Exploitation of Food Feedstock and Waste for Production of Biobutanol

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Abstract

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Nine strains of solventogenic clostridia including the species *C. acetobutylicum, C. beijerinckii, C. pasteurianum,* and *C. saccharoperbutylacetonicum* were tested for the solvents production using potato, maize, and sugar beet molasses as substrates. The solvent concentrations reached in the cultivations with maize and molasses media (15.23 g/l and 13.70 g/l, respectively) looked promising. Based on the screening experiments, the strain *C. acetobutylicum* DSM 1731 was selected for further experiments in the laboratory bioreactor using the maize medium. The results achieved in this batch cultivation (total solvents concentration 12.91 g/l, the yield from maize starch 22%, the solvents formation productivity 0.22 g/l/h, and the ratio of B:A:E approximately 2:1:0) imply the potential of maize as an energetic crop for the biofuel production. In addition, whey protein concentrate was tested as a possible replacement of the usual but expensive media components, i.e. yeast autolysate and/or trypton, and it was confirmed that these substitutes functioned well in the glucose medium.

Keywords: *Clostridium*; acetone-butanol-ethanol (ABE) fermentation; solvents production; maize sugar beet molasses; whey protein concentrate (WPC)

Biobutanol, i.e. 1-butanol produced by solventogenic clostridia from various saccharide containing materials, can be blended either into gasoline in a similar way as ethanol or can be also mixed with diesel fuel. Its use as a fuel additive has some advantages over ethanol: a higher energetic content, limited miscibility with water, lower vapour pressure, and lesser corrosivity. Butanol is produced in the process known as acetone-butanol-ethanol (ABE) fermentation by various species of strictly anaerobic *Clostridium* bacteria, e.g. *C. acetobutyli-cum* or *C. beijerinckii.* During the World War I, this technology was used for the acetone and later also butanol production on industrial scale; in Great Britain and in the period between the World Wars I and II, the ABE enterprises were operated worldwide (e.g. in the USA, Japan, Russia or JAR). Nevertheless, in 1950s and 1960s, when the oil price decreased, the process could not compete with the chemical production of butanol and most of the manufactures were closed. However, nowadays the increasing price of oil together with the effort to

substitute renewable energetic sources for at least part of the consumed ones leads to the renewed interest in the technology mentioned.

Although it is not generally known, the study of ABE process has a long tradition in the Czech Republic, too. Professor Josef Dyr, the first dean of the Faculty of Food and Biochemical Technology of the Institute of Chemical Technology in Prague, contributed significantly to the development of the research in this field (e.g. Dyr & Protiva 1956). Moreover, a small enterprise for the fermentative production of butanol from sugar beet molasses was working in the territory of the distillery in Rájec nad Svitavou in Czechoslovakia in 1950s.

The usual ABE fermentation is believed to be biphasic when the first stage covering lag and exponential growth phases of the batch culture can be characterised as acidogenic and the second one covering late exponential and stationary growth phases is regarded as solventogenic. The major products of the acidogenic phase – acetate, butyrate, CO_2, and H_2 are usually accompanied by small amounts of acetoin and lactate. The onset of the solvents production is stimulated by the accumulation of acids in the cultivation medium together with pH drop. Butanol and acetone are formed partially from the sugar source and partially by reutilisation of the acids formed. Simultaneously, the hydrogen production is reduced to a half in comparison with the acidogenic phase (reviewed in Jones & Woods 1986; Dürre 2008; Lee *et al.* 2008). The old brewmasters, working on ABE industrial fermentation nearly hundred years ago, were persuaded that the solvent production is tightly coupled to sporulation and this phenomenon was proved by finding the gene *spo0A* responsible for both the sporulation and solvent production initiation (Ravagnani *et al.* 2000).

