



Research article

Possibility of using apple pomaces in the process of propionic-acetic fermentation



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ABSTRACT

Background: In 2014, apple production in EU countries amounted to 11.8 million tonnes. A constant increase in the production of these fruits will lead to the accumulation of thousands of tonnes of apple pomace (production waste). The amount of industrial apples is the highest – their proportion on the market is estimated at 50–60%, of which over 95% is processed into juice. The proportion of pomace in the traditional pressing method accounts for 20% of fruits used.

Results: Analysis of the growth dynamics of wild strain *Propionibacterium freudenreichii* T82 in micro-cultures using different carbon sources showed that the highest bacterial growth occurs in an environment with fructose and the most intense biosynthesis of metabolites was found in medium containing only saccharose. It has been found that *P. freudenreichii* T82 used apple pomaces as a source of carbon. Propionic acid biosynthesis reached its maximum value in the 120th hour of cultivation (1.771 g/L). At this time, the content of the acetic acid produced reached the level of 7.049 g/L.

Conclusions: Utilization of by-products is a significant challenge for manufacturing sites and the natural environment. The solution to this problem may involve the use of pomace as a medium component for microorganism cultivation, which is a source of industrially useful metabolites. This study examined the possibility of using apple pomace as a carbon source in the process of propionic-acetic fermentation via wild strain *Propionibacterium freudenreichii* T82 bacteria.

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1. Introduction

In terms of environmental occurrence, bacteria of *Propionibacterium* genus can be classified into two groups: cutaneous (acnes) and classical (dairy) [1]. The first group includes species found on human skin, mucous membranes of the oral cavity and the digestive tract: *Propionibacterium acnes*, *Propionibacterium avidum*, *Propionibacterium propionicum*, *Propionibacterium granulosum*, and *Propionibacterium lymphophilum*. The second group incorporates species of industrial use – propionic acid bacteria (PAB). This group is composed of *Propionibacterium freudenreichii*, *Propionibacterium thoenii*, *Propionibacterium jensenii*, and *Propionibacterium acidipropionici*. They are found, among others, on herbaceous plants, in soil, cattle rumen, feces of ruminants, cheese, dairy products and products of natural fermentation (silage) [1,2].

The *Propionibacterium* spp. belongs to the group of microorganisms characterized by high cultivation requirements. In addition to basic compounds essential for growth (carbon source), this bacterium needs supplementation with specific stimulating substances: trace elements (iron, magnesium, cobalt, manganese, copper), vitamin B7, vitamin B5, or -cysteine hydrochloride [3,4]. Propionic bacteria have been used in the production of cheese, silage food and silage feeding, and they are also used as probiotics in animal nutrition [4,5]. An important characteristic of these organisms is their ability for metabolite biosynthesis, mainly propionic acid (a preservative of food and feed, raw material for production of plastics, herbicides and perfumes), acetic acid and vitamin B12. They exhibit the highest metabolic activity under anaerobic conditions, and are also classified as facultative anaerobes [6].

Currently, propionic acid synthesis only occurs through expensive petrochemical processes [7]. In this respect, there is increasing interest in the production of this metabolite using microorganisms and cheap waste materials. *P. freudenreichii*, which has been awarded GRAS (Generally Regarded As Safe) status by the US FDA (Food and Drug Administration), is the most useful of these bacteria in the biosynthesis of propionic acid on an industrial scale [8,9].

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2.7. Determination of volatile acidity

The principle of the method is based on re-distillation of the volatile acids with steam and then titration of the distillate obtained with NaOH standard solution against an indicator. To a flask of 250 mL, 25 mL of water was added, distillation was carried out with steam for 10 min in a BUCHI Labortechnik AG system. The distillate was heated, and then titrated with 0.1 M NaOH (CHEMPUR) against phenolphthalein (POCH S.A.) as an indicator. The amount of volatile acids was calculated according to the conversion: 1 mL 0.1 M NaOH balances 0.006 g of acetic acid. Volatile acidity was determined at three periods of cultivation: 24, 96 and 120 h.

2.8. Determination of organic acids

The process of propionic and acetic acid biosynthesis was carried out in 500 mL Erlenmeyer flasks containing 250 mL of culture medium with apple pomace inoculated with *P. freudenreichii* T82 inoculation. Experiments were carried out under static conditions for 120 h at 30°C. Samples for analyzes were collected at 24, 48, 96 and 120 h of the process.

