*bioresources.com*

# Characteristics and Kinetics of the Aldonic Acids Production using Whole-cell catalysis of *Gluconobacter oxydans*

Xin Zhou,[a,b] Xing Wang,[a] Rou Cao,[a] Yuheng Tao,[a] Yong Xu,[a,b]* and Shiyuan Yu [a,b]

The characteristics and kinetics of whole-cell catalysis were investigated individually or simultaneously relative to the bio-oxidation of five monosaccharides to corresponding aldonic acids using *Gluconobacter oxydans*. For individual catalysis, 30 g/L glucose could be consumed completely at 3.21 g/L/h in 8 h, and arabinose was the slowest-consumed at 1.18 g/L/h in 8 h. Among five monosaccharides, the yield of xylonic acid was highest at 90.4%, and about 31.0 g/L xylonic acid could be obtained in 24 h. For simultaneous catalysis, a complex substrate competition appeared in the mixed-aldoses solution. The utilization rate of arabinose, galactose, xylose and mannose were repressed distinctly. And glucose exhibited variable inhibitory effects on the remaining four monosaccharides utilization. Higher concentration of mixed aldoses showed a tendency to minimize the difference of glucose inhibition on arabinose, galactose, xylose, and mannose conversion to corresponding acids. Thus, the total concentration and proportion between various aldoses should be properly controlled for a highly efficient production of aldonic acids from lignocellulosic material.

*Contact information: a: College of Chemical Engineering, Nanjing Forestry University, Nanjing, Jiangsu, China; b: Jiangsu Key Laboratory of Biomass-based Green Fuels and Chemicals, Nanjing Forestry University, Nanjing, Jiangsu, China; * Corresponding author: xuyong@njfu.edu.cn*

## INTRODUCTION

Aldonic acids belong to a class of polyhydroxyl acids that are currently generating considerable interest and have significant economic potential as chelators, buffers, and preservatives, and they may also serve as platform reagents for the synthesis of other useful chemicals (Sauer *et al*. 2008; Bozell and Petersen 2010). Aldonic acids are usually derived from oxidization of five common aldoses, including xylose, glucose, mannose, arabinose, and galactose (Pezzotti and Therisod 2006). Thereinto, gluconic acid or gluconate, which is also well known as aldonic acid, is now produced annually at up to 87,000 tons worldwide (Sauer *et al*. 2008). Also, xylonic acid has recently been shown to be a valuable precursor for an industrial synthesis of 1,2,4-butanetriol through microbial fermentation, in addition to its application as a concrete admixture (Niu *et al*. 2003; Chun *et al*. 2006). Arabonic acid, galactonic acid, and mannonic acid have not yet been commercialized because of the issue of environmentally friendly bioconversion technology.

Aldonic acids and other polyhydroxy carboxylic acids are known to be produced by electrochemical or chemical oxidation (Whitfield *et al.* 1993; Lederkremer and Marino 2003). Even so, this method is inaccessible for the commercial production

because of their pollution effects and processing cost (Warwick *et al*. 2004). Up to now, production of aldonic acid with microorganisms or purified enzymes has been reported (Ramachandran *et al*. 2006; Pezzotti and Therisod 2006). There are many kinds of microorganisms that can be used for bio-oxidation of aldoses. It is well known that the stain of *Gluconobacter oxydans* (*G. oxydans*), which belongs to the group of acetic acid bacteria, can catalyze alkane hydroxyl group and aldehyde group to carboxyl acid, by using the dehydrogenase bound to the cytoplasmic membrane, and the membrane-bound dehydrogenases are coupled to the respiratory chain and therefore play an important role in energy generation. In *G. oxydans*, there are various dehydrogenases, such as quinoprotein glucose dehydrogenase, quinoprotein gluconate-5-dehydrogenase which catalyzes the oxidation of D-gluconate and various alcohols, and quinohemoprotein alcohol dehydrogenase. *G. oxydans* also has a cytosolic-oxidizing enzyme system made up of NAD(P)-dependent dehydrogenases, which make only a minor contribution to overall oxidation (Gupta *et al.* 2001; Deppenmeier *et al.* 2002; Hölscher *et al.* 2009).

