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Short Communication

# Process for the successive production of calcium galactonate crystals by *Gluconobacter oxydans*

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ABSTRACT

Galactonic acid and its salts can be potentially used in foodstuffs and as specialty chemicals. So far, the researches on microbial conversion of galactose to galactonate are still scarce. In this study, we initially used *Gluconobacter oxydans* strain NL 71 to convert galactose to galactonic acid via aerobic fermentation by fed-batch and product separation process in shaken flasks, finally 390 g/L galactonic acid could be obtained after 96 h fermentation. To harvest calcium galactonate product, an aeration-agitation bioreactor with product drain port was applied. The lower solubility of calcium galactonate aided its crystallization during fermentation and after 96 h fermentation, approximately 720 g calcium galactonate crystals were produced from 1 L broth. The results showed that successive production of calcium galactonate by a combination of fed-batch and natural crystallization in the process of fermentation was feasible.

## 1. Introduction

Aldonic acids are organic acids with the following chemical formula: $HOCH_2$-(CHOH)n-COOH. They are valuable raw materials in pharmaceutical, food, cosmetics, and construction industries (Dowdells et al., 2010; Toivari et al., 2012). The best known aldonic acid used industrially is gluconic acid, with a yearly consumption of 60,000 tons. The production of various aldonic acids like gluconic acid, lactobionic acid and xylonic acid have been extensively studied and are currently applied industrially with great success (Sauer et al., 2008; Toivari et al., 2012;). Galactonic acid (GalA), a kind of aldonic acid, is chemically similar with gluconic acid and therefore has the potential to be used in similar products and applications, such as sweeteners, intermediates, precursors, dispersion, and retardation (Ramachandran et al., 2006; Zaliz and Varela, 2003). However, the use of GalA is currently limited due to its high cost of production. Nevertheless, it has the potential for much wider use than just a specialty chemical if made available at lower price (Kuivanen et al., 2012).

The microbiological method for aldonic acids production has been widely applied due to its safety, high efficiency and environment protecting (Dowdells et al., 2010; Liu et al., 2014; Zhang et al., 2017). In addition, *Gluconobacter oxydans*, a gram-negative obligate aerobic bacterium, is known for rapidly and incompletely oxidizing sugars and sugar-alcohols (Zhou et al., 2017; Prust et al., 2005) and it has also been reported of being capable of converting galactose to GalA (Švitel and Šturdík, 1994). In this study, we used *G. oxydans* for the incomplete bio-oxidation of galactose to GalA and we found that the calcium carbonate ($CaCO_3$) or calcium hydroxide ($Ca(OH)_2$) that was added to the culture for neutralizing the oxidation reaction, was precipitated as calcium galactonate with increasing concentrations of GalA. The crystallization of calcium galactonate was further aided by its poor solubility in neutral aqueous media. Therefore, an unexpected corollary of our intended bio-production of GalA was the easy separation of calcium galactonate. Thus, the objective of this study was to further the knowledge concerning microbial catalysis of galactose to galactonate.

## 2. Materials and methods

### 2.1. Aerobic fermentation by G. oxydans

The inocula of *G. oxydans* NL71 were prepared in Erlenmeyer shaker flask (sorbitol 50 g/L, yeast extract 5 g/L), and cultured for 24–36 h at 200 rpm and 30 °C (Miao et al., 2015). The fermentation medium was composed of 2 g/L yeast extract, 0.1 g/L $MgSO_4$, 1 g/L $K_2HPO_4$, and 1 g/L $(NH_4)_2SO_4$. For batch fermentation in shaken flasks, different concentrations of galactose (100, 200, 300, 400 and 500 g/L) were loaded as the carbon source. Fed-batch fermentation was conducted in shaken flasks and bioreactor, and 100 g/L galactose was loaded for every batch. The density of the initial *G. oxydans* inoculum was 2 g/L by optical density method (Zhou et al., 2017) and pH was controlled at