The amount of propionic and acetic acid resulting from fermentation was determined. The analysis was conducted using gas chromatography with flame ionization detection (GC-FID). The carboxylic acid fraction was extracted from media utilizing a mixture of hexane and diethyl ether (1/1 v/v). Chromatographic separation was conducted using ZB-WAXplus column (30 m × 0.25 mm × 0.25 μm). Quantitative calculations were made against the internal standard (undecanoic acid – C11:0) using correction factors. Qualitative analysis of the sample was carried out based on retention times compared with standard solutions.

2.9. Statistical analysis

All experiments were performed in triplicates. Mathematical and statistical calculations were performed using computer programs such as Excel 2013 for Windows 10 and STATISTICA 6.0 PL StatSoft, Inc. (2003). For optimization of the composition of carbon sources in the culture medium, we used design of experiment (DoE) methods. In the experiments, simplex plans for the ternary mixtures were used.

3. Results and discussion

An advanced enzyme system allows propionic acid bacteria to utilize a number of carbon sources (saccharides, organic acids). It creates the possibility of the disposal of waste materials containing essential nutrients. Moreover, it gives the opportunity for simultaneous acquisition of valuable metabolites. According to Bergey, *P. freudenreichii* T82 successfully utilizes, among others, glucose and fructose (positive

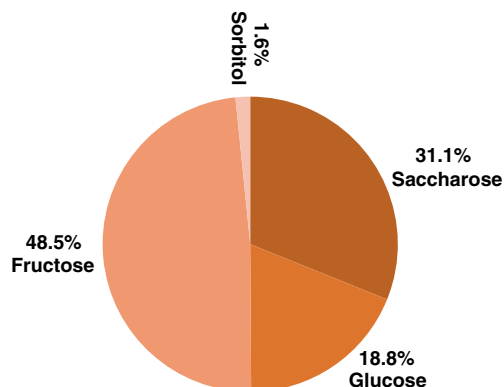


Fig. 1. Sugar profile of medium before cultivation.

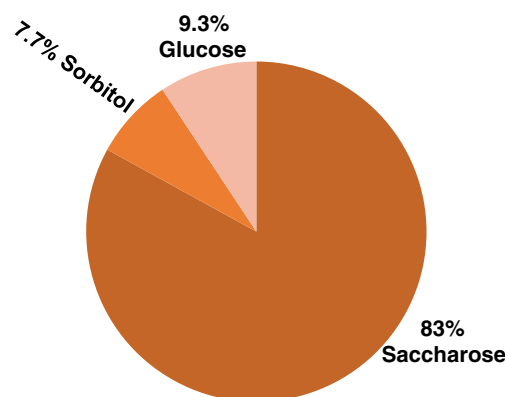


Fig. 2. Sugar profile of medium with the addition of apple pomace after 120 h of *P. freudenreichii* T82 cultivation.

reaction in 90 up to 100% strains), certain strains of this species also have the ability to ferment saccharose (positive reaction in 10 up to 40% strains) [11].

Media prepared under laboratory conditions and containing apple pomace were subjected to an analysis of sugar profiles. Based on chromatographic separation, it was evident that, in this medium, the main saccharide was fructose, which accounted for 48.5% of all carbon sources. The remaining part was composed of saccharose, glucose and sorbitol (Fig. 1). After 120 h of *P. freudenreichii* T82 cultivation, we observed complete and partial consumption of fructose and glucose, respectively. Consequently, an increase in the proportion of saccharose and sorbitol – up to 83% and 7.7%, respectively – was reported in the medium (Fig. 2). The results clearly show that bacteria utilized fructose first. The saccharose molecule is composed of D-fructose and D-glucose linked by a (1 → 2)-β-O-glycosidic bond.

Hydrolysis of this disaccharide into simple sugars is related to, among others, energy expenditure, so that the bacteria more easily utilize available monosaccharides in the environment. It is assumed that propionic bacteria ferment fructose first due to the higher degree of reduction of this sugar compared to glucose and saccharose [12]. After about 60 h of cultivation, inhibition of the growth of *P. freudenreichii* strain T82 was observed, probably due to the reduction in the active acidity of the environment. If optimum pH conditions had been created, it would be possible that even during cultivation bacteria would use other sugars. This would affect an increase in cell yield and maintenance of metabolic activity of these microorganisms, thereby leading to increased production of acids: propionic and acetic [13].

