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Michael Crain-Zamora

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Michael Crain-Zamora

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Idaho National Laboratory
Idaho Falls, Idaho 83415

http://www.inl.gov

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Abstract

Gluconobacter oxydans is an acidophilic bacterium that is used in industry for producing organic acids, such as gluconic acid, from sugars such as glucose. Recently it has been discovered that the organic acids produced by *G. oxydans* has potential to be used in the recovery of rare earth elements (REE), especially gluconic acid. Producing gluconic acid from pure glucose has been deemed costly and so research has been conducted to find more economically friendly feedstock. In this work corn stover, potato peel waste, market rejected fruits, and apple processing and holding pond waters were investigated for their ability to provide *G. oxydans* sufficient sugar to grow and produce organic acid for bioleaching. Substrates such as the corn stover and fruits provided more than 20 g/L of glucose, and the bacterium grew well on all but the apple processing and holding pond water.

Introduction

Gluconobacter oxydans is an industrially important bacterium for producing organic acids, such as gluconic and xylonic acid, from reducing sugars.[1] Reed, D., *et al.* has shown a potentially important application of *G. oxydans'* organic acid production in the bioleaching of rare earth elements from end of life products.[2] Gluconic acid is the primary product of glucose metabolism by *G. oxydans*, and was found to be important in the bioleaching process. It has been estimated however that of the total cost for bioleaching spent fluid catalytic cracking catalyst (FCC) using *G. oxydans* strain B58, approximately 44% stems from the nutrients needed to grow

the bacteria. Additionally, 98% of the cost of nutrients is attributable to glucose (V. Thompson, personal communication). In order to find a cheaper carbon source that produce an organic acid lixiviant comparable to that from glucose, biomass and food waste products were evaluated as substrates for *G. oxydans*.

Lignocellulose hydrolysates makes up 50% of all biomass on earth and is composed mostly of cellulose and hemicellulose while having a relatively low lignin concentration.[3] Biomass waste containing lignocellulose is a byproduct from agricultural products and several promising wastes from wheat, corn, sugar beets, and potatoes may serve as alternatives for glucose as substrate for *Gluconobacter* growth. This waste comes in the form of the stalks from which the corn was grown on, the pulp from sugar beets during sugar refining, or even the peels from potatoes used to make fries and chips. There have been some uses for wastes such as sugar beet pulp in feeding cattle and other animals; however, all of this waste has the potential for other more economically viable applications as well. One waste, wheat straw, is composed of 35-40% cellulose, 20-30% hemicellulose, and <20% lignin.[4] By pretreating straw with dilute acid and saccharifying with enzymes such as cellulase, glucosidase, xylanase, and esterase, as much as 565 mg/g of monomeric sugars can be obtained.[4] These monomeric sugars could then be used to feed *G. oxydans* and produce the organic acids needed for bioleaching.

Teams like Turkia, H., *et al.* have already shown the potential of using wheat straw as a carbon source when they reported producing 18 different organic acids in cultures of *G. oxydans* maintained in media derived from wheat straw hydrolysate supplemented with 5 g/L yeast extract.[3] After incubating these cultures for 40 hours, about 5 g/L of gluconic acid and 15 g/L of xylonic acid were produced. Initial concentrations of glucose and xylose from the hydrolysate were 5.3 g/L and 39 g/L respectively.

Although these materials appear perfect as alternatives to purified glucose two glaring problems must be addressed. One is the presence of large amounts of lignin, which have been known to inhibit enzymatic processes. The other is the need for pretreatments to break up the lignocellulosic materials so that the cellulose and hemicelluloses are open for enzymatic degradation. Dilute sulfuric acid hydrolysis is a popular method for pretreatment of biomass in the laboratory however this process is challenged by corrosion leading to the need for chemical resistant equipment in industrial settings, and the possibility of creating large amounts of acidic water wastes. [5] This has driven some scientists to research pretreatment methods that can be used to keep costs and waste low.[6] The other problem, the presence of lignin and other inhibitory compounds such as glucose itself, can stifle the potential of these feedstocks. These problems have been quantified and suggestions have been made by others for membrane reactor designs that remove glucose during enzymatic hydrolysis.[7] By alleviating the pressure of this one inhibitor, the effects of others may be lessened and in the end more pure glucose can be harvested. Rather than focusing on that though, there are a few other waste products that require less intensive pretreatment, which may in the end be cheaper for lixiviant production while still being readily available.

Once such material is potato peel waste. During potato processing, peels are generated as waste and still contain a good amount of amylose in the form of starch. One group showed that 15% (w/v) solutions of ground dry potato peels subjected to enzymatic hydrolysis using UEB-S amylase and amyloglucosidase could generate 63 to 69 g/L of reducing sugars that *G. oxydans* could use for organic acid production.[8] Compared to other waste products, potato peels show a great potential in being a cheap feedstock due to the need for minimal preparation and ease of enzymatic hydrolysis.

