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Bio-generation of succinic acid by fermentation of *Physaria fendleri* seed polysaccharides

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ABSTRACT

The bio-oils research unit at the national center for agricultural utilization research in Peoria, Illinois produces over 70% of crushed oilseed as press cake annually as tons of seed are crushed for oil. A large amount of this press cake cannot be used as animal feed because of anti-nutritional properties and generally winds up landfilled. *Lesquerella fendleri* now reclassified as *Physaria fendleri* and its press cake belongs in this category of oilseed processed here and its meal is unsuitable as animal feed. Hence the purpose of this study was to explore use of its press cake especially its polysaccharide content as renewable substrate for fermentative production of succinic acid when the crop is commercialized. Success with *P. fendleri* would lead to utilization of similar oilseed press cakes. Succinic acid, an important platform chemical is mainly produced industrially from maleic anhydride, a petrochemical intermediate as substrate. But because most research in succinic acid fermentative production is based on glucose, our goal here was to explore expansion of sugar substrate use beyond glucose so the polysaccharides from oilseed crushes could become renewable feedstuff for succinic acid production. Our initial trials found that the ruminal microorganism strain *Actinobacillus succinogenes* DSM 22,257 was capable of producing succinate using standard sugars known to be components of *P. fendleri* polysaccharides, i.e., glucose, arabinose, galactose, galacturonic acid and xylose. Subsequent trials with hydrolyzates of *P. fendleri* press cake using this strain successfully produced succinate at 93 to >98% conversion. The succinic acid produced was readily quantitatively recovered by solvent extraction of the heterogeneous fermentation broth. The crude extract was purified by recrystallization to >99% purity.

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1. Introduction

In the last decade increased awareness has driven researchers in employing traditionally available but unutilized agricultural resources that were hitherto regarded as waste materials in generating new products. Examples of such resources are post-harvest corn stover that have been transformed into low caloric fiber for the food industry. Many other additives replacing traditional ingredients that were less healthy in human food have also been introduced (Lewis et al., 1987; Kerly et al., 1987; Martel and Gould, 1990). This march to bio-based resources has been accelerated ironically by the marvelous success of the petrochemical industry that has over the century developed chemistries some of which were unintendedly non-innocuous in the environment. Adding to the health cost resulting from pollution from that success is the increasing cost of these needed petro-products. But the burning concern is the uncertainty of their continued availability in the near future. In light of the above concerns, researchers are giving serious thought to resources that could possibly replace many non-renewables and hopefully would also be more environmentally friendly. Presently, bio-ethanol and biodiesel are two obvious examples of this trend (Avci et al., 2013; Qureshi et al., 2013; Rose et al., 2010) and with time other novel products from renewables will follow. There are gradual signs that renewables from new agricultural sources are developing to counter the current debate of "food versus fuel". And this new developing paradigm suggests that the often cited inertia of *cost non-competiveness* of renewables is now broken as the cost of non-renewables continuously increase. In this regard, some important platform chemicals such as succinic acid could be produced from renewables. Succinic acid is a resource that leads to many commodity chemicals as well as monomers and polymers as shown in Fig. 1. The present commercial supply of this C4 diacid has traditionally been from petroleum-based maleic anhydride and its precursors. The biological route on the other hand converts sugars

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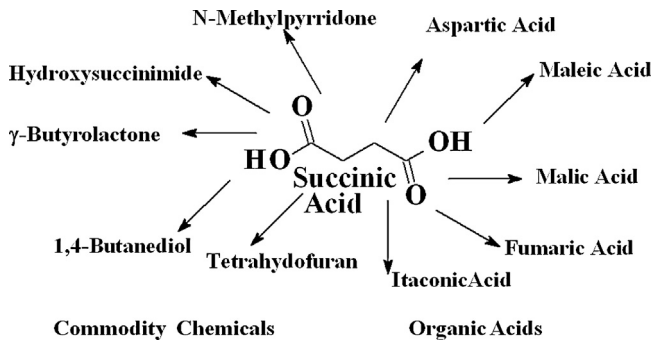

Fig. 1. Succinic acid as platform chemical to organic acids and commodity chemicals.

(glucose) to succinic acid which is a natural component of plant and animal tissues. It is also responsible for modulating many bodily processes. Succinic acid was recognized in 1886 by Robert Koch as an important intermediate in the metabolism of glucose in the tricarboxylic acid (TCA) cycle to produce energy needs that fuel living organisms (Robert, 2006) as abbreviated in Fig. 2. Since the biochemical pathways to succinic acid are known, research in this area has involved screening of series of microorganisms, selecting the most productive candidates and optimizing conditions that maximize yield of the acid in vitro using glucose as substrate (Anon., 2012a,b, 1996, 2013, 2011; Zheng et al., 2009). *P. fendleri* press cake like most oilseed press cakes is rich in polysaccharides that include glucose and could be metabolized by appropriate microorganisms. Our intended goal is to explore expansion of sugar substrate utilization in fermentation to generate succinic acid. This would put to use all the polysaccharides from oilseed crushes as renewable feedstock in the fermentative production of succinic acid. The fermentation option would add immeasurable value to an otherwise large volume of unutilized agricultural byproducts competing for limited landfill space.

