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Ronald H. W. Maas · Robert R. Bakker · Gerrit Eggink · Ruud A. Weusthuis

Lactic acid production from xylose by the fungus *Rhizopus oryzae*

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Abstract Lignocellulosic biomass is considered nowadays to be an economically attractive carbohydrate feedstock for large-scale fermentation of bulk chemicals such as lactic acid. The filamentous fungus *Rhizopus oryzae* is able to grow in mineral medium with glucose as sole carbon source and to produce optically pure L(+)-lactic acid. Less is known about the conversion by *R. oryzae* of pentose sugars such as xylose, which is abundantly present in lignocellulosic hydrolysates. This paper describes the conversion of xylose in synthetic media into lactic acid by ten *R. oryzae* strains resulting in yields between 0.41 and 0.71 g g^{-1}. By-products were fungal biomass, xylitol, glycerol, ethanol and carbon dioxide. The growth of *R. oryzae* CBS 112.07 in media with initial xylose concentrations above 40 g l^{-1} showed inhibition of substrate consumption and lactic acid production rates. In case of mixed substrates, diauxic growth was observed where consumption of glucose and xylose occurred subsequently. Sugar consumption rate and lactic acid production rate were significantly higher during glucose consumption phase compared to xylose consumption phase. Available xylose (10.3 g l^{-1}) and glucose (19.2 g l^{-1}) present in a mild-temperature alkaline treated wheat straw hydrolysate was converted subsequently by *R. oryzae* with rates of 2.2 g glucose l^{-1} h^{-1} and 0.5 g xylose l^{-1} h^{-1}. This resulted mainly into the product lactic acid (6.8 g l^{-1}) and ethanol (5.7 g l^{-1}).

R. H. W. Maas · R. R. Bakker · G. Eggink · R. A. Weusthuis
Biobased Products, Agrotechnology and Food Sciences Group,
Wageningen University and Research Centre,
P.O. Box 17, 6700 AA Wageningen, The Netherlands

R. H. W. Maas (✉) · G. Eggink
Food and Bioprocess Engineering, Agrotechnology and Food Sciences Group, Wageningen University and Research Centre,
P.O. Box 8129, 6700 EV Wageningen, The Netherlands
e-mail: ronald.maas@wur.nl
Tel.: +31-0317-475023
Fax: +31-0317-475347

Introduction

The use of inexpensive and widely available lignocellulosic biomass materials such as wheat straw becomes more and more interesting as potential feedstock for the production of bulk chemicals. Deriving fermentable sugars from lignocellulosic biomass is a major R&D issue in the development of the 'lignocellulose-to-ethanol' production technology. Future prospects show an increasing demand of renewable sources for the production of high volumes of bulk chemicals. The pentose monosaccharide xylose, besides the hexose monosaccharide glucose, is one of the most abundant sugars found in nature. It is the predominant hemicellulosic sugar of hardwoods and agricultural residues, accounting for up to 25% of the dry weight biomass of some plant species (Ladisch et al. 1983). The abundance and ease of isolation of xylose makes it an important potential feedstock for the production of bulk chemicals such as lactic acid.

With a worldwide annually increasing production, lactic acid and its potential derivatives represent an important category of chemicals for industries producing food, chemicals and pharmaceutical products. Lactic acid can be manufactured in a racemic mixture by chemical synthesis and both in racemic mixtures and optically pure forms by microbial carbohydrate fermentation processes (Datta et al. 1995; Zhou et al. 1999). Highly purified, preferably L(+)-lactic acid anhydrous monomer is required for the production of the biodegradable polymer polylactic acid (PLA), which is an environmentally friendly replacement of plastics derived from petrochemical materials. Current commercial industrial processes for the biological conversion of glucose into almost optically pure lactic acid are carried out by homolactic lactobacilli with yields up to 80–90% (Longacre et al. 1997). Homolactic acid bacteria cannot efficiently ferment pentoses and require growth

medium with complex supplements, which adds to the costs of lactic acid production and complicates purification of lactic acid (Skory 2000; Tay and Yang 2002). The separation of the D(−) from the L(+) optical form is also difficult and contributes to the production costs of PLA (Longacre et al. 1997).

The filamentous fungus *Rhizopus oryzae* produces optically pure L(+)-lactic acid and requires a mineral medium composition with some inorganic minerals and ammonium salt as sole nitrogen source (Lockwood et al. 1936; Bai et al. 2003). *R. oryzae* produces mainly lactic acid from glucose with yields of 60–80% and also ethanol, carbon dioxide and minor amounts of malic acid, fumaric acid and citric acid (Longacre et al. 1997; Skory 2004). Product formation depends on cultivation conditions; it has been shown (Skory et al. 1998) that, under oxygen-limiting conditions, product formation shifts from lactic acid to ethanol.