In the apple pomace, chromatographic separation using HPLC indicates the presence of fructose, glucose and saccharose. Therefore,

Table 2
Parameters characterizing the growth of *P. freudenreichii* T82.

Medium	Kinetic parameters						Generation time [min]
	t _{lag} [h]*	t _{log} [h]*	OD _{min}	OD _{max}	ΔOD	μ _{max} [h ⁻¹]**	
I	5	27	0.822	1.896	1.074	0.02	34.66
II	3	29	0.891	2.047	1.156	0.021	33.01
III	3	27	0.722	1.749	1.027	0.022	31.51
IV	3	23	0.858	2.058	1.2	0.022	31.51
V	3	26	0.778	1.918	1.14	0.022	31.51
VI	3	25	0.782	2.017	1.235	0.024	28.88
VII	3	24	0.814	2.018	1.204	0.022	31.51
VIII	4	25	0.860	2.094	1.234	0.023	30.14
IX	3	25	0.753	1.941	1.188	0.024	28.88
X	3	26	0.826	2.058	1.232	0.023	30.14
XI	8	15	0.603	1.51	0.907	0.018	38.51

* lengths of the adaptive (t_{lag}) and logarithmic (t_{log}) phases.

** maximum speed of bacterial growth in the logarithmic phase.

for the purpose of determining the optimal culture medium for the growth of propionic bacteria, we evaluated media containing the above-mentioned carbon source.

The bacterial strain tested demonstrated growth in all the types of media under study. The highest total increase in OD (1.235) was obtained in medium no. VI (Table 2). A similar increase in OD was observed in media nos. IV, VIII and X. Each of these was characterized by the presence of fructose. The lowest efficiency of OD increase was observed in media nos. III (1.027) and XI (0.907). In the media, in which fructose (II) and glucose (I) was the only carbon source, the total increase in OD was 1.156 and 1.074, respectively. A higher increase in OD was found in media in which fructose or glucose were mixed with other sugars. High concentrations of sugar (25 g/L) affected the increase in the osmotic pressure in the environment investigated. It led to the closure of the channels occurring in the cell membrane of bacteria necessary for nutrient uptake from a medium [13,14,15].

According to the literature, immediately after inoculation, a lag phase is observed in which microorganisms adapt to the conditions occurring in the culture medium. Then, the logarithmic growth phase begins in which the number of bacteria increases at a steady rate. The next step is the stationary phase induced by the consumption of nutrients and accumulation of toxic metabolites, in which we observed microbial growth inhibition [16]. Based on the results, we found that the duration of the adaptive (lag) phase in the media investigated ranged from 3 to 8 h. Table 2 shows the kinetic parameters for *P. freudenreichii* T82. Based on these, it was found that the longest phase was observed in medium with apple pomace (XI, 8 h), and the shortest in culture no. II-X (3 h).

In terms of the logarithmic growth phase (log), we observed the opposite trend, which was the longest in medium with fructose (II, 29 h), and the shortest when pomace constituted the carbon source (XI, 15 h). In media I–X, a similarity in terms of the length of the adaptive and logarithmic growth phases was observed. In turn, in medium with pomace, an extension of the duration of the adaptation phase at the expense of the log phase was reported.

The large number of biologically active components found in apple pomace might also be the cause. After medium inoculation, we observed a period in which the lack of bacterial growth was significantly prolonged (8 h), as the culture medium was different from the inoculation medium. It is known that at this time the microorganisms synthesize enzymes essential for growth and metabolic activity in the new environment [16]. The growth of microorganisms in media was inhibited after about 50–60 h, followed by the occurrence of the stationary phase (Fig. 3). This covers the increased intensity of metabolic processes, as evidenced by a simultaneous increase in the production of carboxylic acids.