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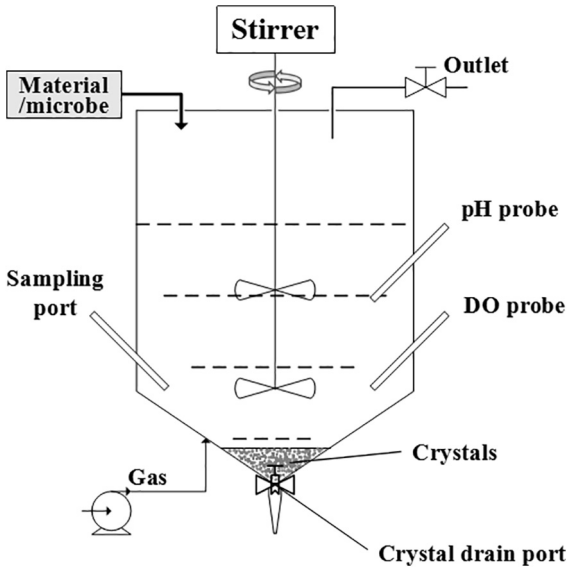


**Fig. 1.** Diagram of the ASBR design for successive production.

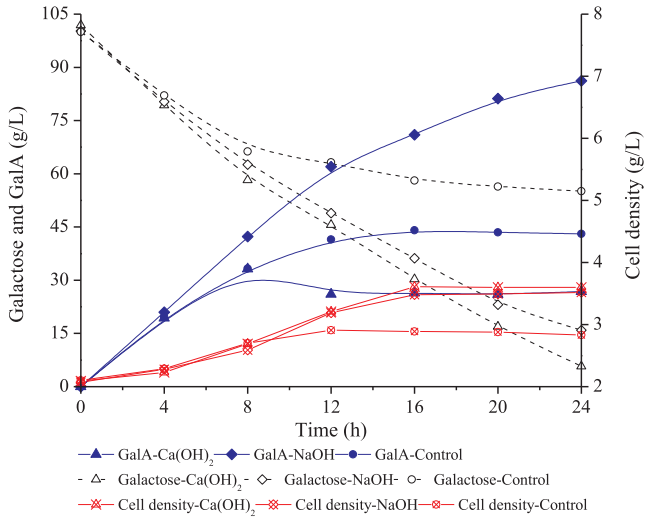


**Fig. 2.** Reaction process of galactose bioconversion in Erlenmeyer shaken flasks. NaOH and $Ca(OH)_2$ were loaded as neutralizers. Recorded concentrations of galactose, GalA and *G. oxydans* cell density.

4.5–6.5 by manually loading $Ca(OH)_2$ or $CaCO_3$. Fermentation was initiated in 250 mL Erlenmeyer shaker flasks containing 50 mL medium at 220 rpm and 30 °C, and continued in an aerated and stirred bioreactor (ASBR) (BioFlo 115, New Brunswick Scientific Co., Inc.), and the vessel was equipped with a drain port at the bottom to collect calcium galactonate. The design of the bioreactor is showed in the Fig. 1. The airflow rate set at 3 vvm and agitation rate in the ASBR with Rushton (6-flat-blade) disc turbine was set manually at 500 rpm. The foam was controlled automatically by on-line addition of organic polyether dispersions as antifoaming agent.

### 2.2. Analytical methods

Inductively coupled plasma mass spectrometry (ICP-MS) (NeXion 300X) was used for the qualitative analysis of GalA and the morphology of GalA crystals was observed microscopically (ZEISS AX10) at 400X magnification. The quantitative analysis of galactose and GalA were carried out using high performance anion-exchange chromatography (Dionex ICS-5000) linked to a CarboPac™ PA 200 column with 60 mM NaOH. GalA yield calculation: the total content of GalA divided by the utilized galactose content and multiplied by 0.918, where 0.918 is the conversion factor for galactose to an equivalent amount of GalA based on the stoichiometric balance.

Each of repetitive assays contained two parallels, and the data averaged.