A number of research groups reported the consumption of xylose by *R. oryzae* NRRL 395 for the production of lactic acid. Park et al. (2004) described the conversion of an enzymatic hydrolysate of waste office paper and artificial media with mixtures of glucose, xylose and cellobiose. The conversion rate of xylose as sole carbon source into lactic acid was 7.3 g l^{-1} d^{-1} and accounted for 28% of the conversion rate of glucose or cellobiose (Park et al. 2004). The conversion of xylose by *R. oryzae* NRRL 395 was also described by Yang et al. (1995) resulting in a lactic acid yield of 0.7 g g^{-1} and with glycerol and ethanol as by-products (Yang et al. 1995). Taherzadeh et al. (2003) reported the cultivation of *R. oryzae* in paper pulp spent liquor to achieve high biomass and ethanol yields. The consumption rate of hexoses was faster than that of pentoses in synthetic media (Taherzadeh et al. 2003).

In the present paper, we report on the conversion of xylose into lactic acid by ten different fungal strains of *R. oryzae*. A selected *R. oryzae* strain was used to convert sugars such as glucose and xylose present in a mild-temperature alkaline wheat straw hydrolysate. As lignocellulosic hydrolysates are complex mixtures with variable sugar concentration and composition, we first studied the effects of different initial xylose and glucose concentrations and mixtures on lactic acid production by *R. oryzae* in synthetic media.

Materials and methods

Fungal strains

Ten *R. oryzae* strains were used: *R. oryzae* CBS 147.22, CBS 128.08, NRRL 395, CBS 539.80, CBS 328.47, CBS 127.08, CBS 321.35, CBS 396.95, CBS 112.07 and CBS 264.28 (CBS—Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands and NRRL—Northern Regional Research Laboratory, Peoria, IL, USA). Strains were grown on potato dextrose agar (Oxoid) at 30°C. Spores were collected after approximately 7 days by washing the mycelium with a sterile solution of 10% (*v*/*v*) glycerol and 0.05% (v/v) Tween 80. The number of spores was determined microscopically using a cell counter (Neubauer, Germany). The stock spore suspension was stored at −80°C.

Inoculum preparation

Spores of ten different *R. oryzae* strains were initially used to inoculate fermentation medium with different carbon sources. We observed germination and growth (~3 to 5 g dry biomass l^{-1}) of the ten tested strains with glucose (120 g l^{-1}) and with a mixture of glucose and xylose (both 10 g l^{-1}). However, attempts to germinate these spores to obtain considerable amounts of mycelial biomass in fermentation medium with xylose (30 g l^{-1}) as sole carbon source failed with six strains. The spores of *R. oryzae* apparently germinate and form mycelial biomass more efficiently in the presence of glucose. It has also been observed by others that the percentage of spore germination of the related fungus *Rhizopus oligosporus* is often determined by the presence of suitable carbon sources (Medwid and Grant 1984). Because the spores of some *R. oryzae* strains were not able to germinate and grow on xylose, another approach was developed. Mycelial biomass was produced in growth medium with 30 g l^{-1} glucose to inoculate fermentation medium with glucose or xylose as sole carbon source. The composition of growth medium per litre was: glucose, 30 g; $(NH_4)_2SO_4$, 1.25 g; KH_2PO_4, 0.6 g; $MgSO_4{\cdot}7H_2O$, 0.25 g; $ZnSO_4{\cdot}7H_2O$, 0.04 g; $Na_2HPO_4{\cdot}2H_2O$, 5.37 g and $NaH_2PO_4{\cdot}H_2O$, 9.64 g. The chemicals, unless indicated otherwise, were purchased from Merck (Darmstadt, Germany). Growth medium was prepared by adding 25 ml of nutrient (4×) stock solution [$(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4{\cdot}7H_2O$ and $ZnSO_4{\cdot}7H_2O$], 50 ml of sodium phosphate (2×) stock solution ($Na_2HPO_4{\cdot}2H_2O$ and $NaH_2PO_4{\cdot}H_2O$) and 25 ml of glucose (4×) stock solution to 250-ml baffled flasks with metal caps which allow gas transfer. Glucose solution served as carbon source and was filter sterilised (cellulose acetate filter with pore size 0.2 μm, Minisart, Sartorius). Nutrient solution and sodium phosphate buffer were sterilised (20 min at 125°C) separately. Growth medium was inoculated with $2{\cdot}10^5$ spores per millilitre and incubated in a rotary shaker with an agitation rate of 150 rpm at 37°C (Innova 4230, Edison, NJ, USA).

The separation of the mycelial biomass from the growth medium (100 ml), was performed by a wash treatment procedure. The medium was removed by 3 min of centrifugation at 2,000 rpm (Centaur 2, Beun de Ronde, Abcoude, The Netherlands) and the fungal biomass pellet was washed with 40 ml of sterile solution of 25 mM sodium phosphate buffer (pH 6.5). The pellet was re-suspended carefully and the centrifugation step was repeated. This washing procedure was carried out twice.