Furthermore, *G. oxydans* exhibits an extremely high tolerance towards lignocellulosic inhibitors, which is crucial for the development of a commercial fermentation (Buchert *et al.* 1988; Buchert and Niemelä 1991). However, little is known about the whole-cell catalysis of the five plant monosaccharides considered in the present study to the corresponding aldonic acids, especially by using the cells of *G. oxydans*. So, catalytic performance of the *G. oxydans* was tested in the shaking flasks, and the reaction characteristics and kinetics were compared between simultaneous or individual catalysis.

Currently, the concept of a biorefinery is receiving much attention as a way to obtain more value from lignocellulosic material (Gallezot 2012; Ragauskas *et al.* 2006). The main polysaccharide components cellulose and hemicellulose can be liberated to the five monosaccharides considered in the present work by pretreatment and enzymatic hydrolysis for the subsequent fermentation to biofuels or bio-based chemicals with microorganisms (Palmqvist and Hahn-Hägerdal 2000; Menon and Rao 2012). Therefore, the production of aldonic acid chemicals by using the lignocellulose as a cost-competitive raw material is promising for biomass biorefinery. Many barriers would appear when pre-hydrolysates that contain inhibitors, unknown colloidal matter, and various aldoses was used as the starting material. Thus, the objective of this paper was to evaluate the competitive inhibition of various aldoses. This paper aims to provide a reference for the co-production of aldonic acids.

## EXPERIMENTAL

### Microorganism

*Gluconobacter oxydans* NL71, domesticated from the strain of ATCC 621, was maintained on the sorbitol-agar colony (sorbitol 50 g/L, yeast extract 5 g/L, agar 15 g/L) at 4 °C.

### Media and Culture Conditions

The inocula of *G. oxydans* NL71 were prepared in 250-mL Erlenmeyer shaken flasks containing 50 mL medium (sorbitol 100 g/L, yeast extract 10 g/L) and cultured for 24-36 h at 220 rpm and 30 °C.

The whole-cell catalysis was carried out in in 250-mL Erlenmeyer shaken flasks containing 50-mL medium, and cultured for 20 to 72 h at 220 rpm and 30 °C. The whole-

cell catalytic medium (g/L) was as follows: yeast extract 5.0, $MgSO_4$ 0.5, $KH_2PO_4$ 1.0, $K_2HPO_4$ 2.0, $(NH_4)_2SO_4$ 5.0. The cell density (dry weight) used in the catalytic medium was 2 g/L. The pH could be controlled at 4.0 to 5.0 by adding $CaCO_3$ powder (Buchert *et al.* 1988). Concentrations of added carbon source (aldose or mixed aldoses) are indicated in the text. The experiments were done in duplicate.

The yield (%) was calculated from the end concentration of aldonic acid (g/L) divided by the initial concentration of the corresponding aldose loading (g/L), and multiplied by the constant of 0.904 for xylonic acid (XylA) and arabonic acid (AraA) and 0.918 for Gluconic acid (GlcA), galactonic acid (GalA), and mannonic acid (ManA) (The constant was the ratio of molecular weight of aldonic acid and aldose). The aldose utilization (%) was calculated from the concentration of consumed aldose divided by the initial concentration of the corresponding aldose loading. The utilization rate (g/L/h) of aldose was calculated from the concentration of consumed aldose divided by the reaction time. The productivity (g/L/h) of aldonic acid was calculated from the concentration of aldonic acid divided by the reaction time.

## Analytical Methods

Aldoses (arabinose (Ara), galactose (Gal), Glucose (Glc), xylose (Xyl), mannose (Man)), and aldonic acids (XylA, GlcA, AraA, GalA, ManA) were determined on a high performance anion-exchange chromatography system (HPAEC-PAD) (Dionex ICS-3000) linked to a CarboPac$^{TM}$ PA 10 column and a pulsed amperometric detector, with NaOH as eluent at a flow rate of 0.25 mL/min and the column temperature was maintained at 30 °C in a cooler/heater. The mobile phase was composed of eluent A(water) and B (100 mM NaOH) according to the following time and composition program expressed as percent (vol/vol): $t$ = 0 to 20 min, A = 94% and B = 6%; t = 20.1-65 min, A = 30% and B = 70%; t = 65.1-80 min, A = 94% and B = 6% (Wang *et al*. 2014).

