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POSSIBLE SUBSTRATES FOR ACETONE–BUTANOL AND ETHANOL FERMENTATION BASED ON ORGANIC BY-PRODUCTS

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An assessment study was carried out on available biomass by-products in Hungary, and also several fruit juice wastes were evaluated for their accessibility to enzymatic hydrolysis. Among the fruit juice residue samples examined the best results were obtained with apple, peach, apricot and tomato. It can be shown that using intensive stirring, higher conversions can be reached. Highest conversion of 98 % - based on carbohydrate content - was obtained with frozen and thawed apple residues.
ABE fermentation of apple pulp hydrolysate supplemented with glucose yielded high solvent concentrations up to 22.5 g/l. For growth and solvent production supplementation by additional nutrients was not essential, however, this accelerated the process. Ethanol fermentation by baker's yeast was carried out on apple pulp hydrolysate supplemented with glucose. This also gave reasonable yields of about 90 % - based on glucose consumption.

Keywords: hydrolysis, biomass, fruit pulp, alternative substrate of ABE fermentation

Introduction

The problem of environmental change caused by greenhouse gas emissions mainly from fossil fuels has motivated research in the utilisation of renewable energy sources. Biomass of various origins is a great resource for energy carriers, not only by direct combustion, but also by biological conversion such as biogas production, ethanol fermentation, and acetone- butanol (ABE) fermentation [7]. In the traditional ABE process, the high cost of conventional "starch" (maize, wheat, millet, rye, etc.) or sugar (molasses) substrates was a major factor affecting the economic viability of the process [7]. ABE fermentation, following its success in the 40's and 50's, and its nearly complete disappearance in the 60's once again became of interest in the 70's after the first oil crisis, when much emphasis was placed on the use of renewable raw materials. This interest and the ability of sugar-fermenting clostridia to utilise many different carbohydrates have stimulated research into the use of alternative cheaper substrates [7].

The use of whey and whey permeate has been studied successfully [8]. Prescott and Dunn [14] have used a number of non-cellulosic, starch-based materials (including potato, rice, jawari, bajra) as substrates with varying success. Cheap low-grade potatoes have been used in the ethanol industry for a long time and proved to be an excellent substrate for batch ABE fermentation after minor pretreatment with starch-liquefying enzyme [3, 12], and could also be used in a continuous process [17]. As early as 1922, Robinson [15] studied the fermentation on various carbohydrates for the production of acetone and butanol. He reported that glucose, fructose, mannose, sucrose, lactose, dextrin, and starch were completely consumed, while galactose, xylose, and arabinose were partially utilised, and glycerol was not fermented. However, Nakhmanichov and Kochkina [11] showed that xylose and arabinose could be fermented completely by most ABE bacteria. Ounine *et al.* [13] reported a relatively high yield of conversion into solvents of 28 % for xylose, close to the maximal value of 32 % for glucose. Wiley *et al.* [22] investigated sulphite waste liquids from the pulp and paper industry containing glucose, xylose, and arabinose as alternative substrates for ABE fermentation. They showed that good yields of solvents were obtained when sulphur dioxide, lignin, and excess calcium were removed from the sulphite liquid. Voget *et al.* [21]

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investigated the use of apple pomace for butanol production and found that 80 % to 84 % of soluble sugars (about 10 % of the dry weight) were consumed. The residue remaining after butanol separation could be used as animal feed. Lignocellulosics are, however, the most abundant renewable resources [6] and are recognised as having great potential as substrates for fermentation, provided that the hemicellulose and cellulose components can be degraded and utilised cheaply and efficiently. Fermentation can be performed by yeasts to ethanol [16] or to acetone-butanol by clostridia [7].

The ABE fermentation by clostridia holds further potential as the butyric acid bacteria are capable of utilising all of the prevalent pentose and hexose sugars present in hemicellulose and cellulose in contrast to conventional baker's yeast *Saccharomyces cerevisiae* used for ethanol fermentation. Lignocellulosic biomass contains hemicellulose in a range of about 20 to 40 % [19] which means that the overall substrate utilisation is higher depending on the content of hemicellulose in the biomass, however, the (hemi)cellulose must be hydrolysed prior to fermentation. Substrates with high lignin content can usually be hydrolysed only after pretreatment under harsh conditions [16], often leading to high levels of substances inhibitory to the fermentation, and to loss of less thermally stable sugars like xylose. This combination of problems leads to poor economic prospects for the saccharification process starting from lignin-rich substrates like wood [5]. These problems have motivated this survey of potentially "easier" substrates which can be readily hydrolysed and are available locally in Hungary. Selected substrates should also be tested in practical hydrolysis and fermentation experiments.