Shake flask experiments

In this study, we used baffled shake flasks instead of controlled stirred fermentors because of the following two reasons. First, our experiments with *R. oryzae* in a submerged controlled stirred fermentor often resulted in heterogeneous growth with mycelium attached around elements in the reactor such as baffles, electrodes and impellers (data not shown). Others also observed that fungal strain, nutrients, substrate, pH, aeration, agitation and concentration of inoculum can influence the growth form of *R. oryzae*. Pellet or clump growth caused sub-optimal growth conditions by a decrease in gas, and mass transfer resulted in long fermentation periods, cell death and low lactic acid yields due to by-product formation such as that of ethanol (Bai et al. 2003). We used baffled shake flasks to achieve reproducible and comparable growth forms of *R. oryzae*. Secondly, an advantage of using a controlled stirred fermentor is that the percentage of oxygen and carbon dioxide of in- and outlet gas can be analysed to determine the respiration rate by *R. oryzae*. Yet, our results showed that due to low gas transfer, probably caused by high viscosity of the fermentation broth, the differences in composition between gasses entering and leaving the fermentor were so small that oxygen consumption and carbon dioxide production could not be determined accurately (data not shown). In shake flask cultures, we used the carbon recovery calculations to estimate the carbon dioxide produced by respiration.

The composition of the fermentation medium used in the shake flask cultures was similar to the growth medium except that sodium phosphate salts were replaced by 52 g l^{-1} $CaCO_3$ (SA Omya, Benelux NV). Calcium carbonate neutralises the lactic acid and regulates the pH between 6.0 and 6.5 but is impure and contains several trace elements. Sugar solution with xylose (Sigma-Aldrich, Germany) or glucose served as carbon source and was filter sterilised (cellulose acetate filter with pore size 0.2 μm, Minisart, Sartorius). The mycelial biomass obtained after the wash treatment procedure of growth medium served as inoculum for fermentation medium [0.1 g mycelial biomass (dry weight) per litre]. The cultures were incubated in a rotary shaker with an agitation rate of 150 rpm at 37°C (Innova 4230, Edison, NJ, USA).

Lignocellulosic hydrolysate

Wheat straw was purchased from a commercial farm located in the northeast of the Netherlands and was used for the manufacturing of a hydrolysate. The wheat straw was mechanically treated by extrusion (rotation of 100 rpm and throughput of 50 kg hr^{-1}) and chemically treated by soaking with 5% calcium hydroxide (pH 9) for 16 h at 75°C. The liquid in pre-treated wheat straw was substituted by fresh water to regain alkali and to reduce the concentration of fermentation inhibitors such as acetic acid, which was reduced to 1 g l^{-1}. This pre-treated wheat straw (dry matter 110 g l^{-1}) was enzymatically hydrolysed with the enzyme cocktails Cellubrix and Novozymes 188 (Novozymes, Denmark) for 24 h at 50°C and pH 4.8. The non-hydrolysed solid particles were removed from the hydrolysate by 3 min of centrifugation at 3,000 rpm (Centaur 2, Beun de Ronde, Abcoude, The Netherlands). The composition of the fermentation medium was 100 ml supernatant of hydrolysate with (per litre): $CaCO_3$, 52 g; $(NH_4)_2SO_4$, 1.25 g; KH_2PO_4, 0.6 g; $MgSO_4 \cdot 7H_2O$, 0.25 g and $ZnSO_4 \cdot 7H_2O$, 0.04 g. Cultivation was performed in 250-ml baffled flasks and the medium was inoculated with $2 \cdot 10^5$ spores per millilitre medium.

Analytical methods

Samples of fermentation medium were heated for 10 min at 70°C to dissolve precipitated calcium lactate. Immediately after heating, the samples were centrifuged for 3 min at 17,400×*g* (Eppendorf 5417C). The supernatant was diluted (1:1, *v*/*v*) with 1 M sulphuric acid. The samples were filtered to remove solids and precipitated proteins (Spartan filter 13, pore size of 0.2 μm, Schleicher & Schuell). Substrates and products were analysed by high-pressure liquid chromatography using an Altech IOA-1000 Organic Acids column with 3 mM sulphuric acid as the mobile phase at 90°C and a flow rate of 0.4 ml min^{-1}. Monosaccharides, polyols and organic acids were detected by a refractive index detector (Waters 2414).

To measure cell dry weight (CDW), residual $CaCO_3$ was removed from biomass by washing approximately 90 ml of fermentation medium with 2 M hydrochloric acid and, subsequently, with distilled water. Broth was filtered on a pre-weighed Whatman filter paper and washed biomass was dried overnight at 50°C before weight analysis. The $CaCO_3$ contained 1.5% (*w*/*w*) insoluble impurities which remained on the filter. The dry weight of the biomass was corrected for this insoluble matter. Because of heterogeneous growth of mycelium and low concentration of biomass, cell dry weight analyses were only performed at the end of the fermentation.