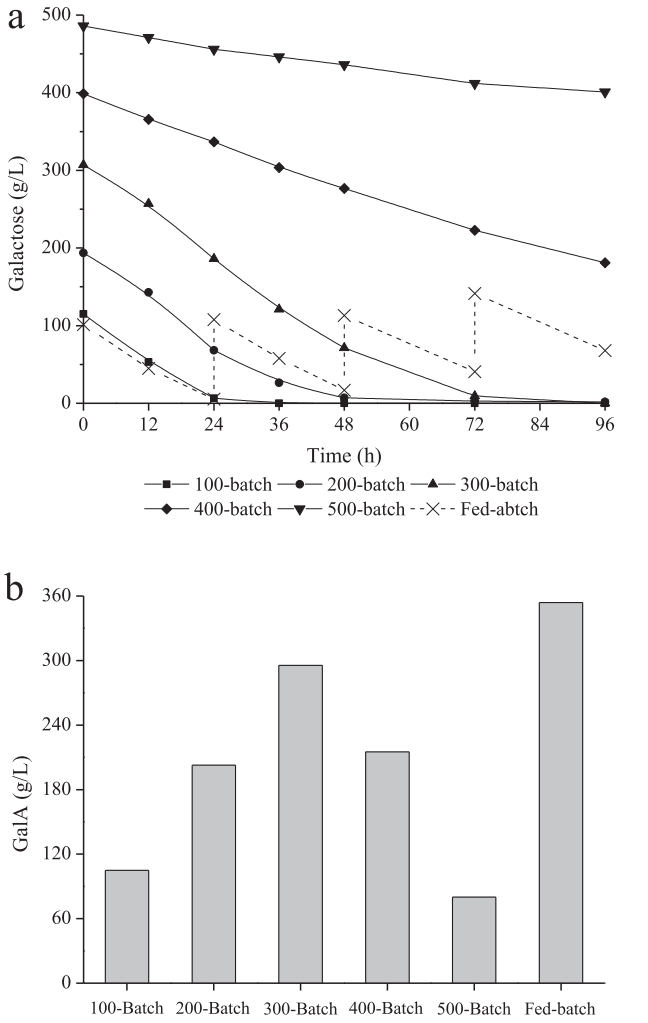


**Fig. 3.** Reaction process of galactose bioconversion in Erlenmeyer shaken flasks. Batch (100–500 g/L) and fed-batch operation (four batch additions of 100 g/L galactose at 0, 24, 48 and 72 h) were investigated. a. The change of galactose. b. The final production of GalA in 96 h.

