Use of Wheat Straw and Chicken Feather Hydrolysates as a Complete Medium for Lactic Acid Production

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Abstract

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Biotechnological production of lactic acid has experienced a boom that is hindered only by the lack of low-cost, abundant material that might be used as a substrate for lactic acid bacteria. Such material should contain not only carbon but also complex nitrogen sources, amino acids and vitamins necessary for the balanced growth of the bacteria. Here, for the first time, a combination of hydrolysates of wheat straw and chicken feathers was used as a complete waste cultivation medium for lactic acid production. It was shown to be a promising substrate for lactic acid production, reducing the medium price by 73% compared with MRS broth, providing more than 98% lactic acid yield and high productivity (2.28 ± 0.68 g/l/h) in a fed-batch process using *Lactobacillus reuterii* LHR14.

Keywords: agricultural waste; fermentation; food industry wastes; lactic acid bacteria

A growing demand for lactic acid, due mainly to its broadened areas of application (food-related or cosmetic applications, biodegradable polymers, green solvents, oxygenated chemicals), has led to its increased production over the last decade. In 2005, the annual world production of lactic acid was reported to be 120 000 t (Datta & Henry 2006), but this had more than doubled to 259 000 t in 2012 and increased further to 367 000 t in 2017 (Jantasee *et al.* 2017). As the commercial price of food-grade lactic acid varies from 1.38 USD/kg (50% lactic acid) to 1.54 USD/kg (88% lactic acid) (Wee *et al.* 2006), the production cost should be no more than 0.9 USD/kg (Jantasee *et al.* 2017). As the price of nutrients significantly influences the final production price, finding a cheap substrate rich in nutrients and that provides for high yields of lactic acid can contribute to economically feasible production.

Lactic acid bacteria have limited abilities to synthesise amino acids and vitamins, and often they are even auxotrophic; therefore, medium for their growth should be supplemented with these nutrients, along with various minerals. MRS medium containing expensive components such as yeast and beef extracts or peptones (which are responsible for up to 50% of the final price of the medium) is usually used for laboratory production of lactic acid but cannot be applied on an industrial scale because of its high price. Although lactic acid is nowadays commercially produced from corn sugars, molasses and whey with homofermentative lactic acid bacteria (Göksungur & Güvenç 1997), various research groups have focused their attentions on identifying a cheap and nutrient-rich substrate, that ideally does not compete with human nutrition. Materials are sought that have a zero or negative price, are rich in

nutrients, locally available within the season, do not contain inhibitors of lactic acid bacteria and yield high levels of lactic acid. Such sources should support homofermentative production of the ʟ-isomer of lactic acid.

In Central Europe, wheat straw can serve as abundant source of carbon and other nutrients. On the other hand, chicken feathers, waste from poultry meat production and whey protein, a by-product of cheese making, can be exploited as sources of nitrogen-rich amino acids.

In the Czech Republic, 3.6–4.3 million t of wheat straw (~60% of total cereal straw) are produced annually (Atea Prague 2017). Although part of this is already used for different purposes (animal bedding, soil fertilisers, mushroom production, building material and energy production), about 15% can be exploited for biotechnological use. Wheat straw is rich in carbon (containing 30–49 % cellulose and 20–50% hemicellulose), but a prerequisite for its use for lactic acid production is a breaking of the recalcitrant structure of the material and the digestion of cellulose and hemicellulose into fermentable saccharides. For straw hydrolysis, different pre-treatment methods can be used (Paulova *et al.* 2012); here, milling followed by alkaline hydrolysis under mild conditions and enzymatic digestion were performed.