Survey of available by-products from agriculture and food industry in Hungary

Hungarian agriculture produces large amount of plant biomass every year. A part of this biomass is a by-product or waste, and its efficient utilisation is not yet solved. The aim of this survey in the agriculture and food industries was to identify potential substrates for ABE fermentation, which should be easy to hydrolyse and available in sufficiently large quantities.

Agricultural by-products

Crop residues considered for collection are from corn, small grains, and grass. They are harvested by combines that leave stubble, and the straw or stalks are returned to the field after harvest [23]. This is a rather large amount

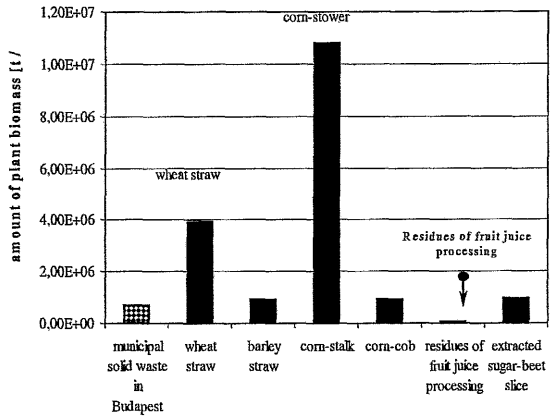

Fig.1 Amounts of various agricultural and food industry wastes in Hungary according to the survey performed in this study (in million tons per year)

of biomass. The amount of corn stalk is tenfold more than the municipal solid waste of Budapest in a year (*Fig.1*).

By-products from the food industry

- Cheese whey has attracted interest as an alternative substrate for ABE fermentation because of its disposal problem, lactose content, and availability in many countries. After the precipitation and removal of casein, whey filtrate contains a relatively low sugar content and is unsuitable for most fermentation processes without prior concentration steps, but it is suitable for ABE fermentation [7]. Maddox *et al.* [8] reported successful utilisation of whey permeate after removal of valuable proteins. In Hungary $435*10^6$ litres of whey is produced annually and only 10 % of this is dried and utilised as animal feed. The ABE fermentation could be a good alternative for the remainder.
- In Hungary, one of the largest companies in the fruit juice processing industry is Rauch Hungary Ltd., which produces annually 10,000 t apple residues (from juice and pulp processing) and apple pomace (from washing and sorting) and 1,500-2,000 t of other fruit waste. Expansion of the production facilities is anticipated.
- At the canning factories there is no problem with wastes yet, because the entire quantity is used as animal feed or is composted.
- According to information from breweries, their by-products are sold as animal feed. However, with the concentration process going on in this industry as in many others, it might become difficult to distribute in large quantities.

Experimental procedure

Substrates for hydrolysis experiments

Industrial residues from the fruit processing industry for use in the comparison experiments were obtained from Rauch Hungary. The comparison experiments were performed with fresh residues in the factory without any treatment or preservation. Peach residue for the assessment effect of re-freezing was stored at –20 °C, thawed and hydrolysed. A portion was refrozen and hydrolysed after the second freeze-thaw cycle.

Enzymatic hydrolysis

Hydrolysis of fruit pulp for the substrate comparison was performed at 3 % dry material in a 0.05 M citrate buffer solution (pH 4.8) supplemented with Pulpzyme HC, 24.7 FPU (Filter Paper Unit) and Novozym 188, 121.7 CbU (Cellobiase Unit) per g dried substrate. Hydrolysis was performed at 50 °C for 5 h in 100ml shake flasks. Samples were collected each hour, centrifuged at 10,000 rpm for 3 minutes and analysed for reducing sugar content by the DNS method.

Enzyme preparations used

Pulpzyme HC is an enzyme preparation with a cellulase and xylanase activity produced in submerged fermentation with a genetically modified *Bacillus* strain (NOVO Nordisk, Denmark). Novozym 188 is a cellobiase enzyme preparation obtained by submerged fermentation with *Aspergillus niger* (NOVO Nordisk, Denmark).