Two parallel assays were performed for each experiment.

## RESULTS AND DISCUSSION
## Characteristics and Kinetics of Individual Catalysis

*G. oxydans* presented a remarkable ability to take up each monosaccharide and catalyze oxidize them individually to the corresponding aldonic acids under aeration culture in spite of their variable concentration of aldonic acids (Fig. 1). The dehydrogenases components that are tightly bound to the bacterial membrane and linked to the cytochrome systems have been shown to control these oxidative reactions under the driving force of oxygen supply (Deppenmeier *et al*. 2002; Gupta *et al*. 2001). In the process of individual catalysis, the highest accumulation of AraA, XylA, GalA, GlcA, and ManA was about 25 g/L, 30 g/L, 29 g/L, 21 g/L, and 17 g/L, respectively. The yields which corresponded to accumulation were 75.3%, 90.4%, 88.7%, 64.3%, and 52.0%, respectively. As shown in Table. 1, Glc was evidently used more rapidly than the other aldoses. In 8 h, 30 g/L Glc could be consumed completely, and the utilization rate of Glc was 2 to 3 times than the other aldoses. For all of the aldoses, the utilization rates were higher than productivity. For instance, in 4 h, the productivity of XylA was only 1.41 g/L/h, which accounts for about 80.6% of the Xyl utilization rate. Namely, the yield of aldonic acid was not matched with the utilization. And it could be found that the formed aldonic acid products tended to decline or even disappear as soon as the corresponding aldose was used up, especially in the case of GlcA.

**Table. 1.** Average Utilization Rate and Productivity of Aldoses and Aldonic Acids in Different Time Periods

| Time (h) | Utilization Rate (g/L/h) | | | | | Productivity (g/L/h) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Glc | Man | Gal | Xyl | Ara | GlcA | ManA | GalA | XylA | AraA |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | 3.68 | 2.67 | 2.21 | 1.75 | 1.28 | 3.14 | 1.98 | 1.24 | 1.41 | 0.78 |
| 8 | 3.21 | 2.25 | 2.17 | 2.41 | 1.18 | 2.54 | 1.67 | 2.08 | 2.24 | 0.94 |
| 16 | - | 1.88 | 1.74 | 1.83 | 1.26 | - | 1.08 | 1.58 | 1.80 | 0.94 |
| 24 | - | - | 1.37 | 1.29 | 1.12 | - | 0.50 | 1.29 | 1.29 | 0.82 |

-, the value of substance was zero and the utilization rate or productivity can't be calculated.
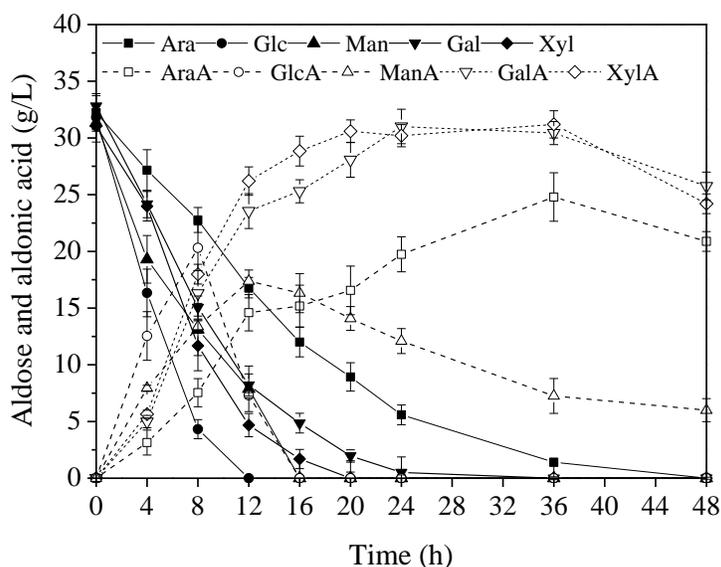