At first, common substrates for butanol fermentation, used on industrial scale, were starch containing agricultural crops like corn, maize, and potatoes, but later the interest was focused on sugar beet and sugar cane molasses as cheap sugar sources (Jones & Woods 1986). In addition, other alternative carbon sources like whey (Ennis & Maddox 1987), apple pomace (Voget *et al.* 1985) or sago (Madihah *et al.* 2001) were tested in laboratory experiments. The feedstock and wastes mentioned can be used once more without any sophisticated pretreatment because clostridia can directly utilise a wide spectrum of mono-, di- and polysaccharides including starch,

saccharose, and lactose. Although at present the preferable sources of biofuels of the so-called second generation are hydrolysates of lignocellulosic materials, the real use of these materials is complicated due to various factors like high expenses necessary for chemical hydrolysis, still inefficient enzyme hydrolysis, low sugar yield, and the formation of compounds that inhibit fermentation (Ezeji *et al.* 2007).

The aim of this work was to test various substrates, namely maize, potatoes, and sugar beet molasses, i.e. agricultural crops and wastes available in the Czech Republic, for the biobutanol production. Moreover, whey protein concentrate was used as a partial or complete substitute for usual but rather expensive components of the cultivation media – yeast extract/autolysate and trypton. Clostridia are known to be amino acids auxotrophs (Jones & Woods 1986) and as such they need complex nitrogen sources supplied in the cultivation medium. It is well-known with the biobutanol fermentative process that the cost of feedstock can raise the end product price by up to 40%, thus the selection of a convenient procedure together with the minimisation of the expenses necessary for the cultivation medium preparation is of key importance.

MATERIAL AND METHODS

Microorganisms. Nine *Clostridium* strains shown in Table 1, maintained as spore suspensions in 1 ml portions in Eppendorf microtubes at 4°C were used. The spore suspensions were prepared from the well sporulated cultures grown in TYA

Table 1. *Clostridium* strains

Strain	Catalog number
C. acetobutylicum	DSM 1731
	LMG 5710
C. beijerinckii	CCM 6218
	DSM 6422
	NCIMB 5022
	NRRL B-466
	NRRL B-592
C. pasteurianum	NRRL B-598
C. saccharoperbutylacetonicum	DSM 14923

medium (see bellow) for 5–7 days after double centrifugation (10 000 min⁻¹, 5 min) followed by resuspending in sterile distilled water.

Cultivation media and fermentation conditions. The following cultivation media were used: TYA medium (Shaheen *et al.* 2000) containing in g/l: glucose 30, yeast autolysate (Imuna Pharm, Šarišské Michaľany, Slovakia) 4, trypton (Fluka, Lyon, France) 12, KH_2PO_4 1, ammonium acetate 6, $MgSO_4 \cdot 7H_2O$ 0.6, $FeSO_4 \cdot 7H_2O$ 0.02; Molasses medium (Shaheen *et al.* 2000) containing in g/l: sugar beet molasses with 20% of saccharose 132, yeast autolysate 10, $(NH_4)_2SO_4$ 2, KH_2PO_4 1; Maize medium (Shaheen *et al.* 2000) containing in g/l: maize having 71% of starch 80, yeast autolysate 10, $CaCO_3$ 2, $(NH_4)_2HPO_4$ 1.8, L-cystein-HCl 0.5; Potato medium (Nimcevic *et al.* 1998) containing in g/l: potatoes having 50% of starch 220. Potatoes were peeled and cut into small cubes; maize was milled to fine powder before the appropriate media preparations. The values of pH of all media were adjusted to 6.5 prior to autoclaving (121°C, 20 min); the potato and maize media were sterilised twice with 24 h delay.

The preliminary screening tests with clostridia using potato, maize, and molasses cultivation media were performed using 50 ml Erlenmeyer flasks, each containing 20 ml of the cultivation medium placed in Merck anaerobic jar, at 37°C for 7 days. After inoculation with 1 ml of spore suspension, the cultures were heat shocked (80°C, 2 min) and subsequently cooled in ice drift for 2 min prior to the cultivation. All experiments were carried out in triplicates. As it is not possible to take samples from Merck anaerobic jar without destroying the anaerobic atmosphere, only the final samples were analysed.

