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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.



## 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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Utilization of by-products of technological processes is one of the important problems for production sites and the natural environment. Therefore, appropriate waste management brings many advantages, among which one can include: reduction in the costs of cleaning and export, increasing levels of hygiene and acquisition of new products. For this reason, there is the constant search for innovative solutions for industrial waste management, e.g. via biotechnological approaches. Waste materials include, among others, by-products from the fruit juice factory — apple pomace. This is a source of many biologically active compounds: saccharides (glucose, fructose, saccharose), proteins, pectins, fiber, vitamins, and organic acids. Therefore, they should be treated as raw materials for further utilization. Biotechnological application of bacteria from the *Propionibacterium* genus may influence the reduction of contamination of the environment, not only through waste reduction, but also via conversion into useful components — propionic and acetic acid.

The objective of this study was to analyze apple pomaces in terms of the potential for their use as a potential source of carbon by *P. freudenreichii* T82 microorganisms in the process of propionic acid and acetic acid biosynthesis.

## 2. Materials and methods

### 2.1. Microorganisms

*P. freudenreichii T82* wild strain derived from the collection of the Department of Biotechnology and Food Microbiology at Warsaw University of Life Science was used in the experiments. Microorganisms were stored at 4–6°C using VL (POCH) liquid medium.

### 2.2. Media

The experiments incorporated culture media which differed in terms of the type and the amount of carbon source. The composition of media is shown in Table 1. The following carbon sources were used: anhydrous glucose (POCH), fructose (POCH), saccharose (POCH), and apple pomace (derived from the production of fruit juices-DÖHLER-Natural Food & Beverage Ingredients). The media were sterilized in an autoclave at 117°C for 20 min, active acidity (pH) was set at 6.8–7 with the use of 25% aqueous ammonia solution.

### 2.3. Inoculum

Culture media inoculation was carried out for 48 h under static conditions at 30°C in 100 mL Erlenmeyer flasks containing 50 mL of VL medium with 2% anhydrous glucose. Before inoculation, media were sterilized in the autoclave at 117°C for 20 min. For inoculation of the appropriate medium, 10 vol% of suspension of proliferating cells in culture inoculation was used. Absorbance of the culture inoculum was set at 0.6–0.8.

### 2.4. Analysis of sugar profiles of media supplemented with apple pomaces

To a 50 mL measuring flask 2 mL of extract and 2 mL of 2% $Ca(OH)_2$ were added to neutralize the environment. The flask was supplemented with distilled water to 50 mL. Before chromatographic separation, the resulting solutions were filtered using PA 0.45 μm syringe filters. For the analysis of media sugar profiles, high performance liquid chromatography was used (Shimadzu, Japan) together with an LC-10 ATV pump, an SIL 20AHT autosampler, a CO-10ASVp oven, a refractive index detector and a 10 μm Carbohydrate Analysis column (3.9 mm × 30 cm, Waters). Separation was performed using isocratic gradient. The eluent constituted a mixture of acetonitrile and water (800/200 v/v), flow rate was established at 1.5 mL/min. Injection volume of the sample was 20 μL. Glucose, fructose, saccharose and sorbitol were identified based on comparisons of retention times with standard solutions using a Shimadzu software.

### 2.5. Evaluation of growth dynamics of P. freudenreichii T82 strain in microcultivation using different carbon sources

Microcultures (medium volume of 300 μL) were grown in Bioscreen C oY AB Ltd., Growth Curves (Helsinki, Finland) were created by an automated analyzer after microbial growth for 120 h at 30°C. For each medium variant, five microcultures were grown. The growth of the tested bacteria was assessed by measurement of changes in optical density (OD) at a wavelength of 420–580 nm, performed automatically every hour. Based on the results obtained, growth curves of *Propionibacterium*, and lengths of the adaptive ($t_{lag}$) and logarithmic ($t_{log}$) phases were evaluated. Moreover, minimum and maximum values of OD in the logarithmic growth phase ($OD_{min\ log}$ and $OD_{max\ log}$) and during the total cultivation time ($OD_{min}$ and $OD_{max}$), were determined. Furthermore, we determined the maximum speed of bacterial growth in the logarithmic phase with the formula: $\mu_{max} = (\ln OD_{max\ log} - \ln OD_{min\ log}) / t_{log}$, the generation time ($g = \ln 2/\mu_{max}$) and the total increase in optical density ($\Delta OD = OD_{max} - OD_{min}$) [10].

### 2.6. Determination of reducing sugars using 3,5-dinitrosalicylic acid (DNS)

The principle of the method is based on the phenomenon that, in basic medium, nitro groups of 3,5-dinitrosalicylic acid are reduced to amino groups, while simultaneously the sugars are oxidized to corresponding acids. The resulting amine derivatives are orange, and measurement of the color intensity is performed at $\lambda = 550$ nm. To 0.5 mL of sample, 1.5 mL DNS was added and the mixture was stored for 5 min at 100°C. Then, after cooling, 8 mL of distilled water was added and 25 min after removal from the bath, the absorbance against a control sample was measured at a wavelength of 550 nm. The control sample consisted of 0.5 mL water, which was then processed in a similar manner to all the remaining samples. Calibration curves were plotted.

**Table 1**
Composition of culture media.

| Substrates g/L | Medium | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | I | II | III | IV | V | VI | VII | VIII | IX | X | XI |
| Glucose | 25 | – | – | 12.5 | 12.5 | – | 16.6 | 4.2 | 4.2 | 8.33 | – |
| Fructose | – | 25 | – | 12.5 | – | 12.5 | 4.2 | 16.6 | 4.2 | 8.33 | – |
| Saccharose | – | – | 25 | – | 12.5 | 12.5 | 4.2 | 4.2 | 16.6 | 8.33 | – |
| Apple pomace | – | – | – | – | – | – | – | – | – | – | 500 |
| Potassium hydrogen phosphate | 1.5 | | | | | | | | | | |
| Potassium hydrogen diphosphate | 2.5 | | | | | | | | | | |
| Yeast extract | 5 | | | | | | | | | | |
| Peptone K | 10 | | | | | | | | | | |
| Biotin | 0.0002 | | | | | | | | | | |
| L-Cysteine hydrochloride | 0.4 | | | | | | | | | | |
| Distilled water | To 1 L | | | | | | | | | | |



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