Due to the low content of nitrogen compounds utilisable by lactic acid bacteria, a medium based on wheat straw should be supplemented with a source of nitrogen that is metabolisable by lactic acid bacteria (Gullon *et al.* 2008). In many studies, yeast extract (Milko *et al.* 1966; Nancib *et al.* 2005; Maas *et al.* 2008), yeast extract combined with peptone (Milko *et al.* 1966; Amrane & Prigent 1997), beef extract (Ohkouchi & Inoue 2007) or corn steep liqueur (Amrane & Prigent 1993; Tsai & Millard 1994; Yu *et al.* 2008) were used, but the costs of the media were considerably increased. Therefore, inexpensive alternatives to these components are preferred as medium supplements. Annually, about 5 million tons of chicken feathers are generated by the poultry industry (Gao *et al.* 2014), and this is used for the production of animal feed supplements such as feather meal (Taskin *et al.* 2012) or disposed of in landfills (Stiborova *et al.* 2016). Since 90–92% of feather is composed of keratin, a protein rich in cysteine, arginine, threonine and hydrophobic amino acids, it can have nutrient potential providing its structure can be effectively broken down. In this study, hydrolysate of chicken feathers was combined with wheat straw to be used as a nutrient source for lactic acid production. In this study, hydrolysates of chicken feathers were combined with wheat straw and used as a nutrient source for lactic acid production.

MATERIAL AND METHODS

Microorganisms. The following strains, obtained from the Department of Dairy, Fat and Cosmetics of the UCT Prague (Czech Republic), were used in this work: *Lactobacillus reuteri* LHR14, *Lactobacillus salivarius* 10A, *Lactococcus lactis* ssp. *lactis* CCDM 617 and *Lactobacillus casei* CCDM 198.

The microorganisms were stored cryopreserved at −70°C in a sterile 30% glycerol solution.

Modified MRS medium. For the screening tests, modified MRS broth was used and contained the following: 20 g/l of glucose (or arabinose, xylose, cellobiose or sucrose), 10 g/l peptone, 10 g/l beef extract, 5 g/l yeast extract, 1 ml Tween 80, 2 g/l K_2HPO_4, 5 g/l sodium acetate, 2 g/l ammonium citrate, 0.2 g/l $MgSO_4 \cdot 7\,H_2O$ and 0.2 g/l $MnSO_4 \cdot H_2O$.

MRS with chicken feather hydrolysate. Degreased chicken feathers were milled using a knife mill (Grindomix GM 200; Retsch, USA) at 10,000 rpm for 20 s and then mixed with 0.6% KOH solution (feather concentration varied from 12–50 g/l). The mixture was hydrolysed for 24 h at 70°C and 150 rpm using a rotary shaker. The hydrolysate was supplemented with all MRS nutrients except for yeast extract, peptone and beef extract. The composition of the hydrolysate was analysed as described previously (Stiborova *et al.* 2016).

MRS with whey protein. Whey protein in the amount of 25 g/l was used in MRS instead of peptone, beef and yeast extracts.

Molasses medium. Molasses was diluted with distilled water to 20 g/l of sucrose and supplemented with 10 g/l peptone, 5 g/l yeast extract and 10 g/l beef extract.

Wheat straw and feather hydrolysate medium. A mixture of milled wheat straw and chicken feathers in 100 ml of 0.6% KOH (concentration of cellulose 40 g/l and feather 30 g/l) was hydrolysed for 24 h at 70°C and 150 rpm. After cooling to 55°C, the pH was adjusted to 5 using 2 ml 10% H_3PO_4, and the material was enzymatically hydrolysed using Cellic CTec2 (Novozyme, Denmark) for 24 h at 55°C on a rotary shaker (150 rpm).

Inoculum preparation. Standard MRS medium (100 ml) was inoculated with one tube of cryopre-

served culture (1 ml) and grown statically at 37°C for 24 hours.

Cultivation in Bioscreen. Cultivations were performed in a Bioscreen C analyser (Labsystems, Finland) using microtiter plates having wells with a cultivation volume of 350 µl, for 24 hours at 37°C. Absorbance was measured every 30 min, at a wavelength of 600 nm.

Cultivation in Erlenmeyer flasks. Erlenmeyer flasks (100-ml) containing 20 ml of medium were inoculated with the pre-cultured inoculum at a ratio of 10%, and the culture was grown statically at 37°C for 24 hours.