**Fig. 1.** Changes in concentrations of five aldoses and their corresponding aldonic acids during individually catalytic reactions

A hexonate such as GlcA could be further oxidized to ketogluconic acid, or be converted to 6-phospho-gluconate, which could be taken into pentose phosphate pathway (Weenk *et al.* 1984). A pentonate such as XylA could be transported though the cytoplasmic membrane for further metabolism by Dahms pathway (Dahms 1974). Thus, the reason for the decline is presumably due to the bacterial utilization of these aldonic acids for cell growth, metabolism or provide the $H^+$ for the formation of energy when aldose has been consumed completely.

## Characteristics and Kinetics of Simultaneous catalysis

Lignocellulosic pre-hydrolysate or enzymatic hydrolysate contains various aldoses; therefore, the competition and repression between the different aldoses are ineluctable in the reaction system of microbial cell catalysis.
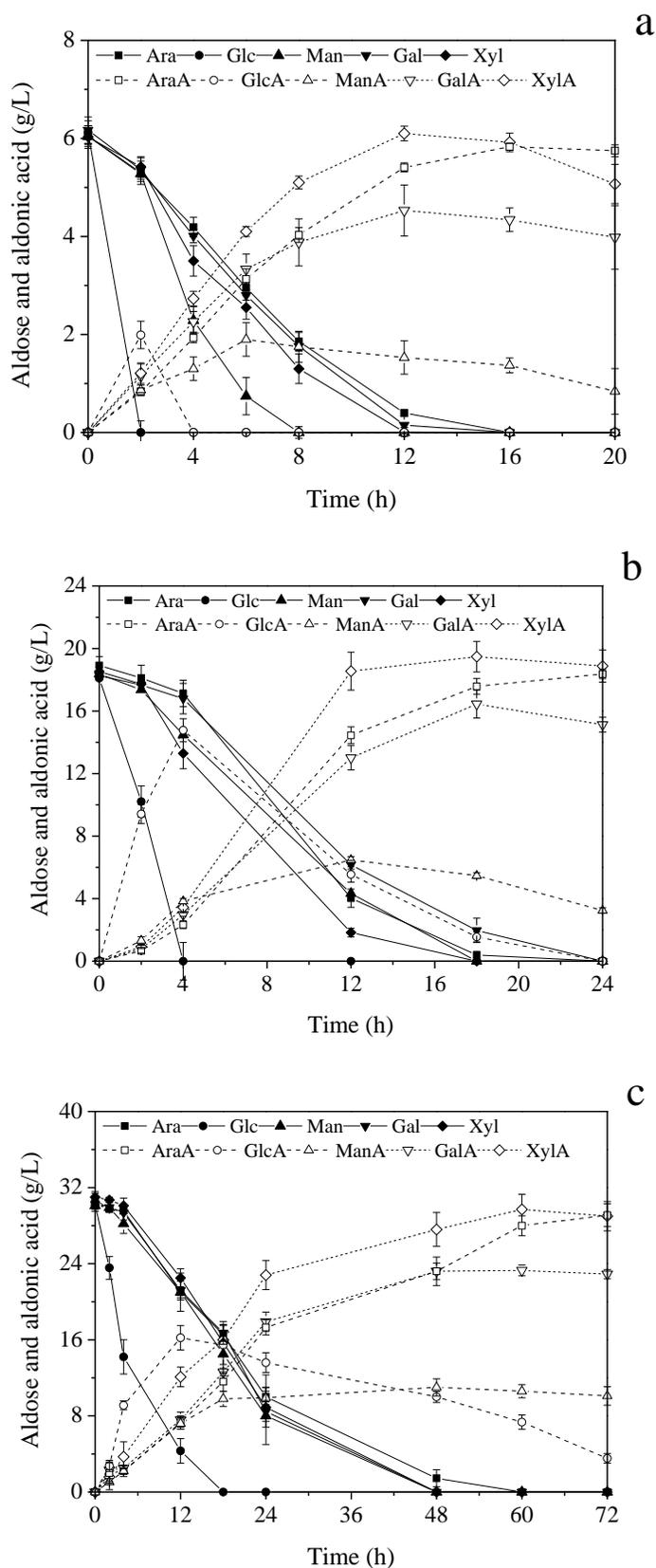
**Fig. 2.** Reaction processes of 6 g/L(a), 18 g/L(b), and 30 g/L(c) five aldoses simultaneous catalysis