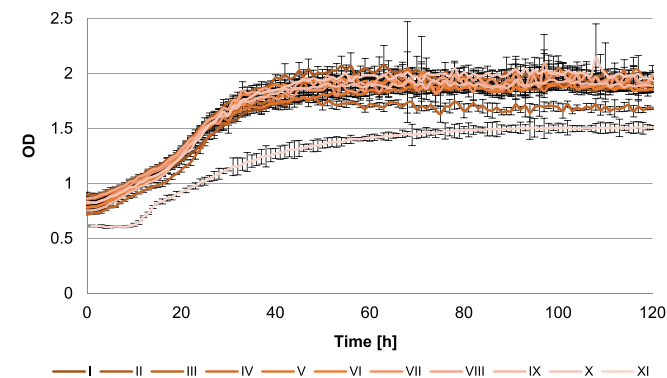


Fig. 3. Changes in OD in the tested media during the cultivation of *P. freudenreichii* T82 bacteria.

The shortest generation time of *P. freudenreichii* T82 was reported in media nos. VI, IX (28.88 min) and VIII, X (30.14 min). Extension of the generation time was observed in the medium with apple pomace (38.51 min). Most likely, this is related to the change in medium composition compared to the inoculation medium and the need to synthesize enzymes by propionic bacteria necessary for the growth and activity in the new environment. The highest maximum speed (μ_{max}), of *P. freudenreichii* T82 strain was demonstrated in media nos. VI, IX (0.024 h⁻¹) and X (0.023 h⁻¹) (Table 2).

Statistical analysis of the growth dynamics of *P. freudenreichii* T82 in micro-cultures with the use of different carbon sources (media I–IX) showed that the highest bacterial growth occurs in an environment with fructose. The addition of glucose and/or saccharose to the medium resulted in a smaller increase in the number of microorganisms, wherein saccharose affected the growth of bacteria more adversely compared to glucose. During the hours analyzed, the weakest microbial growth was observed in medium in which saccharose was the main carbon source (Fig. 4). In addition to evaluation of the growth dynamics, we determined the content of acids formed during the process of fermentation (Fig. 5). It was observed that in the 24th h of cultivation bacteria mainly used glucose for the production of metabolites. In the 96th h, the highest production of acids was found in media in which saccharose and glucose (>3 g/L) constituted the carbon sources.

In the 120th h of the process, the most intense biosynthesis of metabolites (>4.4 g/L) was found in medium containing only saccharose. Based on these results, it was concluded that the use of a sugar mixture affects the reduction of acid production. The higher the percentage of glucose and/or fructose in the medium, the less efficient the biosynthesis of metabolites. The results clearly show that the growth of *P. freudenreichii* T82 is the most intense when fructose is used, whereas the most efficient production of metabolites occurred during the use of saccharose by these bacteria. Intensification of fermentation processes was observed during the stationary growth phase of bacteria.

Fig. 6 shows the course of propionic fermentation in medium containing apple pomace. As a consequence of fermentation, two products emerged: propionic and acetic acid. Wang et al. [17] and Kośmider et al. [18], when using *P. freudenreichii* subsp. *shermanii* and glycerol as the carbon source, apart from the main product of fermentation – propionic acid, also observed two by-products: acetic and succinic acid. The biosynthesis of the additional metabolite in the form of succinic acid is probably dependent on the carbon source used. The proportion of acids changed during the fermentation process. Propionic acid biosynthesis reached its maximum value in the 120th h of cultivation (1.771 g/L). The efficiency of the process was 0.1 g of propionic acid/g of substrate. At this time, the content of the acetic acid produced reached the level of 7.049 g/L. The efficiency of the production of acetic acid per gram of substrate was 0.4 g. The highest increase in the production of both metabolites was found between the 96th and 120th h of cultivation. After the end of the experiment, we observed 1.84 g/L of reducing sugars in the post-culture medium.

Kośmider et al. [18] used the *P. freudenreichii* ssp. *shermanii* strain in the process of periodic fermentation conducted in media supplemented with glucose and glycerol. Total sugar consumption occurred in the 48th h of the process, biosynthesis of propionic acid reached the value of 10.2 g/L with an efficiency of 0.5 g of propionic acid per gram of substrate. The production of acetic acid was 2.4 g/L. In culture in which waste glycerol constituted a carbon source, it was found that the maximum value of the biosynthesis of propionic acid was observed in the 120th h of cultivation (11.6 g/L) – at that time, total consumption of glycerol was reported. After 120 h of the experiment, acetic acid was identified in trace amounts – approximately 0.7 g/L [18]. More propionic acid after the 120th h of fermentation was obtained by Zhu et al. [19]. At that time, the biosynthesis of the metabolite was 15.72 g/L with 1.2 g/L of acetic acid produced.