Cultivation in a laboratory bioreactor. Cultivations were carried out in 1-l Multifors laboratory bioreactors (Infors AG, Switzerland) with 400 ml working volumes in a batch regime, with an inoculation ratio of 10%. Stirring rate was constant at 400 rpm, temperature and pH were controlled at 37°C and 6.5, respectively, and the bioreactor was not aerated. The end of batch culture was indicated by cessation of 10% NaOH solution consumption used for pH control. Feeding of nutrients in fed-batch was realised by a programmable pump as specified in each experiment.

HPLC determination of substrates and products. The determination of concentrations of lactic acid, sources of carbon and energy, ethanol and acetic acid was performed on an Agilent 1200 Series HPLC equipped with a refractometric detector. An IEX H^+ polymer column (Watrex 250 × 8 mm) was used for separation under the following conditions: mobile phase, 5 mM H_2SO_4; flow rate, 0.5 ml/min; column temperature, 80°C.

Determination of optical isomers of lactic acid. The form of the optical isomer was determined using an enzyme kit (D-/L-Lactic Acid Assay Kit; Megazyme, Ireland).
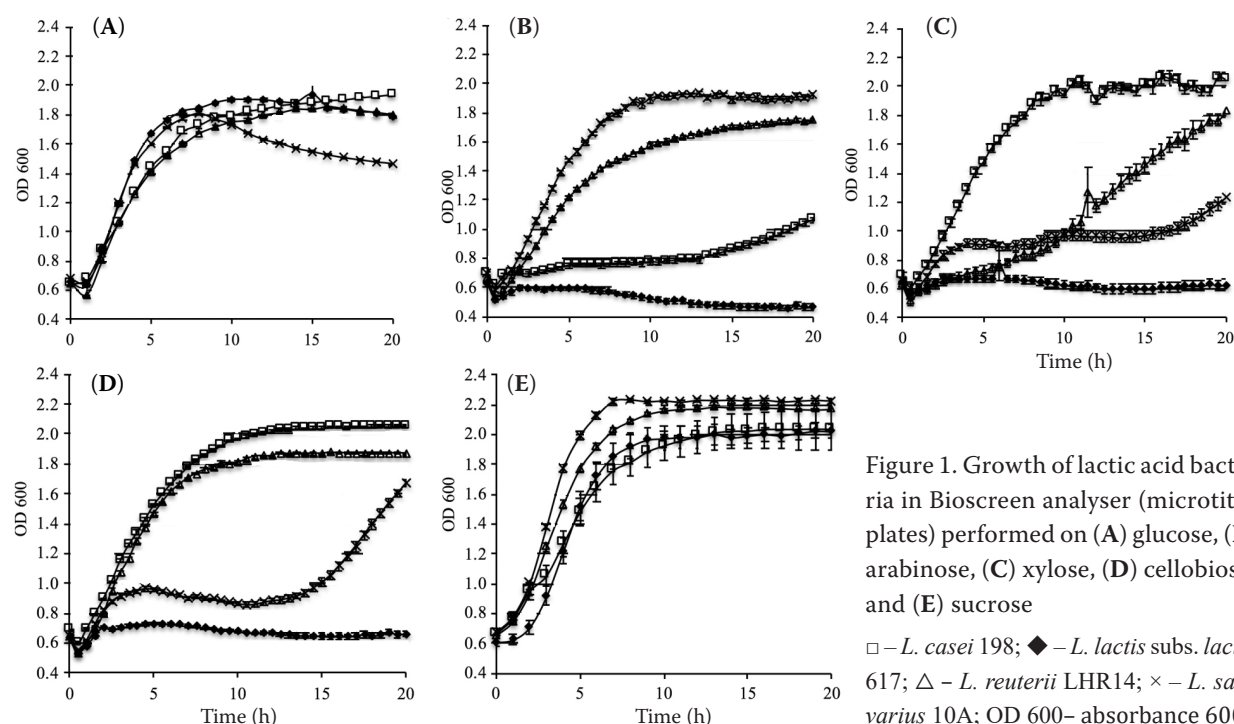
RESULTS AND DISCUSSION