One last possible biomass waste with potential is food waste. According to the Food and Agricultural Organization for the United Nations, roughly one-third, about 1.3 billion metric tons, of food is either wasted or lost. It is estimated that fruits, vegetables, and roots compose half of this number.[9] Grocery stores every day throw out fruits high in sugar because they are deemed unfit for human consumption. Due to their sugar content, these fruits have been identified by other researchers as potential feedstocks for gluconic acid producing bacteria such as *G. oxydans*.[10]

In this work several carbon sources were prepared and tested for their ability to support organic acid production by *G. oxydans*. This was accomplished through the enzymatic hydrolysis of materials such as corn stover and potato peels, as well as the juicing of market rejected fruits: watermelon, nectarine, pluto plum, white peach, baby banana. The resulting substrates were then inoculated with *G. oxydans* and the resulting spent media solutions were used as lixiviants for the leaching of spent FCC catalyst.

Materials and Methods

Corn Stover Hydrolysis

Corn stover pretreated previously in the Idaho National Laboratories was enzymatically hydrolyzed using methods similar to those described by Wolfum *et al*.[11] Corn Stover was prepared as a 10% (w/v) solution using a 0.05 M sodium citrate buffer at a pH of 4.8. Cellic® Ctec2 and Htec2 enzymes (Novozymes®; Franklinton, NC) were added to the solution in 40 mg/g and 4 mg/g amounts respectively. Incubation was then performed at 50 ºC and 200 rpm for two days in a shaker incubator (Innova® 44; New Brunswick Scientific Co., Inc.; Enfield, CT), after which the resulting solution was centrifuged at 7500 x g for one hour and the supernatant was filter-purified using a 0.2 µm filter.

Potato Peel Hydrolysis

Idaho russet potatoes obtained from the local grocery store were peeled and the peels dried at 50 ºC for two days. Peels were then ground and prepared as a 15% (w/v) solution using nanopure water, and the solution was brought to a pH of 7.0 using 1 M HCl. 45 U of alpha amylase (Sigma-Aldrich Co.; St. Louis, MO) was added and the solution was incubated at 80 ºC and 200 rpm for two hours using the same shaker incubator as used for the corn stover. The solution was then allowed to cool to room temperature and then brought down to a pH of 4.5 using 1 M NaOH. 9 U of amyloglucosidase was then added and incubation continued at 60 ºC and 200 rpm for 24 hours, after which the solution was centrifuged at 7500 x g for one hour and filter-purified using a 0.2 μm filter.

Selection and Preparation of Market Reject Fruits

All fruits were obtained from local grocery stores after having been deemed unfit for sale due to excessive blemishes or signs of excessive rot. Fruits were peeled, ground, boiled briefly to inhibit excessive oxidation and liberate as much sugar from fiber, and finally sterilized with 0.2 μm filters. The Baby Banana was prepared as a 25% (w/v) solution while all other fruits were prepared as 38 % (w/v) solutions.

HPLC Analysis of Substrates for Sugar Content

Chemical compositional analysis was determined according to standard biomass procedures developed by the National Renewable Energy Laboratory (NREL). Carbohydrates released in dilute alkaline pretreatment and enzymatic hydrolysis were measured using HPLC (Thermo Fisher Scientific, Ultimate 3000, Waltham, MA, USA) equipped with an Aminex HPX-87H column (Bio-Rad, 300 x 7.8 mm, Hercules, CA, USA) and a Refractive Index (RI) detector.

Preparation of G. oxydans

G. oxydans strain B58 was grown in 25 mL of Pkm media with 10 g/L glucose at 30 ºC and 150 rpm for 29 hours until the optical density (OD) at 600 nm was 0.222. [2,13] At this point *G. oxydans* was presumed to be in the logarithmic growth phase. The culture was then centrifuged and washed with Pkm media, without glucose, before being transferred to 10 mL of Pkm media without glucose to give a final OD of 0.555. OD was measured using a Spectramax Plus 384 spectrophotometer (Molecular Devices, LLC; Sunnyvale, CA).

Incubation of G. oxydans *in Economic Media*

Media was prepared using 5x Pkm diluted with nanopure water (to 1X) and each waste product substrate. For the fruits, corn stover, and potato peels, 20 mL of each were used in Apple processing and holding pond waters (EverFresh; Boring, OR) were used to dilute 5x Pkm to 1x. After media preparation, 1 mL of Pkm media containing bacteria prepared previously, OD_{600} of 0.555, was added to a 10 g/L glucose Pkm control, and all other media, and incubation was conducted at 30 ºC and 150 rpm for 55 hours.

Results

Sugar Content of Waste Product Substrates

Sugar compositional analysis provided the data found in Table 1. Although a 10% corn stover should contain about 37 g/L of glucose, based on the idea that corn stover is 37% cellulose, only 25 g/L was produced. Potato peel waste has also been reported to reach upwards of 60 g/L based on Khawla, B., et al. research.[8] This report conflicts with the resulting 14.83 g/L obtained in this test.

Table 1. Concentrations of sugars in substrates as determined by HPLC analysis.