The batch cultivations in the maize medium were carried out also in a laboratory bioreactor Biostat B with the control unit DCU 1 (B. Braun Biotech International GmbH, Melsungen, Germany). The working volume of the bioreactor (3 l) was inoculated with 300 ml of a vegetative inoculum pre-cultured from spore suspensions in TYA medium for 24 hours. The cultivations were conducted at 37°C, without pH control, with the mixing rate 200 rpm, for 60 hours. Samples were taken every 3 hours.

The experiments focused on partial or complete substitution of yeast autolysate and/or trypton by whey protein concentrate containing 70% of proteins in dry matter (WPC 70, Promil, Nový

Bydžov, Czech Republic) were done in 500 ml Erlenmeyer flasks containing 200 ml of the appropriate cultivation medium, in the anaerobic chamber Concept 400 (Sony Technology Centre, Glamorgam, UK), also at 37°C, for 5–7 days. A certain amount of WPC containing the same quantity of nitrogen as the original medium was added in all cases. The flasks with the cultivation media were inoculated with 10 ml of the vegetative inoculum pre-cultured from spore suspensions in TYA medium for 24 hours. Samples were taken for analyses every 24 h and all these experiments were realised in parallels.

Analyses. The concentrations of glucose, acetate, butyrate, acetone, butanol, and ethanol were determined by HPLC (Agilent 1200 Series, USA) under the following conditions: H^+ column maintained at 60°C, mobile phase 5mM H_2SO_4 in deionised water, flow rate 0.5 ml/min, refractive index detection. The course of sporulation and the state of the culture were observed under a microscope (Olympus BX51, Japan) with a phase contrast.

Calculated parameters. Total ABE concentration – sum of the concentrations of butanol (B), acetone (A), and ethanol (E) formed.

B:A:E – ratio of butanol, acetone and ethanol amounts formed in the cultivation

$Y_{ABE/S}$ – yield of ABE from total amount of the added saccharide expressed in percents formed ABE (g)/substrate (g) × 100

P_{ABE} – ABE productivity in g/l/h formed ABE (g)/cultivation volume (l)/production time (h)

List of abbreviations. A – acetone, B – butanol, E – ethanol, T – trypton, WPC – whey protein concentrate, YA – yeast autolysate.

RESULTS AND DISCUSSION

At first, we started the screening experiments with the aim to find a suitable *Clostridium* strain for each substrate, tested and we also tested the traditional feedstock recommended for the solvent production, i.e. potatoes, maize, and beet molasses that are also available in the Czech Republic. These cultivations with nine clostridial strains (Table 1) were performed in small volumes in Erlenmeyer flasks. From a large number of experiments, we decided to demonstrate only three in each case showing the best solvents productions (Table 2). These screening experiments can be summarised as follows:

Table 2. Summary of screening tests

Clostridium strain	Medium	Acetate	Butyrate	Butanol – B	Acetone – A	Ethanol – E	ABE – total	B:A:E	$Y_{ABE/S}$ (%)
				(g/l)					
C. acetobutylicum DSM 1731	maize	2.48	1.44	10.35	3.97	0.47	14.79	7:3:0	26
LMG 5710		1.73	1.54	10.05	4.42	0.79	15.23	7:3:1	29
C. beijerinckii DSM 6422		1.56	1.58	7.84	3.78	0.45	12.07	7:3:0	21
C. beijerinckii CCM 6218	molasses	1.49	0.45	8.81	2.24	2.65	13.70	6:2:2	21
DSM 6422		2.29	0.43	5.61	1.75	0.19	7.55	7:2:0	11
C. pasteurianum NRRL B-598		2.51	0.42	5.12	1.68	0.34	7.13	7:2:1	11
C. beijerinckii CCM 6218	potato	1.45	1.40	4.73	2.63	0.33	7.69	6:3:0	7
NRRL B-466		0.45	0.73	4.26	2.26	0.30	6.82	6:3:0	6
C. saccharoperbutylacetonicum DSM 14923		1.29	1.26	3.83	2.00	0.35	6.18	6:3:1	6

The best 3 results (averages from 3 parallel cultivations) achieved in the case of each cultivation medium are presented. The cultivations proceeded under anaerobic conditions at 37°C and after 7-day cultivation the whole culture was taken for the analyses

– The highest values of butanol concentration (10.35 g/l), total ABE concentration (15.23 g/l), and yield (29%) were achieved using the maize medium.