Screenings of several strains of lactic acid bacteria were performed in microplates and Erlenmeyer flasks to select the best producers of lactic acid on lignocellulosic hydrolysates. MRS medium containing glucose, xylose, arabinose or cellobiose (to mimic sugars contained in lignocellulosic hydrolysates) and sucrose (sugar contained in molasses used for comparison) were used to support cell growth of different lactic acid bacteria (Figure 1) and lactic acid production (Table 1). Based on these results, *Lactobacillus casei* CCDM 198 and *Lactobacillus reuteri* LHR14 were selected for further experimentation. Both strains preferentially produced the L-lactic isomer (comprising about 85% of the total lactic acid produced) and were able to consume a wide spectrum of saccharides including pentoses. The best productivities and highest specific growth rates were achieved on glucose as expected (Table 2), but a surprisingly high yield of lactic acid (~ 82%) was achieved with *L. reuterii* grown on cellobiose (Table 2). Efficient utilisation of cellobiose (90% yield) was previously reported by ADSUL *et al.* (2007) for the *Lactobacillus delbrueckii* UV mutant, but has also been described for wild strains (BOLOTIN *et al.* 2001; VAN ZANTEN *et al.* 2015). It is not surprising (GOBBETTI *et al.* 2000; BUSTOS *et al.* 2007; ZHU *et al.* 2007; GIVRY *et al.* 2008) that the tested strains were able to ferment pentose sugars (arabinose and xylose) and convert them to lactic acid. Although the yield of lactic acid on pentose sugars is usually considerably lower compared to glucose (GARDE *et al.* 2002), both selected strains were able to use both xylose and arabinose for lactic acid production (Table 2); this ability qualified them as potential candidates for wheat straw hydrolysate fermentation.

The most expensive components of MRS medium are yeast extract, beef extract and peptone. Therefore,

Table 1. Concentrations of lactic acid (g/l) produced by different lactic acid bacteria grown on modified MRS in a batch culture in Erlenmeyer flasks

Source of carbon	*L. reuteri* LHR14	*L. salivarius* 10A	*L. lactis subs. lactic* CCDM617	*L. casei* CCDM 198
Glucose	18.0 ± 0.2	8.7 ± 0.2	17.9 ± 0.3	18.3 ± 0.3
Arabinose	9.4 ± 0.1	7.9 ± 0.2	0.4 ± 0.2	6.6 ± 0.3
Xylose	3.7 ± 0.8	5.9 ± 0.2	0.3 ± 0.1	7.6 ± 0.5
Cellobiose	16.3 ± 1.0	11.3 ± 0.7	0.3 ± 0.1	11.8 ± 0.3
Sucrose	22.2 ± 1.4	13.6 ± 0.8	20.2 ± 0.9	18.5 ± 1.0

The initial concentration of the carbon source was 20 g/l

Figure 1. Growth of lactic acid bacteria in Bioscreen analyser (microtiter plates) performed on (**A**) glucose, (**B**) arabinose, (**C**) xylose, (**D**) cellobiose, and (**E**) sucrose

□ – *L. casei* 198; ◆ – *L. lactis* subs. *lactic* 617; △ – *L. reuterii* LHR14; × – *L. salivarius* 10A; OD 600– absorbance 600

we first attempted to find a cheap nitrogen source to be used later as a supplement for wheat straw. Chicken feather hydrolysate was tested as a possible replacement for complex nitrogen sources in MRS and the data were compared with MRS supplemented with the commonly used whey protein, a by-product of cheese making (Fitzpatrick & O'Keeffe 2001). Initially, several hydrolysates with different starting concentrations of chicken feathers (12–50 g) were used as the only source of nitrogen in MRS to culture both selected strains. As shown in Table 3, the medium based on chicken feather hydrolysate

is poor in phosphorus; therefore, considerably lower growth rates and final concentrations of lactic acid were achieved when the pH was adjusted with hydrochloric acid, compared to experiments with phosphoric acid addition for both tested strains. A high yield (95 ± 2%) of lactic acid on consumed glucose and a high productivity of lactic acid (1.15 ± 0.04 g/l/h) was achieved with *L. reuterii* grown on MRS supplemented with 35 g/l of hydrolysed feather and phosphorus. Although the production of lactic acid, yield and productivity were improved after phosphorus addition also for the second strain tested