Organism, culture maintenance, and growth media

The organism utilised was *Clostridium acetobutylicum* ATCC 824 maintained in spore stock suspension according to Gapes *et al.* [2]. The composition of the medium used for strain maintenance was 60 g/l glucose, 5 g/l yeast extract, 1 g/l $K_2HPO_4 \cdot H_2O$, 1 g/l KH_2PO_4, 1 g/l $MgSO_4 \cdot 7H_2O$, 0.5 g/l $FeSO_4 \cdot 7H_2O$, 0.01 g/l p-amino-benzoic acid, 3 g/l ammonium acetate.

ABE fermentation

Previously hydrolysed apple pulp was used for the ABE fermentation experiments, supplemented by nutrients of the standard semisynthetic medium for the maintenance of the *C. acetobutylicum* ATCC 824 strain according to Gapes *et al.* [2], or for the comparison experiment without supplementation. The sugar concentration was increased to 60 g/l by adding glucose. The experiments were performed in 300 ml sealable glass bottles filled with 150 ml of medium. To ensure anaerobic conditions nitrogen was blown through the solutions for 10 min to remove oxygen from the headspace. Following sterilisation at 121 °C for 16 min the medium was inoculated with 10 ml of a culture of vegetative cells of *C. acetobutylicum* ATCC 824 grown in continuous cultivation in a wheat medium as described by Nimcevic *et al.* [12]. The fermentation experiments were performed at 35 °C.

Ethanol fermentation

The conditions of the experiment were identical to those of the ABE fermentation experiments except for the microorganism used. Commercially available baker's yeast (*Saccharomyces cerevisiae)* was used for the ethanol fermentation. An inoculum culture was grown on semisynthetic medium for 24 hours, and 6 ml of this culture was used to inoculate the apple pulp medium.

Assays

For analysis of the biomass, the first step was an acidic lignin extraction performed according to Hägglund [4]. The sugar content of this acidic supernatant was determined by the phenol-sulphuric acid method [1] and total available carbohydrate concentration was calculated from this. The reducing sugar concentration during enzymatic hydrolysis was estimated colorimetrically using a dinitrosalicylic acid reagent [10]. Glucose concentration was analysed by HPLC or estimated by reflex photometry with Reflolux (Boehringer Mannheim / Roche Diagnostics, Mannheim, Germany).

Results

Comparative hydrolysis experiments with various fruit juice processing by-products

The purpose of these experiments was to identify those by-products of fruit juice processing, which are suitable for ABE fermentation with regard to their sugar and convertible carbohydrate content. The hydrolysis was performed with 12 types of fruit substrates (apple pulp, peach and apricot pulp, juice and pulp processing by-products, black and red currant, raspberry, blackberry, and elder pomaces) in 100 ml shake flasks, using the enzymes Pulpyzime and Novozym. Since a portion of the sugars in fruit processing residues is soluble, the water-soluble sugars were first removed from the samples by cold and hot water washing, followed by filtering. The amounts of dissolved sugars in the filtered

Table 1 Soluble sugar and acid-soluble carbohydrate content of residues from fruit processing by-products

Samples	Soluble sugar (g/100 g substrate dw)	Acid soluble carbohydrate (g/100 g washed substrate dw)
Peach *	9.4	39
Peach **	0.9	28
Apricot *	10	38
Apricot **	2.6	30
Apple pomace	7.1	40
Apple pulp	12	38
Red currant	1.7	35
Raspberry	2.1	38
Blackcurrant	2.1	31
Elder	1.4	21
Tomato	7.6	28
Blackberry	0.5	34

* residues from pulp production
** residues from juice production

water were determined by the phenol-sulphuric acid method. The results, based on 100 g dry weight, are shown in *Table 1*. Together with the carbohydrate contents the dry matter contents were also determined for each sample. The hydrolysis of the samples was performed in 5 h, in suspensions containing 3 % dry weight. Previous experiments [20] have shown that the amount of sugar obtained in 24 h enzyme conversions is approximately 10 % higher than when the hydrolysis is stopped after 5 h.. However, since our goal was the identification of easily convertible wastes, this time period was sufficient and informative enough for making the desired determinations.

The conversion yields reached with enzymatic hydrolysis based on the samples' carbohydrate contents (*Table 2*) proved to be low (~20 %) in the cases of the so-called coloured fruits (red and black currant, blackberry, raspberry, and elder). Among two types of peach and apricot processing wastes, the pressed waste (the remnant of fruit juice processing) is apparently largely composed of stone-seeds, which are difficult to hydrolyse, resulting in a rather low conversion yield. Data from the literature [9] show that the most efficient handling of stone-seeds is combustion. The conversion yields of the by-products of pulping are promising, and these wastes could be useful for fermentation, however, the amounts available are rather small (below 1,000 t/a). The conversion yield of tomato pulp is comparable to those of the apricot and peach and in this case the water-soluble sugar content is rather important as its quantity is similar to that resulting from hydrolysis.