Calculations

The yield of lactic acid ($Y_{p/s}$) is expressed as amount of product (g) synthesised divided by the amount of substrate (g) consumed. Biomass yield ($Y_{x/s}$) is calculated by the amount of biomass (g dry weight) divided by the amount of substrate (g) consumed. Carbon recovery is calculated with respect to moles of carbon produced divided by moles of carbon consumed. Carbon dioxide production could not be measured accurately (see above) and could, therefore, not be included in the carbon recovery calculations. To estimate the amount of carbon dioxide formed, we assumed that all missing carbon in the carbon recovery calculations is in the form of carbon dioxide. The amount of carbon dioxide synthesised in ethanol production can be estimated easily as it is produced in equimolar amounts with ethanol. The amount of carbon dioxide produced in respiration can

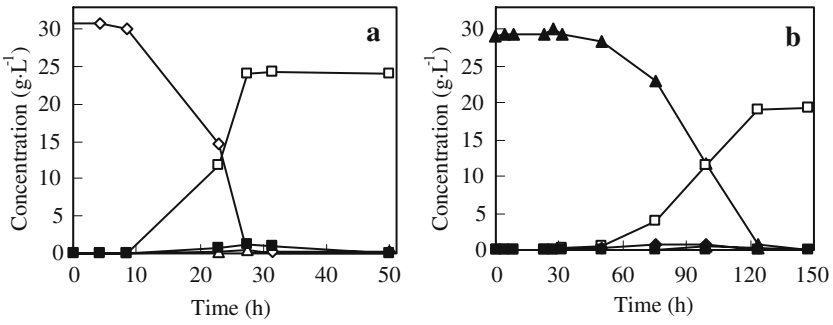

Fig. 1 Typical conversion of **a** glucose (◇) and **b** xylose (▲) into L(+)-lactic acid (□) by *R. oryzae* CBS 127.08. The by-products were xylitol (♦), glycerol (△) and ethanol (■)

then be calculated as the total amount of carbon dioxide formed minus the amount of ethanol produced. For the calculation of the carbon recovery, fungal biomass composition of $CH_{1.72}O_{0.55}N_{0.17}$ with an ash content of 7.5% (*w*/*w*) was used (Carlsen and Nielsen 2001).

Results

Conversion of xylose and glucose by *R. oryzae*

Xylose conversion by ten *R. oryzae* strains was studied in synthetic media in shake flask cultures. All tested strains showed similar fermentation profiles but with different lactic acid concentrations and yields. Figure 1 demonstrates a typical conversion of glucose (a) and xylose (b) by *R. oryzae* CBS 127.08. Table 1 represents an overview of fermentation characteristics of the conversion of glucose or xylose by ten different *R. oryzae* strains. The consumption of xylose started after approximately 30 h of incubation and it was nearly depleted after 120 h. The tested *R. oryzae* strains convert 30 g l^{-1} xylose into mainly lactic acid (10.4 to 21.1 g l^{-1}). By-products such as xylitol (0.3 to 3.8 g l^{-1}) and glycerol (0.2 to 1.2 g l^{-1}) were produced in all cultures and, in some cases, minor amounts of ethanol were formed (0 to 0.6 g l^{-1}). After the depletion of xylose, *R. oryzae* started to utilise xylitol.

With 30 g l^{-1} glucose as the sole carbon source, consumption by *R. oryzae* started after approximately 8 h. Glucose was depleted after 27 h of cultivation and lactic acid was the main product (13.3 to 24.1 g l^{-1}). The by-products were glycerol (0.3 to 0.8 g l^{-1}) and ethanol (0.7 to 2.5 g l^{-1}).

The tested *R. oryzae* strains showed lactic acid yields ($Y_{p/s}$) on glucose from 0.42 to 0.79 g g^{-1} which corresponds to, respectively, 42 and 79% of the theoretical yield. The conversion of xylose resulted in lactic acid yields ranging from 0.41 to 0.71 g g^{-1} and accounted for 41 to 71% of the theoretical yield obtained with glucose (Table 1). In most cases, the biomass yield ($Y_{x/s}$) of *R. oryzae* is higher when cultivated on xylose compared with glucose. The calculations of carbon recovery from cultures grown on xylose were between 56 and 82% in contrast to calculations of cultures with glucose which recover between 74 and 99% of the total carbon used. This result indicates that carbon dioxide produced by respiration, which accounts for the missing carbon, was higher when *R. oryzae* was cultivated with xylose compared to with glucose.

R. oryzae strain CBS 112.07 was selected for the next experiments based on high production of biomass with glucose and xylose as carbon source and high lactic acid yield of 0.85 g g^{-1} with 120 g l^{-1} glucose (data not shown).