Table 2. Cultivation of *Lactobacillus casei* and *Lactobacillus reuterii* on modified MRS in a batch culture in Erlenmeyer flasks

Strain	Source of carbon	Lactic acid (g/l)	Yield (%)	L-Isomer (%)	Productivity (g/lh)	μ_{max} (h^{-1})
Lactobacillus casei CCDM 198	glucose	18.25 ± 0.34	91 ± 2	85	3.04 ± 0.03	0.21 ± 0.01
	sucrose	18.51 ± 1.01	94 ± 3	86	2.87 ± 0.06	0.17 ± 0.01
	arabinose	6.62 ± 0.29	43 ± 3	85	0.42 ± 0.04	0.11 ± 0.00
	xylose	7.55 ± 0.54	43 ± 4	86	0.88 ± 0.06	0.07 ± 0.01
	cellobiose	11.80 ± 0.31	55 ± 3	85	0.79 ± 0.06	0.13 ± 0.01
Lactobacillus reuteri LHR14	glucose	18.00 ± 0.18	90 ± 1	85	2.99 ± 0.02	0.20 ± 0.02
	sucrose	22.19 ± 1.35	111 ± 10	85	2.78 ± 0.17	0.18 ± 0.01
	arabinose	9.39 ± 0.08	49 ± 1	86	0.58 ± 0.01	0.11 ± 0.01
	xylose	16.29 ± 1.01	26 ± 2	85	1.22 ± 0.12	0.14 ± 0.01
	cellobiose	16.25 ± 1.02	81 ± 7	85	1.25 ± 0.10	0.15 ± 0.01

The initial concentration of the carbon source was 20 g/l

Table 3. Production of lactic acid with *Lactobacillus reuterii* and *Lactobacillus casei* 198

	Feather concentration (g/l)	Lactic acid (g/l)	Yield on consumed sugar (%)	Productivity (g/lh)	μ_{max} (h^{-1})
L. reuterii	without phosphorus supplementation				
	12.5	0.13 ± 0.00	52 ± 0	0.01 ± 0.00	0.02 ± 0.00
	20	3.95 ± 0.11	48 ± 1	0.20 ± 0.01	0.07 ± 0.00
	30	4.58 ± 0.27	54 ± 2	0.23 ± 0.01	0.09 ± 0.00
	35	2.04 ± 0.09	25 ± 1	0.11 ± 0.00	0.05 ± 0.01
	with phosphorus supplementation				
	12.5	0.15 ± 0.02	21 ± 0	0.02 ± 0.00	0.01 ± 0.01
	20	7.79 ± 0.21	90 ± 1	0.97 ± 0.03	0.11 ± 0.01
	30	8.65 ± 0.08	60 ± 1	1.08 ± 0.01	0.13 ± 0.01
	35	9.22 ± 0.34	95 ± 2	1.15 ± 0.04	0.15 ± 0.02
	50	8.85 ± 0.26	90 ± 2	1.11 ± 0.03	0.16 ± 0.01
	whey protein	8.13 ± 0.27	99 ± 1	1.02 ± 0.03	0.14 ± 0.01
L. casei 198	without phosphorus supplementation				
	12.5	3.14 ± 0.12	46 ± 1	0.26 ± 0.01	0.06 ± 0.01
	20	3.70 ± 0.34	50 ± 2	0.39 ± 0.03	0.06 ± 0.01
	30	3.54 ± 0.26	56 ± 2	0.44 ± 0.03	0.08 ± 0.01
	35	1.99 ± 0.38	23 ± 1	0.25 ± 0.02	0.06 ± 0.01
	with phosphorus supplementation				
	12.5	5.83 ± 0.44	71 ± 3	0.73 ± 0.02	0.11 ± 0.01
	20	6.82 ± 0.38	61 ± 2	0.97 ± 0.05	0.12 ± 0.01
	30	7.26 ± 0.51	67 ± 4	1.03 ± 0.07	0.11 ± 0.01
	35	6.88 ± 0.29	66 ± 1	0.98 ± 0.05	0.14 ± 0.01
	whey protein	12.62 ± 0.77	87 ± 1	1.81 ± 0.10	0.17 ± 0.01

Samples with use of MRS medium with chicken feather hydrolysate as the only source of nitrogen and different pH adjustments (hydrochloric or phosphoric acid) in a batch culture in Erlenmeyer flasks; the initial concentration of glucose was 20 g/l

(*L. casei*), these were considerably lower compared to *L. reuterii* (Table 3).

