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Production of bioethanol from apple pomace by using cocultures: Conversion of agro-industrial waste to value added product

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ABSTRACT

Direct fermentation of cellulosic biomass to bioethanol has been very promising and hence attracted attention in recent years. In this study, bioethanol production from apple pomace hydrolysate (agro-industrial waste product) was investigated by coculturing *Trichoderma harzianum*, *Aspergillus sojae* and *Saccharomyces cerevisiae* using statistical approaches. Screening and optimization experiments were conducted in order to determine the significant factors and their optimum levels for maximum bioethanol production. Inoculation rates, aeration and agitation speed were considered as factor variables and bioethanol production as response variable. Highest bioethanol (EtOH) concentration and ethanol yield on total reducing sugar content ($Y_{P/S}$) were 8.748 g/L and 0.945 g/g, respectively. Optimum conditions were 6% (w/v) inoculation rates of *T.harzianum* and *A.sojae*, and 4% (v/v) inoculation rate of *S.cerevisiae* with vented aeration method and agitation speed of 200 rpm. To best of our knowledge to date, no reports are available in literature regarding the coculturing of *T.harzianum*, *A.sojae* and *S.cerevisiae* for bioethanol production. Therefore, this study will serve as a base line of initial studies in this field. The method can create a renewable alternative feedstock for fossil fuel production and suggest a feasible solution to multiple environmental problems simultaneously creating a sink for waste utilization.

1. Introduction

During the last decades the search for new alternative and renewable energy resources has increased rapidly, as a response to the increase in population that caused growing energy demand for transportation and industry and a huge consumption of limited fossil fuels. Dramatic raises in oil prices and global warming reached threatening limits. Hence, there has been a tremendous urge in extending the use of biofuels and biomass derived energy, since it can be obtained from sustainable resources [1]. Biomass, which refers to living and recently dead biological materials, is an infinite and renewable feedstock for production of biofuels [2]. However, in order to become a future alternative fuel source some properties are required. First of all, the potential candidate must decrease greenhouse gas emissions, decrease energy consumption, slow down global warming by capturing and storing CO_2 and provide efficient energy utilization. Furthermore, its production technology must be clean with regard to the environment and be economically feasible [3]. Besides, non-food feedstocks can also be used in the production of alternative fuels in order to prevent some concerns and ethical problems related to their usage. Bioethanol proved itself as an attractive low-cost alternative to replace fossil fuels with its biorenewable nature carrying all of the features required.

Fruit juice industry is one of the biggest industries in the world that forms a large quantity of wastes, such as peel, seed, pomace, rags, kernels etc. In 2009, Europe produced 11.3 billion liters of fruit juice products followed by North America with 9.5 billion liters [4]. Apple pomace is one of the wastes resulting from the food industry and contains peel, seeds and remaining solid parts formed after juice extraction. Pomace represents approximately 25–35 % of the weight of the fresh apple processed [5]. According to FAOSTAT (Food and Agriculture Organization of the United Nations Statistical Databases), total world production of apple was approximately 76 million tons by the year 2011 [6] and apple pomace constituted approximately 8 million tons [7] which causes important environmental problems. Due to its composition (richness in carbohydrates, dietary fibres and minerals, high fermentable sugar

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content), it holds a great potential to be used as raw material for the microbial production of value added products such as bioethanol. In fact pomaces are easy to obtain, harsh and expensive methods are not necessary. Within this context, utilization of apple pomace for production of bioethanol can lead the way of producing value added products from similar agro-industrial wastes and provide an alternative solution to the accumulation of waste of the fruit juice industry which is a primary environmental problem. The new technological improvements in biotechnology based on alternative biomass sources will play an important role in solving the problem related to growing energy demands.

S.cerevisiae is the most commonly used microorganism for bioethanol production because of its high production rate, but it cannot use xylose for fermentation which is one of the main sugars present in lignocellulosic biomass, especially in fruit pomaces. Various filamentous fungi, such as certain *Trichoderma* and *Aspergillus* species, have been reported to produce bioethanol as the main fermentation product from lignocellulosic biomass, directly [8,9]. These fungi are thought to contain two biological systems: one system produces cellulase enzyme for degradation of cellulose to fermentable sugars under aerobic conditions; the second system produces ethanol under anaerobic conditions [10]. However, although *Trichoderma* and *Aspergillus* are able to utilize five of the lignocellulosic sugars (glucose, mannose, galactose, xylose and arabinose) and the ability of direct fermentation of lignocellulose to bioethanol, they do not produce bioethanol with high yield and high rate. Therefore, in order to increase the fermentation yield, utilization of cocultures could be a convenient way of producing bioethanol from agricultural residues.