Table 1 Fermentation characteristics of ten *R. oryzae* strains with an initial xylose concentration of 30 g l^{-1} or glucose concentration of 30 g l^{-1} as the sole carbon source

R. oryzae strain	Fermentation time (h)		$Y_{p/s}$ (g lactic acid g^{-1})		$Y_{x/s}$ (g CDW g^{-1})		Carbon recovery (% C-mol)	
	Xyl	Gluc	Xyl	Gluc	Xyl	Gluc	Xyl	Gluc
CBS 147.22	124	27	0.71	0.74	0.05	0.06	79	91
CBS 128.08	124	27	0.42	0.69	0.10	0.05	56	89
NRRL 395	124	23	0.61	0.42	0.18	0.13	82	74
CBS 539.80	124	27	0.51	0.74	0.09	0.03	61	90
CBS 328.47	124	27	0.44	0.71	0.09	0.12	79	96
CBS 127.08	124	27	0.67	0.79	0.11	0.03	81	91
CBS 321.35	100	27	0.60	0.73	0.12	0.10	75	90
CBS 396.95	100	27	0.57	0.73	0.12	0.18	71	99
CBS 112.07	124	23	0.41	0.61	0.14	0.15	65	86
CBS 264.28	75	27	0.65	0.73	0.12	0.08	80	86

After the indicated fermentation time, a percentage of 95–100% of xylose and 99–100% of glucose was consumed. The initial biomass concentration was 0.1 g l^{-1}

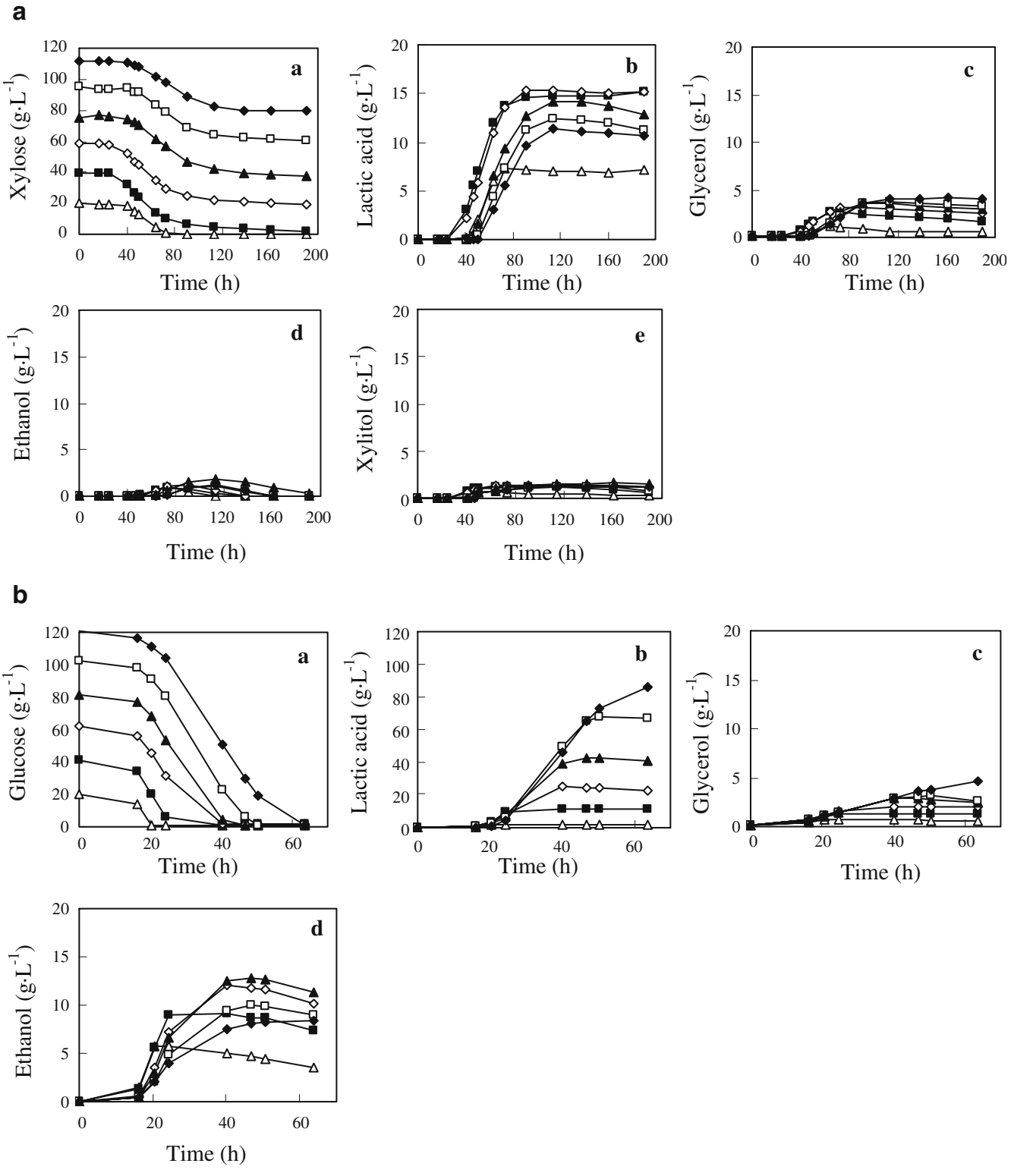

Fig. 2 **a** Consumption of (*a*) xylose and formation of (*b*) lactic acid, (*c*) glycerol, (*d*) ethanol and (*e*) xylitol by *R. oryzae* CBS 112.07. Initial concentrations of xylose were 20 (△), 39 (■), 58 (◇), 76 (▲), 95 (□) and 111 g l^{-1} (♦). **b** Consumption of (*a*) glucose and formation of (*b*) lactic acid, (*c*) glycerol and (*d*) ethanol by *R. oryzae* CBS 112.07. The initial concentrations of glucose were 20 (△), 41 (■), 62 (◇), 82 (▲), 102 (□) and 121 g l^{-1} (♦)