The results are quite promising with the apple residues, the apple pulp, as well as the pulp preparation residues from peach and apricot. These by-products are also available in significant quantities above 10,000 tons per year. Therefore, further hydrolysis experiments were carried out with this group of fruit by-products.

Table 2 Conversion of insoluble carbohydrate to soluble reducing sugar by enzymatic hydrolysis of washed fruit residues, given as the ratio (in percent) of reducing sugar after hydrolysis to total available carbohydrate content

Examined residues	Conversion (%)
Peach *	47
Peach **	21
Apricot *	46
Apricot **	23
Apple pomace	63
Apple pulp	73
Red currant	16
Raspberry	21
Blackcurrant	17
Elder	30
Tomato	46
Blackberry	28

* residues from pulp production
** residues from juice production, containing the stones

Hydrolysis experiments in a stirred system, with the most readily convertible fruit processing by-products

These 250 ml experiments were performed at 50 °C with 3, 6, 9, and 12 % dry weight content suspensions under mild stirring (100-150 min^{-1}) for 24 hours in order to determine the maximum amount of sugar that can be reached, which is an important factor for further experiments. The more efficient stirring increased the effectiveness of the hydrolysis. By increasing the initial amount of substrate, the maximum sugar concentration reached also increased (*Table 3*). In the case of suspensions containing 12 % dry weight, the highest sugar concentration, 46 g/l, was obtained from apple residues, while the lowest, 9 g/l, from tomato juice processing by-products. At the same time, with the increase in the amounts of sugar obtained, the conversion values decreased. However, the decrease between the 3 % and the 12 % samples was less than 10 % (*Table 4*). Among the conversion values obtained with the 5 h and the 24 h hydrolysis, even with the mixtures containing larger amounts of initial matter, the difference was smaller than 10 % (*Fig.2*), thus in future ABE fermentation experiments it would be practical to inoculate the hydrolysate after 5 hour hydrolysis. Statistical tests showed no significant difference at a 95 % confidence level in the total amount of sugar extracted when the original, unwashed sample is directly hydrolysed, when the sample is first washed and only then hydrolysed. The amount of extractable sugar also does not change when using fresh, dried, or powdered samples. Freezing and thawing of the samples does not spoil the degradability, moreover it shows a positive effect on the enzymatic hydrolysis. (*Table 5*).

Table 3 Reducing sugar concentrations of enzymatic hydrolysates starting from substrates at varied dry matter contents

	Sugar concentration (g/l)			
Examined residues	3%	6%	9%	12%
Apple pulp	12	24	36	46
Apricot pulp	8.3	16	24	33
Peach pulp	9.0	16	25	32
Tomato	3.0	5.3	7.6	9.3

Table 4 Conversions efficiency of enzymatic hydrolysis starting from substrates at varied dry matter contents

	Conversion of carbohydrate (%)			
Examined residues	3%	6%	9%	12%
Apple pulp	98	96	94	91
Apricot pulp	67	65	65	63
Peach pulp	70	64	64	62
Tomato	45	42	40	38

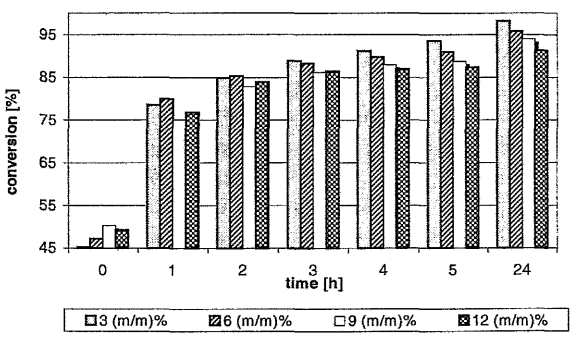

Fig.2 Conversion of insoluble carbohydrate to soluble reducing sugar in hydrolysis of apple pulp, in relation to initial dry matter content of the substrate (in mass percent). Conversion is calculated as in *Table 2*

ABE fermentation on hydrolysed apple pulp

ABE fermentation experiments were performed on enzymatically hydrolysed apple pulp. The apple pulp was supplemented with glucose to elevate its sugar concentration to 60 g/l, which was necessary because the ABE bacteria used require a minimal sugar concentration to initiate solvent production. In addition to the glucose, the medium was also supplemented with nutrients as used in the semisynthetic glucose media [2]. A comparative experiment with an unsupplemented medium was done without adding the nutrients.