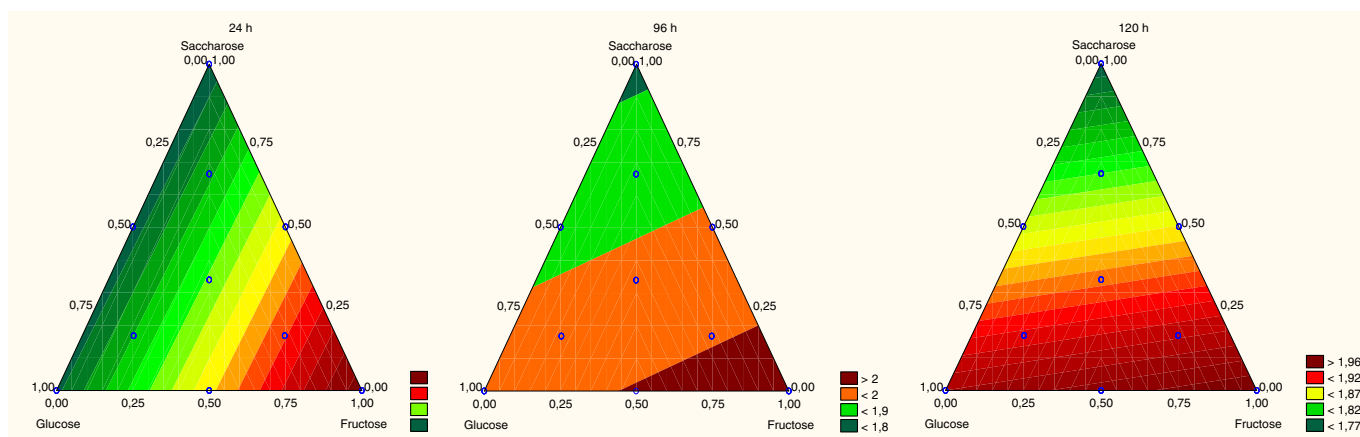


Fig. 4. Contour plot demonstrating an increase in OD in *P. freudenreichii* T82 bacterial culture depending on the composition of the carbon source in medium. The results obtained for medium I–X according to Table 1 were taken into account.

In comparison to the study conducted by Kośmider et al. [20], the amount of propionic acid using apple pomace is low (1.771 g/L). On the other hand, a considerably higher amount of acetic acid was produced. The waste material used contains various compounds. A rich composition of medium may lead to decreased efficiency of the fermentation process by extending the adaptive phase. After medium inoculation, a period in which no growth is observed occurs. This results from the need to adapt bacteria to new cultivation conditions. Cultivation conditions used in the study may be a reason for the decreased production of propionic acid. Propionic acid bacteria exhibit the highest activity in neutral and oxygen-free environments. In studies conducted by Kośmider et al. [20], the active acidity of media was stabilized at the level of 6.7–7 for the whole period of the experiment.

In the experiment with apple pomace, the pH of the environment was established once (6.7–7) – before the cultivation. Thus, we evaluated the possibility of *P. freudenreichii* T82 for growth and biosynthesis of metabolites at diverse pH levels (<7). Inhibition of bacterial growth occurred after about 60 h, when active acidity reached the value of 6. After 120 h of cultivation, the pH of the environment was 4.3. Despite the acidic pH, bacteria exhibited metabolic activity (the highest production of metabolites occurred between the 96th and 120th h of the experiment); however, inhibition of bacterial growth was also observed. The experiment was conducted under conditions of low oxygen supply, and thus a shift of fermentation processes toward biosynthesis of acetic acid was observed (aerobic conditions favor the production of acetic acid), which explains the increased production of this metabolite.

Excessive production of acetic acid leads to inhibition of the biosynthesis of propionate (and vice versa), which results from the need by bacterial cells to maintain balanced redox potential [20]. It should also be noted that the use of waste material prolongs propionic fermentation. In the study conducted by Kośmider et al. [20] and Himmi et al. [21], glucose fermentation was already completed by the 48th h of cultivation, then complete consumption of the carbon source was observed. The use of waste glycerol resulted in an extended time period of the process of up to 120 h. At that time, we observed a complete re-fermentation of the carbon source in medium. The amount of propionic acid produced from these two nutrients differs by 1.4 g/L in favor of glucose.