Effect of xylose concentration on lactic acid production by *R. oryzae*

The xylose concentrations in lignocellulosic hydrolysates can vary depending on the origin of the raw material, the efficiency of mechanical and chemical treatment and enzymatic hydrolysis. To study the effect of xylose concentration on lactic acid production, *R. oryzae* CBS 112.07 was cultivated in fermentation medium with different initial concentrations of xylose in a range of 20 to 111 g l^{-1} (Fig. 2a). The consumption of xylose started after approximately 25 to 40 h. Increasing initial xylose concentrations (20 to 111 g l^{-1}) resulted in longer lag phases and lower consumption rates. After 90 h of incubation, xylose in the culture with an initial concentration of 20 g l^{-1} was completely depleted. The other *R. oryzae* cultures with initial xylose concentrations above 40 g l^{-1} showed consumption of approximately 40 g l^{-1} xylose. After this amount of xylose was consumed, the substrate consumption rate decreased approximately tenfold. Although sufficient substrate was available and slowly consumed, neither lactic acid nor any other fermentation product was produced (Fig. 2a). This suggests that *R. oryzae* switched to respiratory metabolism and converted the available xylose slowly to carbon dioxide. To determine if this phenomenon is specific for xylose, similar experiments were performed with glucose as substrate. To exclude an osmolarity effect in the comparison between xylose and glucose, the same molar concentrations were used for both sugars, resulting in somewhat higher amounts of glucose (g l^{-1}) compared with xylose. The profile of xylose uptake and product formation was not observed when *R. oryzae* CBS 112.07 was cultivated on different initial concentrations of glucose. Available glucose (20 to 121 g l^{-1}) was consumed completely starting after 16 h of incubation and lactic acid production rates were significantly higher compared with fermentation on xylose (Fig. 2b).

Independent of the initial xylose concentration (20, 39, 59, 76, 95 or 111 g l^{-1}), *R. oryzae* CBS 112.07 consumed a maximum of approximately 40 g l^{-1} xylose and produced 11 to 15 g l^{-1} lactic acid which resulted in yields ($Y_{p/s}$) around 0.4 g g^{-1} (Table 2). On the other hand, *R. oryzae* consumed glucose concentrations of 20, 41, 62, 82, 102 and 121 g l^{-1} and produced 2, 12, 25, 43, 67 and 86 g l^{-1} lactic acid, respectively, which resulted in lactic acid yields of 0.09 to 0.72 g g^{-1} (Table 2). The by-products of fermentation with xylose were xylitol, glycerol and ethanol and with glucose as substrate, glycerol and ethanol (Fig. 2a, b). At the end of the fermentation, the biomass concentrations of cultures with different initial xylose concentrations were between 3.9 and 6.9 g l^{-1}, whereas the cultures with different glucose concentrations ranged between 2.3 and 4.4 g l^{-1}. This resulted in biomass yields ($Y_{x/s}$) of 0.12 to 0.23 g g^{-1} xylose and of 0.04 to 0.12 g g^{-1} glucose. Again the carbon recovery of cultures with xylose was between 63 and 85% and with glucose between 82 and 94% (Table 2), indicating that respiration was higher when *R. oryzae* was cultivated with xylose compared to with glucose.

Table 2 Fermentation characteristics of different initial concentrations of xylose and glucose by *R. oryzae* CBS 112.07

Initial concentration (g l^{-1})	Produced lactic acid (g l^{-1})	$Y_{p/s}$ (g lactic acid g^{-1})	Produced cell dry weight (g l^{-1})	$Y_{x/s}$ (g CDW g^{-1})	Carbon recovery (% C-mol)
Xylose					
19.8	7.2	0.36	4.4	0.23	78
39.3	15.2	0.40	4.4	0.11	63
58.5	15.2	0.38	6.9	0.18	73
75.6	14.1	0.39	4.3	0.11	75
95.1	12.4	0.40	6.4	0.18	85
111.3	11.3	0.39	3.9	0.12	81
Glucose					
20.4	1.7	0.09	2.3	0.12	82
40.8	11.7	0.30	2.7	0.07	83
61.9	25.0	0.41	2.5	0.04	88
81.9	42.5	0.53	2.9	0.04	91
102.3	68.2	0.67	3.9	0.04	94
121.0	85.9	0.72	4.4	0.04	93

Conversion of glucose/xylose mixtures in synthetic media by *R. oryzae*

Lignocellulosic hydrolysates often contain mixtures of hexose and pentose sugars. To study the effect of sugar mixtures on lactic acid production by the ten different strains of *R. oryzae*, a synthetic medium with glucose (10 g l^{-1}) and xylose (10 g l^{-1}) was used. All tested cultures showed consumption profiles where glucose first depleted followed by xylose (data not shown). *R. oryzae* CBS 147.22, CBS 127.08 and CBS 264.28 produced mainly ethanol whereas the other strains produced mainly lactic acid. Strains NRRL 395 and CBS 396.95 produced ethanol during glucose consumption and lactic acid during xylose consumption.