· The results showed that five monosaccharides could be catalyzed simultaneously to the corresponding aldonic acids in various aldoses concentrations (30, 90, and 180 g/L) by using *G. oxydans* (Fig. 2). For simultaneous catalysis of five aldoses, the Glc could be consumed rapidly in different concentrations. By contrast, Xyl, Ara, Gal, and Man were difficult to utilize when Glc was present. According to the study of Zhang *et al*. (2013), the oxidization of Xyl to XylA is also attributed to the membrane-bound glucose dehydrogenase (*mgdh*) (Zhang *et al*. 2013). Therefore, we inferred that the reason might be caused by the selectivity of *mgdh* which hampered the utilization of the other four aldoses.

· The utilization rate of Xyl, Ara, Gal, and Man began to rise until the concentration of Glc declined by a wide margin. As can be seen in Fig. 2a, 2b, under the condition of low mixed-aldose concentration, the utilization rate of aldoses was different except for Glc. With ascending concentration of loading-aldoses, the utilization rate of Xyl, Gal, Man, and Ara tended to be consistent, which is most likely because the process is influenced by the enzymatic properties of dehydrogenase (Gupta *et al.* 2001). It was inferred that the type of Xyl, Gal, Man, and Ara dehydrogenase action was likely to be similar. The oxidization of Xyl, Gal, Man, and Ara might be all from the *mgdh.* With the increase of substrate, the reaction velocity would reach the maximum value ($V_{max}$).
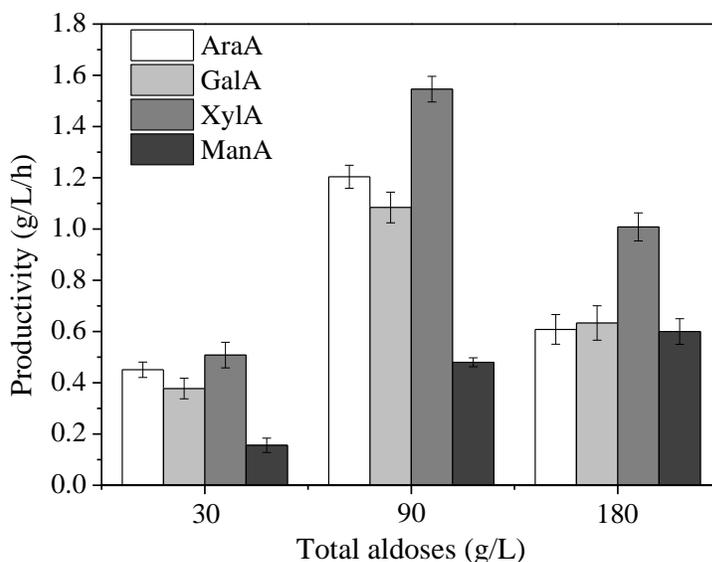


**Fig. 3.** The effect of total aldose concentrations on aldonic acids productivity (in 12 h)

Under the condition of same inoculum density, when the total concentration of aldoses was beyond 90 g/L, the simultaneously catalytic rate of aldonic acid (GlcA was consumed completely in 12 h and not shown in the Fig. 3) was generally higher than the condition of 30, 180 g/L mixed-aldose concentration, except for Man (Fig. 3). Generally, the catalytic rates are particularly associated the multifunction dehydrogenase or specific dehydrogenase. Namely, under the same condition, the reaction velocity was proportional to concentration of substrate. With the increase of substrate, the reaction velocity would reach at $V_{max}$ when all of the enzyme had become bound with substrate. That to say, dehydrogenase might be saturated with aldoses when the loading of aldoses reached 90 g/L.