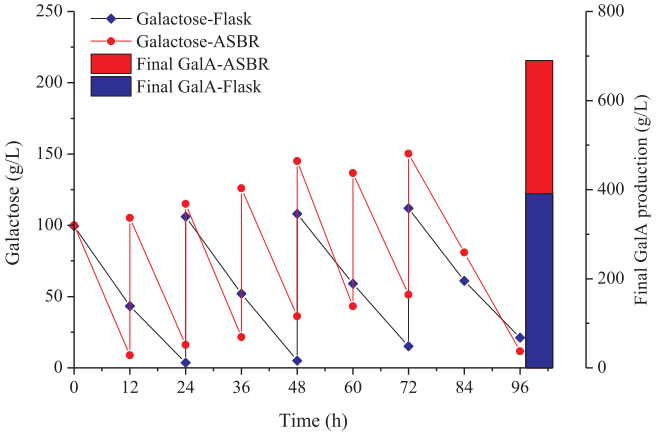


**Fig. 4.** Reaction process of fed-batch unites with product separation operation in Erlenmeyer shaken flasks and ASBR.

## 3. Results and discussion

### 3.1. Effect of different neutralizers on the fermentation reaction of G. oxydans

The neutralizer plays a significant role in increasing GalA titers. As shown in Fig. 2, in the case which absence of any neutralizing agent, the production of GalA was noticeably limited, moreover the cell growth was also inhibited as depicted in Fig. 2. In the case of pH regulation, *G. oxydans* could effectively convert galactose. There were however differences in the neutralizing efficacy of NaOH and $Ca(OH)_2$. The galactose utilization ratio was significantly higher with $Ca(OH)_2$ and only 5.0 g/L of galactose remained after 24 h of fermentation. At the same time point, 17.0 g/L galactose remained in the broth for NaOH regulation assay. Surprisingly, when $Ca(OH)_2$ was used for pH control, the GalA concentration in the broth was always lower than 30 g/L. In addition, we observed that a great mass of crystallized substances was formed during fermentation. The photograph showed that the crystals is rod-like (photograph not shown) and the crystals that were between 200 and 300 μm in length comprised more than 80%. Meanwhile, the crystals were also analyzed by ICP-MS. And the main peak had a fragmentation of the molecular ion $[M - H]^-$ at m/z 195.05 (data not shown), which matches the theoretical value of GalA. Calcium galactonate, like calcium gluconate, is only slightly soluble in water (20 °C) but dissolves instantly in acid (Bao et al., 2003). The calcium galactonate crystals could dissolve in the solution with pH below 3.0. Thus, in the fermentation end-point of $Ca(OH)_2$ as neutralizer, calcium galactonate could be re-dissolved into the broth by adjustion of hydrochloric acid. Then GalA could be analyzed by chromatography ant its final concentration (approximately 97 g/L) was higher than the result (82 g/L) of NaOH neutralization. All observations indicated that calcium galactonate could be separated automatically in the process of fermentation. Therefore, the end-product could be moderately controlled to improve the separation of products and obtain successive harvests of calcium galactonate crystals.

### 3.2. Influence of fermentation mode on calcium galactonate production

In this study, we also tested and compared two processes (batch and fed-batch) in GalA production. With batch operation in the Erlenmeyer shaken flask, a certain amount of galactose could be utilized completely by *G. oxydans* as part of regular metabolism. However, the bioconversion efficiency decreased significantly with continuous increase in substrate content as shown in Fig. 3a. Batch fermentation experiments showed that limiting substrate concentration during fermentation resulted in a maximum GalA production of 300 g/L. Fed-batch process has already been used in the production of a wide variety of products with the aim of increasing the yields of desired metabolites by avoiding substrate inhibition. Thus, we used substrate feeding via fed-batch fermentation to improve GalA production. As shown in Fig. 3b, the productivity was improved greatly even at the total substrate loading of 400 g/L galactose. Moreover, the maximum GalA concentration obtained using this method was about 350 g/L.

### 3.3. Successive production of calcium galactonate crystals in ASBR

While, it could be found that the cell catalytic capacity could not be recovered completely in the high galactose loading medium. This might be due to abundant crystals (calcium galactonate crystal contains five moleculars crystal water) or limited oxygen transfer rate in the flask, both of which would negatively impact the catalytic activity of *G. oxydans*. Based on that outcome, we conducted another fed-batch fermentation with a step of crystals removal. Before loading the next batch of galactose, the crystals were harvested through a filter funnel (G2, 10–15 μm porosity) and the residual galactose and *G. oxydans* cells were retained in the broth for successive fermentation. The broth volume was reconstituted to the initial volume (50 mL) by adding distilled water before starting the next batch of fermentation. With the crystals removal step, there was a significant increase in GalA production and the total amount of accumulated GalA was about 390 g/L, indicating a significant enhancement in *G. oxydans* fermentative capability as shown in Fig. 4. As an obligate aerobic bacterium, *G. oxydans* heavily depends on adequate oxygen supply for bio-oxidation. Therefore, we conducted the fermentation process in an ASBR which equip with a drain port at the bottom of the vessel. The rationale is that the sedimentation rate of calcium galactonate is markedly higher than *G. oxydans* cells, the product can be harvested from the drain port while the cells remained in the broth. The broth volume was maintained at the initial volume (1 L) by adding distilled water. The results showed in Fig. 4, 700 g galactose was loaded in 1 L broth and finally 720 g calcium galactonate was harvested and about 30 g/L GalA was still dissolved in the broth. That's to say, approximately 690 g/L GalA could be produced in 96 h. Taken together, successive GalA production was markedly improved by a combination of fed-batch and product removal in ASBR.