Another experiment where *R. oryzae* CBS 112.07, pregrown on xylose, was cultivated in fermentation medium with a mixture of glucose and xylose (both 30 g l^{-1}) showed that glucose was first completely utilised followed by consumption of xylose (Fig. 3a). The consumption of glucose began after 11 h of incubation and after 23 h the glucose was depleted and consumption of xylose started. The rate of substrate consumption was significantly higher during glucose consumption (1.8 g l^{-1} h^{-1}) compared with xylose consumption (0.6 g l^{-1} h^{-1}) which is similar to results obtained by cultivation on individual carbon sources. The main fermentation products were lactic acid (33.4 g l^{-1}), glycerol (1.9 g l^{-1}) and ethanol (2.1 g l^{-1}) and, during xylose conversion, xylitol (0.5 g l^{-1}) was produced. Lactic acid production rate during glucose consumption was 0.7 g l^{-1} h^{-1} and during xylose consumption 0.4 g l^{-1} h^{-1}. In case of cultivation in fermentation medium with 100 g l^{-1}

glucose and 30 g l^{-1} xylose (Fig. 3b), a similar profile was observed where glucose was first consumed (3.2 g l^{-1} h^{-1}) followed by xylose (0.3 g l^{-1} h^{-1}). Due to the higher concentration of glucose, start of xylose consumption shifted to 35 h of incubation. This indicates that the consumption of xylose by *R. oryzae* is repressed by glucose and is called bi-phasic or diauxic growth.

Conversion of a lignocellulosic hydrolysate

Wheat straw was pre-treated by mild-temperature alkaline treatment followed by an enzymatic hydrolysis which resulted in a hydrolysate that contained a mixture of different monosaccharides released from (hemi)-cellulose. Glucose (19.2 g l^{-1}) and xylose (10.3 g l^{-1}) were the main monosaccharides present in the hydrolysate. *R. oryzae* CBS 112.07 was cultivated in the hydrolysate and substrate consumption and product formation were followed. Glucose consumption started after 13 h of incubation and xylose after 24 h. As with the glucose/xylose mixtures in synthetic media, glucose was first completely utilised followed by depletion of xylose (Fig. 4a). In the lignocellulosic hydrolysate, glucose (2.2 g l^{-1} h^{-1}) was also utilised faster than xylose (0.5 g l^{-1} h^{-1}). Lactic acid (3.7 g l^{-1}) and ethanol (5.7 g l^{-1}) were the main fermentation products during glucose consumption and lactic acid (3.1 g l^{-1}) and xylitol (1.5 g l^{-1}) during xylose consumption (Fig. 4b). Lactic acid yield was 0.23 g g^{-1} total sugar, whereas the ethanol yield was 0.19 g g^{-1} total sugar.

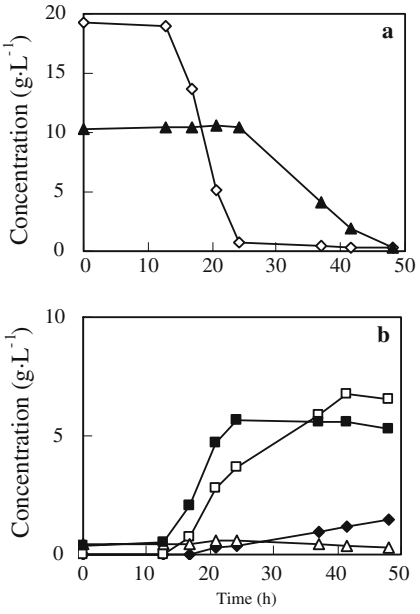

Fig. 4 Fermentation of mild-temperature-alkaline-treated wheat straw hydrolysate by *R. oryzae* CBS 112.07 **a** Substrate consumption of glucose (◇) and xylose (▲). **b** Product formation of lactic acid (□), xylitol (♦), glycerol (△) and ethanol (■)

Discussion

Lignocellulosic materials are cheap and widely available and, therefore, potentially interesting feedstocks for the production of organic acids such as lactic acid (Tsao et al. 1999). Both hemicellulose and cellulose can be hydrolysed into fermentable monosaccharides such as glucose and xylose. The biodegradable polymer PLA can be commercially competitive to petrochemical-derived plastics if both hexose and pentose sugars will be converted into lactic acid. Others described that commercial bioconversion of lignocellulose to ethanol also requires efficient fermentation of sugar mixtures including xylose (Hinman et al. 1989). Without the conversion of xylose, the product yields are low. Because lignocellulosic hydrolysates are complex media with sugar mixtures, it is necessary to study first the conversion of sole carbon sources in synthetic media by *R. oryzae*.