– It was demonstrated (Table 2) that it is necessary to use different clostridial strains for different substrates. It seems that some strains were originally selected for starch substrates (e.g. *C. acetobutylicum* DSM 1731) and others for saccharose (e.g. *C. beijerinckii* CCM 6218) which is in accordance with the previously published findings (Jones & Woods 1986).

– From nine *Clostridium* strains tested, only four (*C. saccharoperbutylacetonicum* DSM 14923, *C. beijerinckii* DSM 6422, *C. beijerinckii* CCM 6218, and *C. pasteurianum* NRRL B-598) succeeded in growing in the beet molasses medium, i.e. to utilise saccharose as the carbon source. The highest butanol and total ABE concentrations reached in the molasses medium (8.81 g/l and 13.70 g/l, respectively) looked also promising.

– Although potatoes were mentioned to be an excellent substrate for the ABE fermentation without any pretreatment or hydrolysis (Nimcevic *et al.* 1998) our results obtained with this substrate did not confirm these findings. The possible cause is not easy to assume because the clostridial strains used evidently formed amylolytic enzymes necessary for the starch utilisation as demonstrated with the maize medium. A possible explanation may be the lack of some essential nutrient (e.g. an amino acid or a vitamin) because the potato medium as the only one in this study did not contain yeast autolysate.

Based on the screening experiments (Table 2), it was decided to continue with the maize cultivations carried out in a laboratory bioreactor with the strain *C. acetobutylicum* DSM 1731 (Figure 1). This figure shows the concentrations of all compounds determined with the exception of ethanol, whose concentration was very low throughout the fermentation, reaching maximum value of 0.5 g/l at the end of the cultivation. The following cultivation parameters were attained: maximum total ABE concentration 12.91 g/l, the yield ($Y_{ABE/S}$) 22%, ABE productivity 0.22 g/l/h and the ratio of B:A:E approximately 2:1:0. Suprisingly, the total ABE concentration and the yield values were lower (by about 15% and 25%, respectively), than those obtained with the cultivations in Erlenmeyer flasks. The
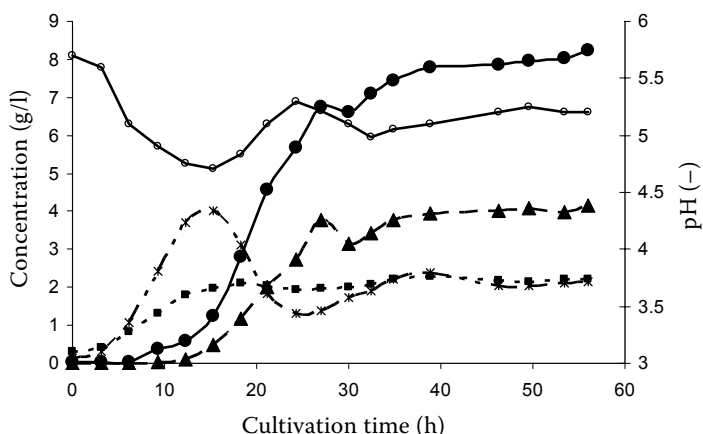
Figure 1. Solvents and acids production by *C. acetobutylicum* DSM 1731 in the maize cultivation medium

The cultivation proceeded in the laboratory bioreactor at 37°C, without pH regulation
Concentrations of individual compounds are marked with $-●-$ for butanol, $-▲-$ for acetone, $-■-$ for acetate, and $-✳-$ for butyrate. Values of pH are marked with the following symbol $-○-$

different cultivation times (168 h and 60 h for the flask and bioreactor experiments, respectively) and completely different cultivation conditions could provide the explanation for this unexpected phenomenon. Nevertheless, this cultivation was quite comparable with those performed by Shaheen *et al.* (2000). In this work, 18 strains of four clostridial species (*C. acetobutylicum, C. saccharobutylicum, C. sacharoperbutylacetonicum* and *C. beijerinckii*) were grown in the maize medium with the composition identical to that in our experiments and at the end of fermentation total ABE concentration varied from 6.8 g/l to 19.6 g/l, the yield being in the range from 8.5% to 24.5%. In general, with the batch cultivation it is possible to obtain total ABE concentrations lying in the range from 12 g/l to 20 g/l, yields of total ABE varying in the interval 30–40% and ABE productivity 0.3–1.2 g/l/h if a common unmodified *Clostridium* strain is used (Ezeji *et al.* 2005); our results are near the lower level of the intervals mentioned.