## 4. Conclusions

The results obtained in the present study demonstrated that *G. oxydans* was capable of efficiently converting galactose to GalA. We devised a successive GalA preparation method which combined fed-batch and product online separation operation in the ASBR system, and boosted GalA production. We put forward this strategy for obtaining pure GalA at the industrial level in a cost-competitive manner.

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## References

Bao, J., Koumatsu, K., Arimatsu, Y., Furumoto, K., Yoshimoto, M., Fukunaga, K., Nakao, K., 2003. A kinetic study on crystallization of calcium gluconate in external loop airlift column and stirred tank for an immobilized glucose oxidase reaction with crystallization. Biochem. Eng. J. 15, 177–184.

Dowdells, C., Jones, R.L., Mattey, M., Bencina, M., Legisa, M., Mousdale, D.M., 2010. Gluconic acid production by *Aspergillus terreus*. Lett. Appl. Microbiol. 51, 252–257.

Kuivanen, J., Mojzita, D., Wang, Y., Hilditch, S., Penttila, M., Richard, P., Wiebe, M.G., 2012. Engineering filamentous fungi for conversion of D-galacturonic acid to L-galactonic acid. Appl. Environ. Microbiol. 78, 8676–8683.

Liu, H., Ramos, K.R., Valdehuesa, K.N., Nisola, G.M., Malihan, L.B., Lee, W.K., Park, Si Jae, Chung, W., 2014. Metabolic engineering of *escherichia coli* for biosynthesis of D-galactonate. Bioprocess Biosyst. Eng. 37, 383–391.

Miao, Y., Zhou, X., Xu, Y., Yu, S., 2015. Draft Genome Sequence of *Gluconobacter oxydans* NL71, a strain that efficiently biocatalyzes xylose to xylonic acid at a high concentration. Genome Announcements 3, 15.

Prust, C., Hoffmeister, M., Liesegang, H., Wiezer, A., Fricke, W.F., Ehrenreich, A., Gottschalk, G., Deppenmeier, U., 2005. Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. Nat. Biotechnol. 23, 195–200.

Ramachandran, S., Fontanille, P., Pandey, A., Larroche, C., 2006. Gluconic acid: properties, applications and microbial production. Food Technol. Biotechnol. 44, 185–195.

Sauer, M., Porro, D., Mattanovich, D., Branduardi, P., 2008. Microbial production of organic acids: expanding the markets. Trends Biotechnol. 26, 100–108.

Švitel, J., Šturdík, E., 1994. D-galactose transformation to D-galactonic acid by *Gluconobacter oxydans*. J. Biotechnol. 37, 85–88.

Toivari, M.H., Nygard, Y., Penttila, M., Ruohonen, L., Wiebe, M.G., 2012. Microbial D-xylonate production. Appl. Microbiol. Biotechnol. 96, 1–8.

Zaliz, C.L.R., Varela, O., 2003. Straightforward synthesis of derivatives of D- and L-galactonic acids as precursors of stereoregular polymers. Tetrahedron Asymmetry 14, 2579–2586.

Zhang, H., Han, X., Wei, C., Bao, J., 2017. Oxidative production of xylonic acid using xylose in distillation stillage of cellulosic ethanol fermentation broth by *Gluconobacter oxydans*. Bioresour. Technol. 224, 573–580.

Zhou, X., Zhou, L., Xu, Y., 2017. Improvement of fermentation performance of *Gluconobacter oxydans* by combination of enhanced oxygen mass transfer in compressed-oxygen-supplied sealed system and cell-recycle technique. Bioresour. Technol. 244, 1137–1141.