The results obtained from this study showed that ten tested *R. oryzae* strains converted xylose and glucose mainly into lactic acid with yields which agree very well with data reported by other researchers (Yang et al. 1995; Kosakai et al. 1997; Zhou et al. 1999; Park et al. 2004). Carbon recovery calculations showed differences between *R. oryzae* cultures with glucose (74–99%) and xylose (56–82%). These results suggest that, during xylose metabolism, relatively more substrate is respired into carbon dioxide, which has two possible explanations. First, a longer fermentation time enabled *R. oryzae* to take oxygen over a longer period resulting in a relative higher contribution of respiration to cellular metabolism. Secondly, others described xylose-fermenting yeasts such as *Pichia stipitis*, *Candida shehatae* and *Pachysolen tannophilus* which follow the oxido-reductive route via xylitol in the conversion of xylose to xylulose (Hahn-Hägerdal et al. 1994). In these xylose-converting cultures, respiration is required to balance the cofactors NADH and NAD^+ playing a role in the xylitol into xylulose conversion.

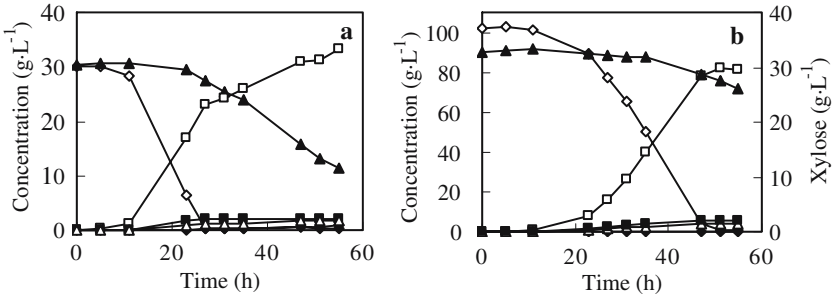

Fig. 3 Conversion of sugar mixtures with **a** 30 g l^{-1} glucose and 30 g l^{-1} xylose and **b** with 100 g l^{-1} glucose and 30 g l^{-1} xylose by *R. oryzae* CBS 112.07. Substrate consumption of glucose (◇) and xylose (▲) and product formation of lactic acid (□), xylitol (♦), glycerol (△) and ethanol (■)

More respiration results in higher energy generation accompanied by higher amount of biomass. More respiration and production of the intermediate xylitol also indicates that *R. oryzae* converts xylose through the oxido-reductive route but this requires further research.

To achieve an efficient process with lignocellulosic hydrolysate as feedstock, variable sugar concentrations should be completely converted into products such as lactic acid. Therefore, the capability of *R. oryzae* CBS 112.07 to convert different initial xylose and glucose concentrations in synthetic media was tested. *R. oryzae* stopped converting xylose into lactic acid after approximately 40 g l^{-1} xylose was converted while carbon source was still in excess. This was not the case with different glucose concentrations where all glucose was converted into lactic acid. Moreover, at the end of the fermentation, biomass concentrations obtained with xylose were higher than biomass concentrations with glucose as substrate. This suggests that a nutrient, other than carbon source, was limiting the conversion of xylose. To convert higher concentrations of xylose, medium optimisation is required and may contribute to higher productivity and lactic acid yield.

Lignocellulosic hydrolysates were also simulated by mixing xylose and glucose in synthetic media. Subsequent or simultaneous utilisation will influence the lactic acid productivity and, consequently, the total fermentation time required in converting sugars. When the ten *R. oryzae* strains were exposed to a mixture of glucose and xylose, diauxic growth was observed with consumption of glucose first followed by xylose consumption. As shown in Fig. 3a, b, the start of xylose consumption is determined by the presence of glucose. Therefore, we can conclude that biphasic growth occurs due to repression of xylose metabolism by glucose.

In mild-temperature-alkaline pre-treated wheat straw hydrolysate, glucose and xylose were converted subsequently with rates of, respectively, 2.2 and 0.5 g l^{-1} h^{-1} resulting into the products lactic acid and ethanol. These consumption rates agreed very well with results achieved in synthetic media, indicating that the level of inhibitors such as acetic acid (1 g l^{-1}) present in the wheat straw hydrolysate was not inhibiting the sugar consumption rates by *R. oryzae*. Due to the formation of high-concentration by-products, a relatively low lactic acid yield (0.23 g g^{-1}) was obtained. The production of the by-product ethanol by *R. oryzae* is probably due to limited oxygen transfer in the viscous hydrolysate (Skory et al. 1998). Usage of a controlled tank reactor such as an airlift fermentor can enhance gas and nutrient transfer and improve the lactic acid yield.

From the results obtained in this study, we can conclude that the filamentous fungus *R. oryzae* is an excellent microbial producer of L(+)-lactic acid from glucose. Furthermore, *R. oryzae* also converts xylose into mainly lactic acid but with a lower rate and lower yield. To produce lactic acid by *R. oryzae* from wheat straw with conversion of glucose and xylose in an economically feasible process, the consumption rate and yield of xylose must improve. The effect of higher xylose concentrations on lactic acid production and the influence of oxygen on the xylose conversion by *R. oryzae* will be further studied.

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