Figure 1 clearly shows that the acids (acetic and butyric) formation preceded the solvents (mainly butanol and acetone) production, and that part of the formerly produced butyrate was subsequently consumed. Moreover, the solvents production and butyrate consumption were probably triggered by pH drop from the value 5.7 to 4.8 after approximately 12 h from the beginning of cultivation (Figure 1) while the onset of the solvents production was accompanied with a little delayed spore formation as seen under the microscope. The opinions on the pH value necessary for the solvent production start vary, usually pH value under 5.5 is recommended (Jones & Woods 1986; Terracciano & Kashket 1986) but it seems more probable that the concentrations of dissociated acetic and butyric acids produced or added in the

milieu play an important role in the regulation of the solvent production. It was demonstrated that very low extracellular concentrations of the acids – 10–20mM, i.e. about 1 g/l, can cause a switch from the acidogenic to the solventogenic metabolism (Jones & Woods 1986; Terracciano & Kashket 1986). However, in our case the total acids concentration at the metabolic switch point was about 6 g/l.

Recently, it has been calculated (Qureshi & Blaschek 2001) that corn can be an economically profitable substrate for biobutanol production employing even a usual batch cultivation process and distillation for ABE recovery, however, the assumed ABE yield was 42%. In the Czech Republic, the main portion of maize is grown for cattle feeding but at the same time, the size of cattle herds diminishes every year. As the important goals of the biofuels production are, beside others, also the support of farmers and maintenance of arable land areas, maize can be seen as an energetic crop, too. Moreover, in the Czech Republic, the areas seeded with genetically modified (GM) maize (variety MON 810), resistant against the most significant maize pest *Ostrinia nubilalis*, have been increasing till this year (Křístková 2008). The yields of GM maize are up to 30% higher in comparison with the conventional maize while the most important argument against its growing is the question whether or not its consumption by either humans or animals is coupled with some health risks. In the case of GM maize use for the industrial purposes like biobutanol production, these reasons are groundless.

Since clostridia are often amino acids auxotrophs, yeast extract (or yeast autolysate) and/or trypton are usually added into the cultivation medium as a complex nutrient source. However, the prices of

these supplements are quite high and therefore different alternatives of complex nutrient sources have already been searched for, e.g. corn steep liquor (Qureshi & Blaschek 2001). We have decided to try a whey protein concentrate (WPC) that can be easily gained via ultrafiltration of common dairy waste (whey). The WPC used was produced by the dairy factory Promil (Nový Bydžov, Czech Republic) and contained 70% of protein in the dry matter and no lactose. The results of the experiments, in which the components of TYA cultivation medium, yeast autolysate or trypton or both, were replaced by WPC, are shown in Figure 2. All cultivations were run with the strain *C. pasteurianum* NRRL B-598 that was selected due to its