In addition, *G. oxydans* is an obligate aerobe. The metabolism of aldoses in *G. oxydans* is a close-coupling bio-oxidation reaction of the dehydrogenation, and the cellular respiration chain that depends heavily on the oxygen supply. Normally, the viscosity of solution would increase with ascending loading of aldoses, and it would impose inhibitory effects on the dissolution of oxygen. Accordingly, it would influence the mass transfer of the oxygen and the dissolve oxygen. Namely, the respiratory chain and catalytic reaction also would be suppressed (Garcia-Ochoa and Gomez 2009). Then the final result was that the co-enzyme regeneration (PQQ/NADH) was trapped and the catalytic performance of aldoses declined (Hardy *et al*. 1993; Galar and Boiardi 1995). It was also observed that the Man was not similar to the other Xyl, Gal, and Ara. The productivity and yield of the ManA was enhanced with ascending loading of aldoses. The reasons for the enhancement might be that the Man was taken in catabolism of cell and was partly used for cell growth, while Glc was not sufficient for metabolism of *G. oxydans*. However, under the condition of high concentration, the selectivity of Glc or GlcA took precedence of the other aldoses and they would be consumed preferentially for metabolism.

## Kinetic Comparison of Individual and Simultaneous catalysis

The results for the utilization rates of the five aldoses (in 12 h) in individually and simultaneously catalytic system are shown in Fig. 4. In the individually catalytic system, the utilization rate of Glc was about 2.5 g/L which is similar to the simultaneously catalytic system at the utilization of 2.2 g/L. The Glc was not influenced by the other aldoses. However, in contrast with Glc, the utilization rate of Ara, Gal, Xyl, and Man were repressed distinctly in simultaneously catalytic system. Compared with the other aldoses, the performance of Xyl inhibition was the most prominent, and the utilization rate of Xyl in simultaneously catalytic system in 12 h only accounted for about 33.1% of the individually catalytic system. In like manner, for Gal, Man and Ara, the utilization rate in simultaneously catalytic system accounted for 47.3%, 45.6%, and 61.3% of the individually catalytic system.
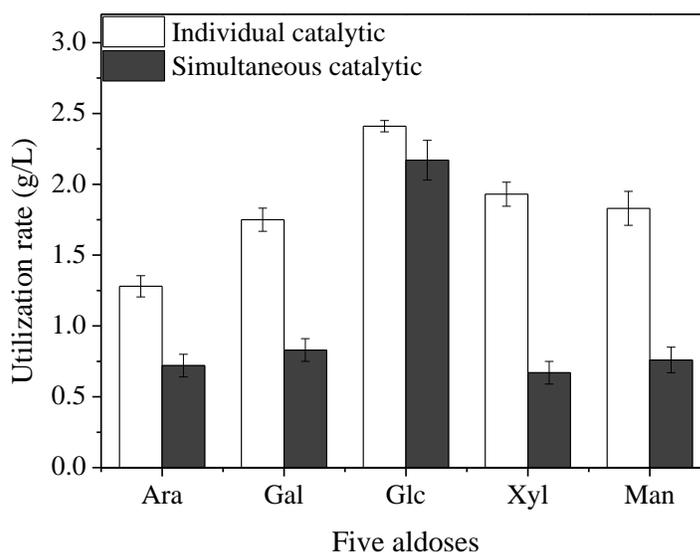


**Fig. 4.** Comparison of five aldose utilization rates in individually catalytic and simultaneously catalytic system (in 12 h)

The reason for inhibition in individually catalytic system might stem from co-competition of the various aldoses and the inhibition of the high substrate concentration, which inhibited the bacteria performance. Therefore, when using lignocellulosic pre-hydrolysate or enzymatic hydrolysate as material, to reach near-theoretical yield of aldonic acid and technical economic, it is necessary to control the total concentration, proportion, and terminal time of reaction.

