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Use of dry-milling derived thin stillage for producing eicosapentaenoic acid (EPA) by the fungus *Pythium irregulare*

Yi Liang [a], Xuefei Zhao [b], Megan Strait [c], Zhiyou Wen [a,*]

[a] Department of Food Science and Human Nutrition, Iowa State University, Ames, IA 50011, USA
[b] Department of Agricultural and Biosystems Engineering, Iowa State University, Ames, IA 50011, USA
[c] Biology Department, Central College, Pella, IA 50219, USA

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ABSTRACT

This study was to explore the use of thin stillage, a major byproduct in dry milling corn–ethanol plants, for production of eicosapentaenoic acid (EPA) by the fungus *Pythium irregulare*. Thin stillage contains various compounds that were ideal for fungal growth. Thin stillage concentration and temperature played important roles in fungal growth and EPA production. When 50% thin stillage was used in a stepwise temperature shift culture process, the cell density reached 23 g/L at day 9 with EPA yield and productivity of 243 and 27 mg/Lday, respectively. The fungal biomass contained 39% lipid, 28% protein, 30% carbohydrate, and 3% ash. The fungal culture also generated a nutrient-depleted liquid by removing organic compounds in the raw thin stillage. The results collectively showed a new use of thin stillage by feeding to the fungus *P. irregulare* for producing omega-3 fatty acids.

1. Introduction

The fuel ethanol production from corn in the United States has expanded rapidly over the past years. According to Renewable Fuel Association, the annual corn fuel ethanol production has reached 13 billion gallons in 2010 (http://www.ethanolrfa.org/pages/statistics). Currently, the majority of corn derived fuel ethanol is produced using a dry milling process. In this process, after ethanol is distilled from fermented beer, the residual ethanol-free slurry (whole stillage) is separated into a solid fraction (wet distillers grains) and a liquid fraction (thin stillage) (Liu, 2011). While part of the thin stillage is recycled to slurry the corn meal at the beginning of the dry milling process, the majority of thin stillage is sent to an evaporator to be condensed into a syrup-like paste with about 35% solids content. This condensed syrup is usually combined with the wet distiller's grains and dried into dried distillers grains with solubles (DDGS), which are primarily used as animal feed. During the whole downstream process, evaporation of the thin stillage is an energy-intensive process and a major cost to the ethanol plant. DDGS, as animal feed, also have only moderate value for the ethanol producers. Therefore, developing an efficient way to use thin stillage and increase the product value will enhance the economy of the dry milling ethanol production chain.

Thin stillage from a dry milling plant contains various unfermented components of the grains (e.g., fibers, oil and proteins) and yeast cells (Kim et al., 2008). These compounds are ideal nutrients for microorganisms (Dowd et al., 1993; Kim et al., 2008). For example, thin stillage was used as a carbon source for growing the fungus *Ganoderma lucidum* to produce polysaccharides (Hsieh et al., 2005). van Leeuwen et al. (2010) performed a thorough study of using thin stillage for growing a variety of fungi, which can be potentially used as animal feeds, human food and sources of nutraceuticals. The authors recently reported using thin stillage for growing oleaginous fungus *Mucor circinelloides* in an air-lift bioreactor which led to a high biomass concentration and high oil content (Mitra et al., 2012). Ahn et al. (2011) also reported using thin stillage to grow bacterium *Clostridium pasteurianum* for butanol production. Using anaerobic digestion for treating thin stillage for improving water quality and energy efficiency in dry milling ethanol plant was also investigated (Alkan-Ozkaynak and Karthikeyan, 2011).

Using thin stillage to produce eicosapentaenoic acid (EPA, C20:5, n-3) by fungal fermentation provides another outlet for this currently underutilized material. EPA is an important omega-3 polyunsaturated fatty acid that possesses many health-promoting properties such as prevention of human cardiovascular disease, cancer, schizophrenia, and Alzheimer's disease (Simopoulos, 1999). The omega-3 rich biomass is also an ideal aquafeed that can be used as a substitute for fish oil/fish meal in the aquaculture industry. Currently, commercial microbial production of EPA is still facing the challenges of high production cost. As a result, fish oil is still

* Corresponding author. Tel.: +1 515 294 0426; fax: +1 515 294 8181.
E-mail address: wenz@iastate.edu (Z. Wen).

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the predominant commercial EPA source, despite various limitations associated with fish oil such as odor/taste problems, heavy metal contamination, and limited supply.

Various researchers have used less expensive agricultural byproducts for growing the fungus *Pythium irregulare*, which is a good EPA producer. These byproducts include crude soybean oil and soymeal waste (Cheng et al., 1999), crude glycerol from a biodiesel plant (Athalye et al., 2009), and rendered animal proteins (Liang et al., 2011). However, these materials have only one function for microbial growth. For example, crude glycerol serves as a carbon source while rendered animal proteins serve as a nitrogen source for the microorganism; there is still a need to supplement other nutrients to the growth medium, which may eventually increase the medium cost. In contrast, thin stillage can be used as the sole source for the fungal growth due to its complete nutrient composition for the microorganisms. The objective of this work is to test the feasibility of using thin stillage as a nutrient source for EPA production by *P. irregulare*. The fungal culture process will also be evaluated as an effective way of treating thin stillage to prepare nutrient-depleted "clean" water that can be recycled.