stable growth and solvents production observed in many experiments (data not shown). The TYA medium was selected for these tests because its composition has already been optimised for the solvents production (Shaheen *et al.* 2000). In the cases of the original TYA medium components (yeast autolysate and trypton) replacement with WPC (Figure 2B–D), the butanol concentrations reached were higher and glucose utilisations was better in comparison with the cultivation in TYA medium (Figure 2A). Acetate concentrations shown in Figure 2 reflected both the acetate added into the medium in the form of ammonium acetate and the acetate produced. From the corresponding curves it was clear that acetate was also consumed.
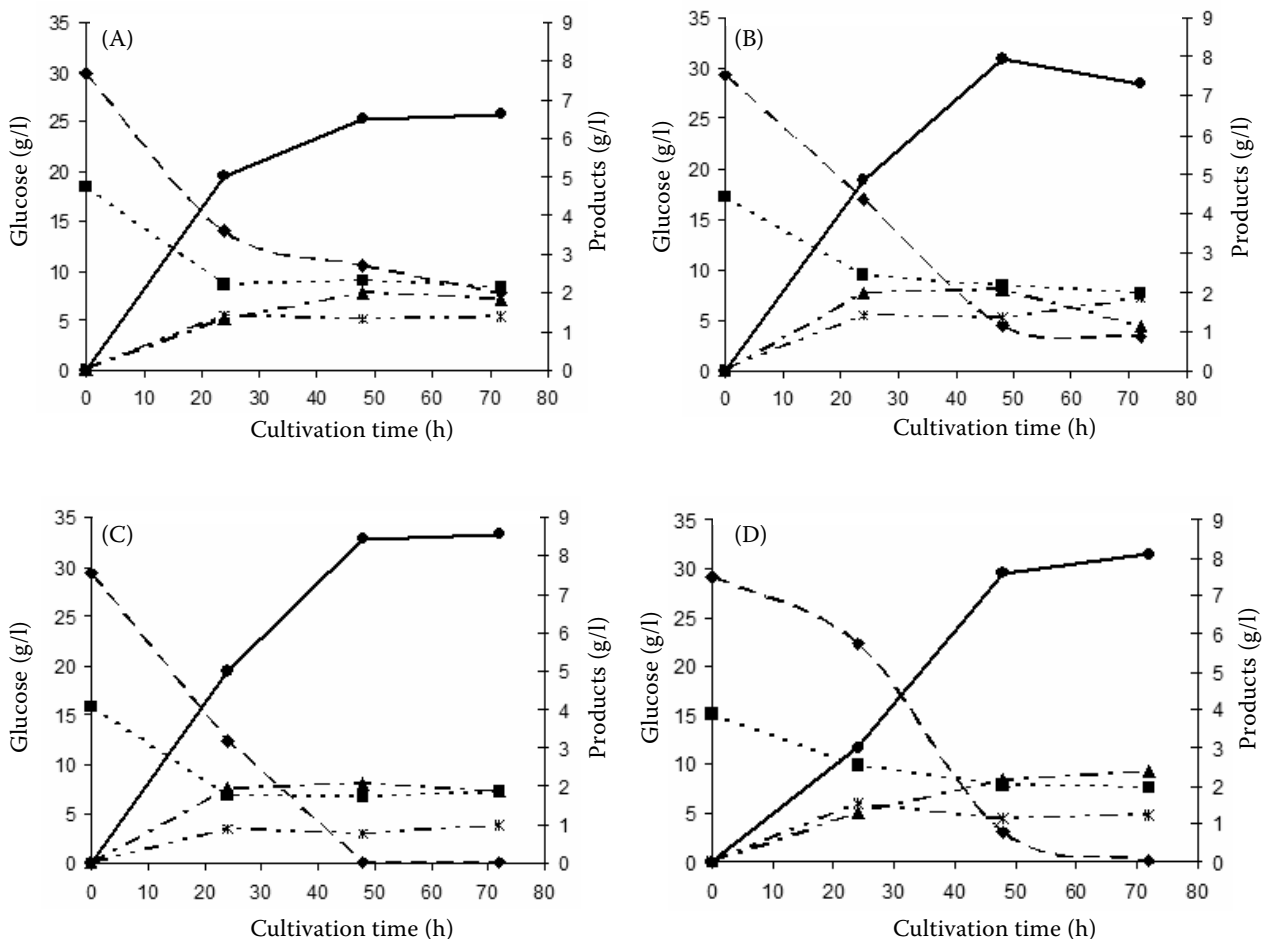
The experiments were carried out in Erlenmeyer flasks placed in an anaerobic chamber at 37°C, for 72 hours. The media in the flasks were inoculated with 10 ml of vegetative culture pre-cultured 24 h in TYA medium. Abbreviations T, WPC, and YA stand for trypton, whey protein concentrate, and yeast autolysate, respectively. Concentrations of individual compounds are marked with −♦− for glucose, −●− for butanol, −▲− for acetone, −■− for acetate, and −✳− for butyrate

Figure 2. Comparison of acids and solvents production by *C. pasteurianum* NRRL B-598 in TYA medium (A), TYA medium with WPC instead of YA (B), TYA medium with WPC instead of T (C), and TYA medium with WPC instead of both YA and T (D)

When WPC was used as the substitution for both yeast autolysate and trypton, slower fermentation start was probably used by the lag growth phase prolongation (Figure 2D).