In terms of acetic acid, the difference was 1.6 g/L (also in favor of glucose). The use of pomace also resulted in the extension of fermentation, in the 120th h of the process in the post-culture medium, we still found 1.8 g/L of sugars. It is possible that, despite the decreasing environmental pH, fermentation might extend by consecutive hours, until depletion of all the available compounds from the culture medium. Longer fermentation times with the use of waste materials are probably caused by the need for the adaptation of bacteria to culture medium conditions.

4. Conclusions

Waste substrates are much cheaper than glucose or other carbon sources and therefore may constitute an alternative for the production of propionic and acetic acid using microbiological approaches. The

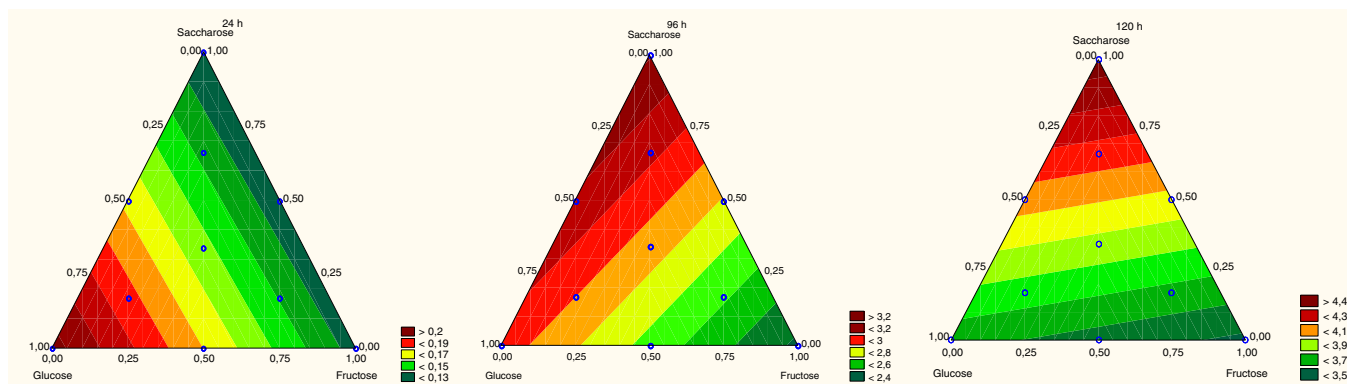


Fig. 5. Contour plot demonstrating the production of acids in *P. freudenreichii* T82 bacterial culture depending on the composition of the carbon source in medium. The results obtained for medium I–X according to Table 1 were taken into account.

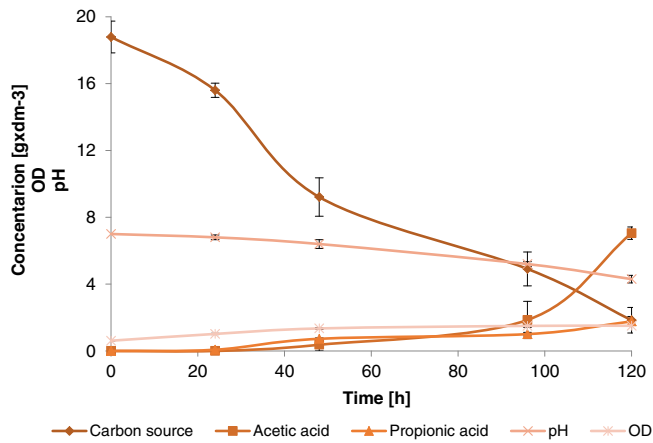


Fig. 6. Propionic fermentation using apple pomace as the carbon source.

perspectives for the use of apple pomace in biotechnological processes seems to be attractive; however, this still requires further studies concerning, among others, selection of strains, optimization of cultivation conditions (e.g. suitable carbon sources, biostimulants) or regulation of the biosynthesis of propionic fermentation metabolites. In our experiments, we demonstrated the possibility of using apple pomace as a potential carbon source.

Conflict of interests

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ejbt.2016.07.004>.

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