Sample	Cellobiose (g/L)	Glucose (g/L)	Xylose (g/L)	Galactose (g/L)	Arabinose (g/L)	Fructose (g/L)
Watermelon	0.79	12.05	0.02	0.15	0.24	20.88
Nectarine	29.55	7.93	0.20	0.05	0.13	8.43
Pluto Plum	3.82	20.00	0.24	0.18	0.21	20.04
Baby Banana	16.44	8.86	0.00	0.07	0.11	8.18
White Peach	20.52	3.84	0.23	0.04	0.05	4.56
Corn Stover	5.79	25.18	9.81	0.15	0.69	0.79
Potato Peel	0.80	14.83	0.00	0.14	0.00	0.54
Everfresh Pond	0.00	0.00	0.00	0.02	0.00	1.65
Everfresh Apple	0.00	0.00	0.00	0.07	0.01	0.67

Growth of G. oxydans *on Waste Products*

Growth of *G. oxydans* was assessed by OD measurements at 600 nm. Based on glucose content, it was thought that the corn stover would be the most like the glucose control in growth and organic acid production, as measured by pH, with the pluto plum being close to it. Conversely, the apple processing and holding pond water substrates were not expected to support much growth at all considering their lack of glucose, or any other measured sugar. This was not the case entirely as corn stover, while being close in OD to glucose, had a higher ending pH (Table 2). The processing and holding pond waters were found to have the lowest growth of all the substrates.

Table 2. General growth of *G. oxydans* after 55 hours. The pH was measured to gauge the ability of the bacteria to utilize each substrate for relative organic acid production. Optical density (OD) was used to compare the general growth of bacteria using each media, Glucose (10 g/L) containing Pkm media was used for the control.

Substrate	Initial Glucose Conc. (g/L)	Initial pH	Final pH	Final OD_{600}
Glucose	10.00	4.92	2.27	0.694
Watermelon	4.82	5.64	2.86	1.472
Nectarine	3.17	4.26	3.07	1.336
Pluto Plum	8.00	3.45	2.75	0.864
Baby Banana	3.54	5.29	2.97	0.952
White Peach	1.54	4.32	3.50	0.815
Corn Stover	10.07	4.74	2.89	0.710
Potato Peel	5.93	5.18	3.14	1.030
Apple Processing Water	0.00	3.12	2.94	0.277
Apple Holding Pond Water	0.00	4.78	4.04	0.231

Discussion

The purpose of this work was to show that *G. oxydans* could grow on glucose-containing substrates with a relative cost cheaper than pure glucose and to determine if any of these substrates could be used by the bacterium in producing a lixiviant effective for bioleaching.

From the initial screening of sugar levels in each substrate, it was found that corn stover and potato peel substrates did not yield as much glucose as anticipated. For the corn stover it is possible that more time was needed for the cellulase and hemicellulase to break down the material; hydrolysis was allowed to proceed for only 2 days, rather than the recommended 5 days.[11] On the other hand however, lignin, a known inhibitor to enzyme activity, is known to be found in corn stover and may have prevented full hydrolysis of the cellulose. As for the potato peels it is possible that insufficient enzyme was used for the residual amylose on the peels. In the future efforts should be taken to optimize substrate preparation.

The growth screening of each substrate produced some interesting results. As stated previously, it was thought that with corn stover an addition resulting in a medium containing 10 g/L glucose, the same concentration found in the control, the two treatments would exhibit similar growth and end pH values. However the corn stover medium ended with a pH of 2.89,

while the glucose control treatment had a pH of 2.27. After going over the procedures for enzymatic hydrolysis, it was postulated that a possible cause for a higher pH was the presence of 0.5 M sodium citrate buffer used to make the aqueous corn stover solution. It was originally added to keep the solution at a pH of 4.8 for the enzymes, but may have also contributed resistance to the lowering of pH in the medium. Therefore, it may be beneficial to test the effects of a lower buffer concentration on hydrolysis pretreatment of corn stover, or see if the procedure can be done without a buffer at all.

Another unexpected result was the extreme growth and low pH of the culture provided with the watermelon solution, considering that it had only half the amount of glucose as the control. Upon further inspection of Table 1 however, it was found that the watermelon had a high amount of fructose, about 20 g/L, which upon dilution leaves the watermelon Pkm media with about 8 g/L of fructose. This could have allowed *G. oxydans* to thrive and produce other organic acids that would drive the pH down. This finding hints that glucose may not be the only sugar important for low pH organic acid production.

The goal of preparing cheap substrates for *G. oxydans* and finding one that could replace glucose as the main ingredient in organic acid production has been met. A few substrates such as corn stover and market rejected fruit like watermelon have proven to grow the bacteria well and produce low pH lixiviant. Suggestions for follow on research include investigating how to prevent pretreatment buffers from interfering with lixiviant production by *G. oxydans* grown on corn stover and comparing different fruits with equal sugar concentrations. Additionally, the different spent media must still be tested for their effectiveness in bioleaching end-of-life materials.

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