2. Methods

2.1. Thin stillage source

Thin stillage samples were obtained from Lincolnway Energy (Nevada, IA), a dry-milling corn ethanol manufacturer. The samples were collected in 1-L Nalgene HDPE bottles and stored in a −20 °C freezer. Prior to use, the frozen samples were thawed and homogenized. The thawed samples were characterized for total solids, COD (chemical oxygen demand), pH, total carbohydrate, reducing sugar, glycerol, lactic acid, acetic acid, nitrogen, and phosphorus contents.

2.2. Microorganism, media and culture conditions

The fungus *P. irregulare* (ATCC 10951) was used. The fungus was grown on agar plates (containing 30 g/L glucose and 10 g/L yeast extract) for 5 days at 25 °C. The agar plates were then washed with distilled water containing glass beads to dislodge the mycelium. The mycelium suspension was stored at 4 °C prior to use as inoculum. The inoculum size was 10% of the total culture medium. The formulation of 30 g/L glucose + 10 g/L yeast extract was used as the control medium for this research. A formulation of 30 g/L glycerol + 10 g/L yeast extract was tested as another control medium in the feasibility study (Section 3.2) as glycerol was the major carbon source in the thin stillage.

In the study of EPA production from thin stillage, the fungal cells were grown in media containing different concentrations of thin stillage. The pH was adjusted to 7.0 before autoclaving the media at 121 °C for 15 min. The cells were grown in 250-mL Erlenmeyer flasks, each containing 50 mL medium, and incubated in an orbital shaker set at 200 rpm. The temperature was set to the desired levels based on experimental design. For each experimental condition, three replicates were used and the standard deviation was calculated.

2.3. Analyses

2.3.1. Cell dry weight

The fungal mycelium from each flask was harvested by filtering the spent medium through a stainless steel wire mesh screen with a nominal size of 495 microns. The majority of the solid particles in thin stillage medium passed through the screen, while large particles retained by the screen were manually removed from the screen. There was still a small amount of particles attached on the biomass surface; therefore, distilled water was used to thoroughly wash the biomass to remove these particles. The washed biomass was then transferred to a pre-weighed tube, and freeze-dried to determine the cell dry weight.

2.3.2. Thin stillage characterizations

Total solids, total COD, total Kjeldahl nitrogen (TKN), nitrate, ammonium, total phosphorus, and orthophosphate were determined according to standard methods (APHA 1995). Total carbohydrate was determined by phenol–sulfuric acid method (Dubois et al., 1956). Reducing sugars were determined by the dinitrosalicylic acid method (Ghose, 1987). Glycerol was determined by a free glycerol determination kit (Sigma). Lactic acid and acetic acid were analyzed by a Dionex ion chromatograph ICS 3000 system with an analytical column (Dionex IonPac®ICE-AS1 4X250mm, P/N 064198). The column temperature was set at 19 °C, and the eluent (1.0 mM heptafluorobutyric acid) flow rate 0.120 mL/min. The concentrations were determined by comparing peak areas with standard samples.

2.3.3. Proximate analysis

The freeze-dried fungal biomass was subjected to proximate analysis. The lipids of the biomass were extracted and quantified according to the Folch method (Folch et al., 1957). The crude protein content was estimated by measuring the TKN and multiplying by the conversion factor of 6.25. The carbohydrate was estimated by subtracting lipid, protein and ash contents from the dry biomass.

2.3.4. Fatty acid analysis

Freeze-dried fungal biomass and raw thin stillage were analyzed for their fatty acid compositions. The fatty acid methyl esters (FAME) were prepared according to the procedures described previously (Pyle et al., 2008). The fatty acid profile was analyzed by a Varian GC-450 gas chromatograph equipped with a flame ionization detector and a SGE SolGel-Wax capillary column (30 m × 0.25 mm × 0.25 μm). The fatty acids were identified by comparing the retention times with those of standard fatty acids and quantified by comparing their peak area with that of the internal standard (C17:0) (Liang et al., 2011).

3. Results and discussion

3.1. Thin stillage characterization

The chemical characteristics of thin stillage indicate a variety of organic compounds are contained in the thin stillage (Table 1). Thin stillage contains around 6.5% total solids with a total COD concentration of 112 g/L and a pH of 4.5. The carbohydrate, nitrogen and phosphorus are derived from the corn kernel remnant and the residuals of yeast cells; while glycerol, acetic acid, and lactic acid are the metabolic byproducts of the yeast cells during ethanol fermentation. Table 1 indicates that thin stillage can provide various nutrients such as carbon, nitrogen, and phosphorus for fungal growth. For example, the glycerol has been reported as an ideal carbon source for the fungus *P. irregulare* (Athalye et al., 2009). Our earlier studies have also shown that yeast extract is a suitable nutrient source for supporting the growth of *P. irregulare* by providing not only nitrogen but a variety of growth promoting factors such as B vitamins (Liang et al., 2011). Therefore, thin stillage is believed to have a similar function as yeast extract to support the growth of *P. irregulare* since some soluble compounds from the dead yeast cells after dry milling fermentation are preserved in thin stillage.