The fermentation was inoculated with vegetative cells grown in continuous cultivation on a starch medium. The maximum average product concentrations during the fermentation are presented in *Table 6*. On the supplemented medium the sugar was almost completely consumed within 42 h, its concentration decreasing from 60 g/l to 1,2-3,7 g/l. Coupled with the decrease in

Table 5 Effect of refreezing of peach residue on conversion effectivity of enzymatic hydrolysis starting from varied dry matter contents in the substrate

Time (h)	Sugar concentration [g/l] S=3%	S=3% refreezing	S=12%	S=12% refreezing
0	1.8	3.0	3.5	18
1	9.6	12	31	41
2	10	14	32	42
3	11	15	32	45
4	13	17	37	50
5	14	17		
5	14	17	39	54
24	17	18	48	56

Table 6 Results of ABE fermentation using enzymatically hydrolysed apple pulp and effect of medium supplementation on performance

Product, Substrate	Maximum product concentration at completed medium (g/l)	Maximum product concentration at not completed medium (g/l)
Butanol	11	9.6
Ethanol	3.0	1.0
Acetone	6.6	4.8
Butyric acid	0.02	0.13
Acetic acid	1.4	1.3
Remaining sugar	2.3	20

Start of solvent formation: - before 18 h for supplemented medium; - after 24 h for unsupplemented medium. Max. solvent concentration was reached - at 42 h for supplemented medium; - at 66 h for unsupplemented medium.

the sugar, the products (acetone, butanol, ethanol) appeared in the broth and reached their maximum concentrations after just 42 h when the sugar was almost completely consumed (95 % of the initial glucose). On the uncompleted medium, the maximum product concentrations were somewhat lower (*Table 6*). Glucose was not consumed completely - only about 70 % was consumed. The fermentation proceeded more slowly, solvent formation started later and maximum product concentrations were reached later.

Ethanol fermentation experiments on apple pulp with Saccharomyces cerevisiae

The fermentation experiments were also performed on hydrolysed apple pulp with commercially available baker's yeast. In order to run both fermentation experiments under comparable conditions, the solutions were supplemented with enough glucose to reach the sugar concentration of 60 g/l. As in the ABE experiments the medium contained all the necessary nutrients. Following sterilisation, the media was inoculated for one night with a *Saccharomyces cerevisiae* inoculum and the fermentation was performed at 35 °C for 46 h. The sugar concentration gradually decreased from the initial value of 60 g/l and was completely consumed by the 46th hour, at which point the maximum ethanol concentration was reached as well

(24.4, 24.8 and 28.3 g/l in three parallel experiments). This is close to the theoretical maximum yield (0.51 g/g glucose).

Conclusions

There is large quantity of agricultural and food industrial by-product, which could be potential substrates for ABE fermentation. Among the 12 different fruit processing residues there are 4 different by-products (apple, apricot, peach and tomato) which are worth further examination. The conversion yields of the apple processing by-products are the most promising, and the nearly 100 % conversion of the technological wastes is especially noteworthy. The experiments also show that in the case of apple residues the amount of sugar obtainable by washing is also significant. In these cases significantly better conversions are obtained, when the apple wastes are initially washed with water, which can be explained by product inhibition (β-glucosidase). Apple processing by-products are promising both in the ethanol and the ABE fermentation experiments. The results showed that the previously hydrolysed apple pulp contain no inhibitors that could interfere with or block the activities of the microorganisms. Supplementation of the hydrolysate with additional nutrients is not essential for growth and solvent production, however, it accelerates the fermentation process. The strain examined is able to utilise up to 96 % of the sugar extracted during the hydrolysis and during the anaerobic fermentation it is able to produce the desired products acetone, butanol, and ethanol in amounts close to the theoretical yields. Yeast ethanol fermentation on the hydrolysates is also possible, and the yields are reasonable (ca. 90 % based on glucose).

The pomaces of the berries, blackberry, red and black currant, raspberry, and elder were not easily accessible to enzymatic hydrolysis. Their conversion values are low, therefore they cannot be recommended as ABE fermentation substrates based on these results despite their potentially high carbohydrate contents.

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