## CONCLUSIONS

1. *G. oxydans* has a preferable capability to individually catalyze the formation of five aldonic acids, and the yield of XylA, GlcA, AraA, GalA, and ManA was 75.3%, 90.4%, 88.7%, 64.3%, and 52.0%, respectively, by using 30 g/L substrate. In addition, Glc was the fastest-utilized at 3.21 g/L/h compared with the slowest of Ara at 1.18 g/L/h, and GlcA achieved the highest productivity value of 3.14 g/L/h while AraA reached the lowest value of 0.78 g/L/h.

2. When the broth contained various aldoses, *G. oxydans* also could catalyze all of aldoses to aldonic acids simultaneously. However, the catalytic performance of Ara, Gal, Glc, and Xyl was impacted markedly by Glc when its concentration was more than 10 g/L. And when the total amount of aldoses was about 90 g/L, all yield of aldonic acids reached their highest value.

3. To reach near-theoretical yield, it is important to control the concentrations and the proportions of all kinds of monosaccharide components from the lignocellulosic hydrolysate or enzymatic hydrolysate as material.

## ACKNOWLEDGMENTS

## REFERENCES CITED

Buchert, J., Puls, K., and Poutanen. (1988). "Comparison of *Pseudomonas fragi* and *Gluconobacter oxydans* for production of xylonic acid from hemicellulose hydrolyzates," *Appl. Microbiol. Biot.* 28(4-5), 367-372. DOI: 10.1007/BF00268197

Buchert, J., and Niemelä, K. (1991). "Oxidative detoxification of wood-derived inhibitors by *Gluconobacter oxydans*," *J. Biorechnol.* 18(1-2), 1-12. DOI: 10.1016/0168-1656(91)90231-J

Bozell, J. J., and Petersen, G. R. (2010). "Technology development for the production of biobased products from biorefinery carbohydrates-the US Department of Energy's "Top 10" revisited," *Green Chem.* 12(4), 539-554. DOI: 10.1039/B922014C

Chun, B.W., Dair, B., Macuch, P. J., Wiebe, D., Porteneuve, C., and Jeknavorian, A. (2006). "The development of cement and concrete additive: Based on xylonic acid derived via bioconversion of xylose," *Appl. Biochem. Biotechnol.* 131(1-3), 129-132, 645-658. DOI: 10.1385/ABAB:131:1:645

Dahms, A. S. (1974). "3-Deoxy-D-pentulosonic acid aldolase and its role in a new pathway of D-xylose degradation," *Biochem. Biophys. Res. Commun.* 60(4), 1433-1439.

Deppenmeier, M., Hoffmeister, C., and Prust. (2002). "Biochemistry and biotechnological applications of *Gluconobacter* strains," *Appl. Microbiol. Biotechnol.* 60:233–242. DOI: 10.1007/s00253-002-1114-5

Galar, M. L., and Boiardi, J. L. (1995). "Evidence for a membrane-bound pyrroloquinoline quinone-linked Glucose-dehydrogenase in *Acetobacter diazotrophicus*," *Appl. Microbiol. Biotechnol.* 43(4), 713-716. DOI:10.1007/BF00164778

Gallezot, P. (2012). "Conversion of biomass to selected chemical products," *Chem. Soc. Rev.* 41(4), 1538-1558. DOI: 10.1039/C1CS15147A

Garcia-Ochoa, F., and Gomez, E. (2009). "Bioreactor scale-up and oxygen transfer rate in microbial processes: An overview," *Biotechnol. Adv.* 27(2), 153-176. DOI: 10.1016/j.biotechadv.2008.10.006

Gupta, A., Singh, V. K., Qazi, G. N., and Kumar, A. (2001). "*Gluconobacter oxydans*: its biotechnological applications," *J. Mol. Microb. Biotech.* 3(3), 445-456.

