oil spread method d [mm]	Hemolytic activity
4. Brewery effluent	T-1	37.73±1.05	+	4.67	+
	T′-1	42.73±0.58	–	17.67	+
	I′-1a	33.05±0.17	+	6	+
6. Brewery effluent	T-1	34.43±0.30	+	4.33	+
	T′-1	39.21±0.98	–	4	+
	I′-1a	31.54±0.57	+	10	+
10. Molasses	T-1	26.64±0.43	+	28.33	+
	T′-1	29.25±0.93	+	40	+
	I′-1a	28.45±0.54	+	40	+
21. Fruit and vegetable decoction	T-1	31.55±0.91	–	2.33	+
	T′-1	36.55±0.28	–	2.67	+
	I′-1a	30.82±0.64	–	6.67	+

Among the potential biosurfactant producing microbes, *Bacillus* species are known to produce cyclic lipopeptides including surfactins, iturins, fengycins and lichenysins as the major classes of biosurfactants [11, 23]. Surfactin, one of the most effective surfactants produced by *Bacillus* strains, showed a ST of 25 mN/m, IT < 1.0 mN/m and CMC of 0.025 g/dm^3 [23].

In the present investigation, it was shown that three *Bacillus* strains were capable of growing and producing biosurfactants in media composed of brewery effluents, molasses and fruit and vegetable decoction from processing factory at 30 °C.

Medium composition is critical in determining properties of biosurfactants. Makkar and Cameotra [8] cultivated *B. subtilis* MTCC 2423 and 1427 under thermophilic conditions using molasses as a carbon source; the biosurfactant production lowered

the ST of the medium to 29 and 31 mN/m for MTCC 2423 and 1427, respectively. Potato substrates were evaluated as a carbon source for surfactant production by *B. subtilis* ATCC 21332 [12, 24]; ST dropped from 71.3 to 28.3 mN/m, and CMC of 0.10 g/dm^3 was obtained. In addition, Nitschke and Pastore [13] used a cassava-flour processing effluent as a substrate for surfactant production by *B. subtilis* LB5a and *B. subtilis* ATCC 21332, which reduced ST of the medium to 25.9 and 26.6 mN/m, respectively. Joshi et al. [7] studied biosurfactant production using molasses and cheese whey under thermophilic conditions by four *Bacillus* strains. ST was reduced to 34–37 mN/m for isolates grown under both static and shaken modes.

4. CONCLUSION

Bioconversion of industrial wastes into useful products (biosynthesis) has the potential of being a source of new materials and can convert industrial wastes into commercial products, and still reduce pollution.

Our preliminary investigation confirms that the three *Bacillus* strains can grow on various waste products (brewery effluents, molasses and fruit and vegetable decoction) as organic media (microbiological nutrients) and can produce biosurfactants. Replacing traditional microbiological media with agroindustrial wastes as substrates for biosurfactant production holds great potential. Moreover, this will reduce many management problems of processing industrial waste. The isolation and characterisation of biosurfactants produced by *Bacillus* strains growing on various wastes will be taken into consideration in the future research. However, the use of wastes as feedstock for bioprocesses generates new analytical and methodological difficulties concerning the measurement of the product accumulated. To overcome the problem new methods should be developed based on the waste used [18].

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