Compared to whey protein, *L. reuterii* (Table 3) exhibited 12% higher production using chicken feather hydrolysate prepared from 35 g/l of feather and a slightly higher productivity (1.15 ± 0.03 g/l/h), while the yield on consumed sugar was in 9% lower. As reported (Altaf *et al.* 2007; Mawgoud *et al.* 2016), corn, soy or red lentil hydrolysates are often successfully used as cheaper alternatives to peptone, beef and yeast extracts, but there is a conflict with human nutrition which can be eliminated by the use of chicken feather, a which is not usable for the human diet. Mild alkali treatment (Patakova *et al.* 2017) was chosen for the preparation of the feather hydrolysate with the intention of integrating the prepared product into the previously optimised process of wheat straw hydrolysis (Jaisamut *et al.* 2013), and this substrate was used for lactic acid

production using both selected strains in the next set of experiments. The data were compared with results achieved using molasses supplemented with peptone, yeasts and beef extracts as a rich cultivation medium. As seen in Table 4, a combined hydrolysate of wheat straw and chicken feathers seems to be a promising substrate for the cultivation of *L. reuterii*; using this medium, the productivity (1.16 ± 0.03 g/l/h) and final concentration of lactic acid (10.44 ± 0.36 g/l) produced by *L. reuterii* LH124 were higher compared to the rich molasses medium used as a control. This medium was then used for cultivation of *Lactobacillus reuteri* LHR14 to evaluate the process of lactic acid production in parallel tests in laboratory bioreactors run in fed-batch mode. Initially, the strain was grown in batch using only a combination of wheat straw and chicken feather hydrolysates. The end of the batch (at 6 h) was indicated by the cessation of feeding of the alkaline solution used for pH control;
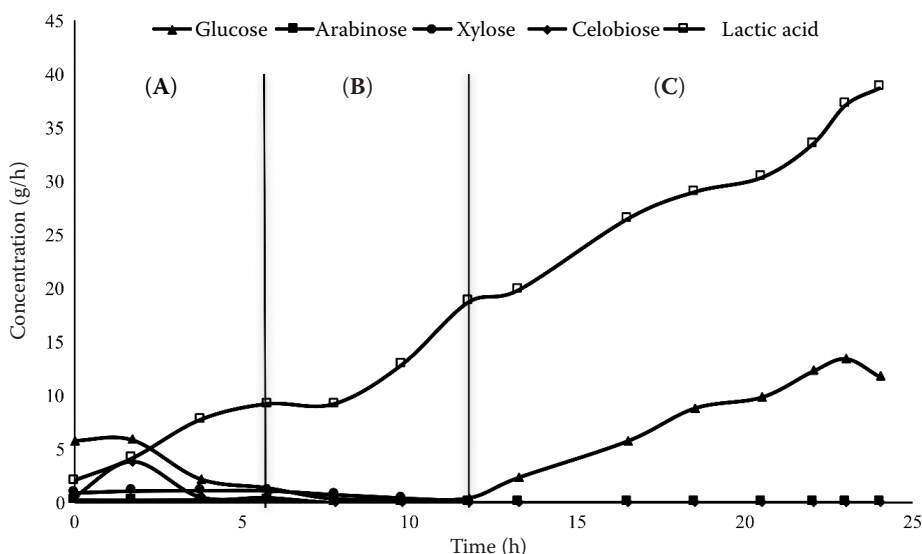
Figure 2. Production of lactic acid in a bioreactor: (**A**) – batch, (**B**) and (**C**) – fed-batch regime with *L. reuterii* LHR124 using a combined hydrolysate of wheat straw and chicken feathers with a concentrated glucose feed