Hardy, G. P., Teixeira de Mattos, M. J., and Neijssel, O. M. (1993). "Energy conservation by pyrroloquinoline quinol-linked xylose oxidation in *Pseudomonas putida* NCTC 10936 during carbon-limited growth in chemostat culture," *FEMS Microbiol. Lett.* 107(1), 107-11. DOI:10.1111/j.1574-6968.1993.tb06012.x

Hölscher, T., Schleyer, U., Merfort, M., Bringer-Meyer, S., Görisch, H., and Sahm, H. (2009). "Glucose oxidation and PQQ-dependent dehydrogenases in *Gluconobacter oxydans*," *J. Mol. Microbiol. Biotechnol.* 16(1-2), 6-13. DOI: 10.1159/000142890

Lederkremer, R. M., and Marino, C. (2003). "Acids and other products of oxidation of aldoses," *Adv. Carbohyd. Chem. Bi.* 58(1), 199-306. DOI: 10.1016/S0065-2318 (03)58005-2

Menon, V., and Rao, M. (2012). "Trends in bioconversion of lignocellulose: Biofuels, platform chemicals & biorefinery concept," *Prog. Energ. Combust.* 38(4), 522-550. DOI: 10.1016/j.pecs.2012.02.002.

Niu, W., Molefe, M. N., and Frost, J. W. (2003). "Microbial synthesis of the energetic material precursor 1,2,4-butanetriol," *J. Am. Chem. Soc.* 125(43), 12998-12999. DOI: 10.1021/ja036391+

Palmqvist, E., and Hahn-Hägerdal, B. (2000). "Fermentation of lignocellulosic hydrolysates. I: Inhibition and detoxification," *Bioresource Technol.* 74(1), 17-24. DOI: 10.1016/S0960-8524(99)00160-1

Pezzotti, F., and Therisod, M. (2006). "Enzymatic synthesis of aldonic acids," *Carbohyd. Res.* 341(13), 2290-2292. DOI: 10.1016/j.carres.2006.05.023

Ragauskas, A. J., Williams, C. K., Davison, B. H., Britovsek, G., Cairney, J., Eckert, C. A., FrederickJr, W. J., Hallett, J. P., Leak, D. J., Liotta, C. L., Mielenz, J. R., Murphy, R., Templer, R., and Tschaplinski, T. (2006). "The path forward for biofuels and biomaterials," *Sci.* 311(5760), 484-489. DOI:10.1126/science.1114736

Ramachandran, S., Fontanille, P., Pandey, A., and Larroche, C. (2006). "Gluconic acid: properties, applications and microbial production," *Food Technol. Biotechnol.* 44(2), 185-195.

Sauer, M., Porro, D., Mattanovich, D., and Branduardi, P. (2008). "Microbial production of organic acids: Expanding the markets," *Trends Biotechnol.* 26(2), 100-108. DOI: 10.1016/j.tibtech.2007.11.006

Wang, X., Xu, Y., Lian, Z. L., Yong, Q., and Yu, S. Y. (2014). "A one-step method for the simultaneous determination of five wood monosaccharides and the corresponding aldonic acids in fermentation broth using high-performance anion-exchange chromatography coupled with a pulsed amperometric detector," *J. Wood Chem. Technol.* 34(1), 67-76. DOI: 10.1080/02773813.2013.838268

Warwick, P., Evans, N., Hall, T., and Vines, S. (2004). "Stability constants of uranium(IV)-α-isosaccharinic acid and gluconic acid complexes," *Radiochim. Acta* 92(12), 897-902. DOI: 10.1524/ract.92.12.897.55106

Weenk, G., Olijve, W., and Harder, W. (1984). "KetoGluconate formation by *Gluconobacter* species," *Appl. Microbiol. Biotechnol.* 20(6), 400-405. DOI: 10.1007/BF00261942

Whitfield, D. M., Stojkovski, S., and Sarkar, B. (1993). "Metal coordination to carbohydrates. Structures and function," *Coordin. Chem. Rev.* 122(1-2), 171-225. DOI: 10.1016/0010-8545(93)80045-7

Zhang, M. H., Wei, L. J., Zhou, Y., Du, L. Q., Imanaka, T., and Hua, Q. (2013). "Genetic analysis of D-xylose metabolism pathways in *Gluconobacter oxydans* 621H," *J. Ind. Microbiol. Biotechnol.* 40(3), 379-388. DOI 10.1007/s10295-013-1231-4