To the best of our knowledge, nobody has ever tried to use WPC in ABE fermentation. The only reference dealing with WPC use for microbial nutrition concerned the replacement of the yeast extract in the medium for lactic acid production but in this case WPC was hydrolysed with a proteolytic enzyme prior to its use (Heriban *et al.* 1993). Although whey as the carbon source was tested for the solvents production many times (e.g. Qureshi & Maddox 2005), in these cases yeast extract was usually added to the whey medium as a complex nutrient source. We tried to replace not only the yeast autolysate but also trypton with WPC but unlike trypton WPC was not enzymatically digested and the used strain *C. pasteurianum* NRRL B-598 had to form its own proteolytic enzymes for WPC utilisation. Nevertheless, solventogenic clostrida should posses the abilities to form proteolytic and peptidolytic enzymes (Jones & Woods 1986). However, the yeast autolysate or extract is certainly added to the medium not only because of amino acids but also as a source of other unspecified nutrients (vitamins, etc.) necessary for a good clostridial growth and solvent production. Therefore, despite the fact demonstrating that both the yeast autolysate and trypton may be replaced by WPC, it appears necessary to perform long-term experiments in order to prove that this step does not result in the so-called strain degeneration which is the state of solvents production inability (Jones & Woods 1986).

CONCLUSIONS

The screening experiments with the traditional substrates for biobutanol fermentative production, i.e. potatoes, maize, and sugar beet molasses, successfully followed up the experiments performed at the Department of Fermentation Chemistry and Technology, ICT in Prague more than 50 years ago. Total ABE concentrations reached in the maize and molasses media cultivations (15.23 g/l and 13.70 g/l, respectively) demonstrated the potential of both feedstocks for their use in the biofuel industry. In the case of sugar beet, further experiments, are planned for the future, that will test not only molasses but also thin/thick sugar beet juice for

the butanol production. Sugar beet is a crop grown in the Czech Republic for the last 160 years which provides high yields and can be used in the non-food field for the biofuel production.

Maize cultivation medium was also used in a batch biorector cultivation, and the cultivation parameters (total ABE concentration 12.91 g/l, yield from maize starch 22%) lay within the ranges of the values commonly achieved in a simple batch cultivation. However, our cultivation suffered from the product inhibition caused by the solvent toxicity toward the bacterial cells. The product inhibition resulted in a low solvent concentration which increases the product separation expenses. Nevertheless, this main obstacle to the economically profitable ABE fermentation process can be overcome by efficient removal of the solvents from the cultivation medium coupled with the cultivation. The solvent removal can be performed in various ways; in the particular case of the maize medium, gas stripping would be probably the process of choice because membrane processes like pervaporation would suffer from fouling. Based on the demonstrated results, it can be concluded that the first step for the utilisation of maize as an energetic crop has been done.

Both sweet and acid whey are produced by dairies in large volumes therefore whey can be considered a waste product and, as a consequence of this fact, it is cheap. Possible exploitations of whey in biotechnologies have already been investigated by a number of researchers. We were interested in whey protein concentrate that was richer in nitrogen than whey itself. WPC is gained from whey by ultrafiltration and is usually sold as a food supplement recommended for a quick growth of the muscle mass. The aim of this work was to examine whether yeast extract/autolysate and trypton could be replaced by WPC for clostridia and the answer is yes. This finding is important for the reduction of cultivation medium cost in the case of utilisation of any feedstock because a complex nitrogen source must always be present in the medium for a good clostridial growth and solvent production.

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