within this time period, 9.22 g/l of lactic acid was produced (Figure 2A). Then, the feeding of a concentrated solution of glucose (366 g/l) to mimic the feeding of concentrated wheat straw hydrolysate was carried out with a constant feed rate (6 ml/h). The feed rate was calculated to be slightly below the maximum rate of glucose consumption (2.20 ± 0.3 g/h) achieved in previous tests with this strain and substrate. Within the first 13 h of fed-batch, all glucose was consumed at a rate of 2.16 ± 0.04 g/h, as expected (Figure 2B); it then gradually accumulated in the second half of the fed-batch (Figure 2C). This was accompanied by a decreased rate of glucose consumption (1.36 ± 0.27 g/h) in this period. On the other hand, the lactic acid production rate and productivity were stable within the fed-batch (1.35 ± 0.41 g/h and 2.28 ± 0.68 g/l/h) and were not influenced by glucose accumulation. At the end of feeding, 38.7 g/l of lactic acid were obtained. The cause of glucose accumulation might be nitrogen

limitation if there are only saccharides in the feed stream. Therefore, the same experiment was then repeated, but the fed-batch was carried out by feeding the combined hydrolysate of wheat straw and feathers (same composition as used for batch), and the feed rate was increased to 53 ml/h to deliver the same amount of sugars as previously. In this case, glucose was fully consumed within the process and the glucose consumption rate was stable within the fed-batch (0.72 ± 0.02 g/h) as were the lactic acid production rate (0.72 ± 0.22 g/h) and productivity (1.38 ± 0.23 g/l/h), suggesting that nutritional requirements were satisfied. On the other hand, the final concentration of lactic acid produced in the process was only 11.24 g/l because of the large increase in volume; this was caused by feeding diluted substrate which resulted in the dilution of the product.

The bioreactor experiments proved that the medium consisting of a combination of wheat straw and chicken feather hydrolysates prepared by simultane-

Table 4. Production of lactic acid with *Lactobacillus reuterii* and *Lactobacillus casei*

Type of waste	*L. casei* CCDM 198				*L. reuterii* LHR14			
	lactic acid (g/l)	yield on consumed sugar(s) (%)	productivity (g/lh)	μ_{max} (h^{-1})	lactic acid (g/l)	yield on consumed sugar(s) (%)	productivity (g/lh)	μ_{max} (h^{-1})
Wheat straw & feather hydrolysates	9.66 ± 0.21	72 ± 1	1.02 ± 0.05	0.10 ± 0.01	10.44 ± 0.36	98 ± 1	1.16 ± 0.03	0.09 ± 0.00
Molasses & complex nitrogen sources	14.56 ± 0.23	82 ± 0	1.32 ± 0.02	0.18 ± 0.01	9.45 ± 0.56	95 ± 1	0.95 ± 0.09	0.15 ± 0.01

Samples with Batch culture in Erlenmeyer flasks using waste materials

ous alkali hydrolysis contains enough nutrients for bacterial production of lactic acid when phosphorus in the form of phosphoric acid was supplemented. To the best of our knowledge, this is the first attempt to use such a medium, originally proposed in our patent for ethanol or butanol production (Patakova *et al.* 2017), for lactic acid fermentation. Medium combining chicken feather hydrolysate prepared by acid hydrolysis and glucose has now been used to test the growth of several bacterial strains (Taskin & Kurbanoglu 2011), among others also of *Lactobacillus delbrueckii* ssp. *bulgaricus*, but the low biomass yields suggest nutrient limitation.

CONCLUSIONS

Wheat straw and chicken feather hydrolysates prepared by simultaneous mild alkali treatment proved to be suitable substrates containing all the nutrients supporting the growth of lactic acid bacteria if supplemented with phosphorus. Medium composed solely from wheat straw and chicken feather hydrolysates seems to be a promising substrate for lactic acid production using *L. reuterii* LHR14 and provides for high yields of lactic acid (over 98%). Using this medium, the substrate price is reduced by 73% compared with commonly used MRS. In a fed-batch process, a stable, high rate of lactic acid production can be achieved if nitrogen limitation is avoided. On the other hand, exploitation of this substrate still has one drawback: the feeding stream needs to be more concentrated; otherwise, a high dilution of the product in the bioreactor occurs.

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