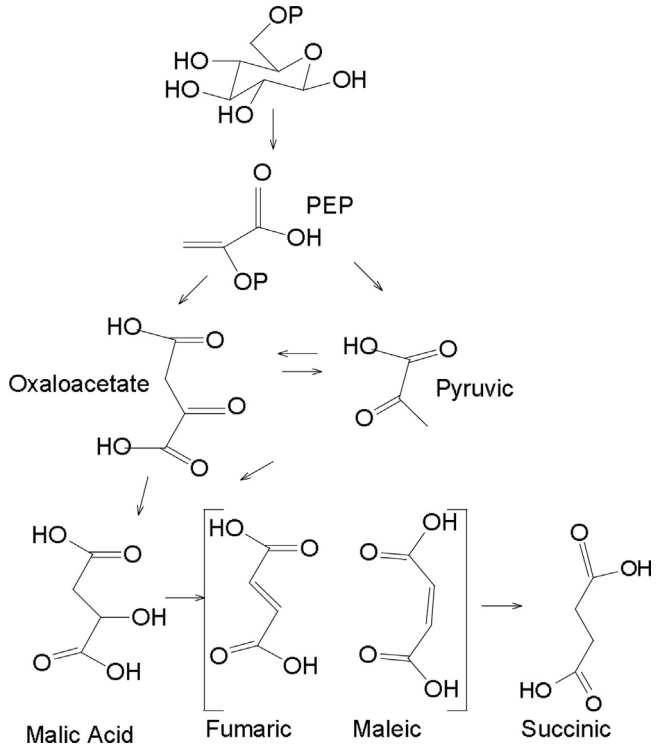

Fig. 2. Abbreviated TCA cycle, natural pathway to succinic acid from glucose-6-phosphate.

2. Materials and methods

2.1. Materials

P. fendleri press cake was obtained from seed crushed for oil in the Bio-Oils pilot plant at the National Center for Agricultural Utilization Research, Peoria, IL. Defatted press cake (kilogram quantities) was obtained by Soxhlet extraction of residual oil from the seed meal above with reagent grade hexane. The microorganism used was *Actinobacillus succinogenes* (DSM 22,257) from the USDA-ARS culture collection NRRL B-59377. Sulfuric acid [H_2SO_4], barium hydroxide [$Ba(OH)_2$], calcium hydroxide [$Ca(OH)_2$], magnesium carbonate [$MgCO_3$], sodium fumarate [$Na_2(O_2C)_2C_2H_4$] were purchased from Fisher Scientific (Chicago, IL). Brain heart infusion (BHI) was purchased from Becton, Dickinson and Company, (Sparks, MD) and brain heart infusion agar, HiVeg Media (Special infusion agar, HiVeg), was purchased from Media Laboratories Pvt. Ltd. India. K_2HPO_4, NaCl, $(NH_4)_2SO_4$, $CaCl_2$, $MgCl_2.6H_2O$, diethyl ether and hydrochloric acid, bacto peptone, bacto yeast and bacto agar, vitamin B6, lipoic acid, thiamin, riboflavin, niacin, 1,4-napthoquinone, pantothenate, B12, biotin, folic acid, methyl butyric acid, valeric acid isobutyric acid, xylose and haemin were procured from Sigma-Aldrich (St. Louis, MO).

2.2. HPLC analytical procedures

Sugars and organic acids were analyzed by high-performance liquid chromatography (HPLC). The separation system consisted of a solvent delivery system (LC-20 Prominence, Shimadzu, Kyoto Japan) equipped with an auto sampler, a refractive index detector, a UV detector and a computer software based integration system (LC solutions 1.23 SP1, Shimadzu). An ion moderated partition chromatography column (Aminex HPX-87H with a Carbo-H microguard cartridge, Bio-Rad Laboratories Inc., Hercules, CA) was used. The column was maintained at 65 °C, and the samples were eluted with 15 mM nitric acid (made with Milli-Q filtered water) at a flow rate of 0.6 mL/min. All solutes were identified and quantified by comparison to retention times of authentic sugar and succinic acid standards. Also substrates and products were analyzed by HPLC. A second separation system used consisted of a solvent delivery system (P2000 pump, Thermo Scientific, Waltham, MA) equipped with an auto sampler (717 plus, Waters Chromatography Division, Millipore Corp., Milford, MA), a refractive index detector (410 differential refractometer, Waters) and a computer software based integration system (Chromquest 4.0, Thermo Scientific). An ion moderated partition chromatography column (Aminex HPX-87P with Deashing and Carbo-P micro-guard cartridge from Bio-Rad Laboratories Inc., Hercules, CA) was used. The column was maintained at 85 °C and the sugars were eluted with Milli-Q filtered water at a flow rate of 0.6 mL/min; injection volume was 20 μL. Peaks were detected by refractive index and were identified and quantified by comparison to retention times of authentic sugar standards (Zheng et al., 2009).

2.3. Fourier transform infrared spectrometry (FT-IR)

FT-IR **s**pectra were measured on an Arid Zone FT-IR spectrometer (ABB MB-Series, Houston, TX) equipped with a DTGS detector. Solid samples (1.00 mg) of analyte was homogenized with 300 mg of spectroscopic grade dry KBr and pressed at 2400 psi to form a transparent disc for FT-IR analysis. Absorbance spectra were acquired at 4 cm^{-1} resolution and signal-averaged over 32 scans. Interferograms were Fourier transformed using cosine apodization for optimum linear response. Spectra were baseline corrected, scaled for mass differences and normalized to the methylene peak at 2927 cm^{-1}.