This study investigates the bioethanol production from apple pomace hydrolysate using the cocultures of *T.harzianum*, *A.sojae* and *S.cerevisiae,* in order to create a renewable and low cost alternative feedstock for fossil fuel production and to highlight a feasible solution to multiple environmental problems by reducing the accumulation of agro-industrial waste products.

2. Materials and methods

2.1. Apple pomace and its hydroylsation

Apple pomace, composed of almost just peels of approximately 1 cm^2 particles, was obtained from "Konfrut Fruit Juice Concentrates and Purees, Denizli, Turkey" in ice bags and stored until usage at −20 °C in plastic packages. It did not require any chopping before use.

According to previous studies conducted by Ucuncu et al. [11], temperature of 110 °C, 40 min, 4% phosphoric acid and 1:10 solid/liquid ratio (w/v) were determined as optimum hydrolysis conditions of apple pomace. Hydrolysates were filtered, pH was adjusted to 5.0, using 6N NaOH and sterilized at 121 °C for 15 min.

2.2. Microorganism and media

Total of three strains, two fungi and one yeast were used in fermentation experiments. The fungal strains, *T.harzianum* NRRL 31396 and *A.sojae* ATCC 20235, were kindly provided by Paul J. Weimer from USDA-ARS-US Dairy Forage Research Center, Madison, United States and from Food Engineering Department of İzmir Institute of Technology (IZTECH), İzmir, Turkey, respectively. The yeast, *S.cerevisiae* NRRL Y-139, was obtained from Molecular Biology Laboratory of IZTECH.

T.harzianum was incubated at 30 °C until well sporulation (5–7 days) on Malt Extract Agar (MEA) petri dishes and slants containing (g/L): malt extract, 30; peptone, 3; and agar, 15. The pre-activation of *A.sojae* cultures was done on YME (Yeast Malt Extract) agar medium containing (g/L): malt extract, 10; yeast extract, 4; glucose, 4; and agar, 20 and activation in molasses agar slants containing (g/L): glycerol, 45; molasses, 45; peptone, 18; NaCl, 5; agar, 20; and stock solutions (mg/L): $FeSO_4.7H_2O$, 15; KH_2PO_4, 60; $MgSO_4$, 50; $CuSO_4.5H_2O$, 12; and $MnSO_4.H_2O$, 15) incubated at 30 °C for one week (until well sporulation). Spores of both *T.harzianum* and *A.sojae* were harvested using 5 ml of Tween80-water (0.02% v/v) and collected in sterile falcon tubes. Spore counts were performed using Thoma bright line haemocytometer (Marienfield, Germany). *S.cerevisiae* was propagated at 30 °C for 48 h on YPD (Yeast Extract-Peptone-Dextrose) media containing % (v/v): glucose, 2; peptone, 2; yeast extract, 1; and agar, 2. A loop-full of 48 h-old single colony was transferred from a fresh YPD agar plate into 250 mL Erlenmeyer flask containing 50 mL of YPD broth media and incubated at 30 °C and 150 rpm in basic orbital shaker for 48 h, in order to construct the growth curve by measuring the viable cell counts and optical densities using a Varian Cary Bio 100 spectrophotometer at 600 nm.

2.3. Fermentation

2.3.1. Aerobic growth

A.sojae was grown in 250 mL Erlenmeyer flasks containing 50 mL molasses broth media. Initial spore count was adjusted to approximately 1×10^7 spore/mL and used for the inoculation of the flasks which were incubated at 30 °C in a 200 rpm rotary shaker based on a study conducted by Skory et al. [9]. Incubation time was determined as 48 h in order to obtain larger pellets. *T.harzianum* was grown on MM (minimal medium) which was the YNB (Yeast Nitrogen Base medium) of Wickerham and Burton [12] with glucose as carbon source. Flasks were inoculated with spores (1×10^7 spore/mL) and incubated at 30 °C in a 150 rpm rotary shaker. *S.cerevisiae* was grown until reaching the log phase in a 150 rpm rotary shaker on YPD broth media at 30 °C.

2.3.2. Anaerobic fermentation

The mycelial mass coming from aerobically grown cultures was added into the anaerobic fermentation media, which was the apple pomace hydrolysate. 40 mL hydrolysate was added into 50-mL Erlenmeyer flasks in order to leave ~20% of the culture flask volume as air space. Fermentation experiments were conducted for 5 days at 30 °C. Samples were taken within certain time intervals, centrifuged at 6000 g for 15 min. The supernatants were stored at −18 °C for further analysis.

2.4. Assays

The amount of bioethanol in the supernatant was determined using HPLC (High Pressure Liquid Chromatography) equipped with RI (refractive index) detector and an Aminex HPX-87H column with an appropriate guard column (Bio-Rad, USA) at a flow rate of 0.6 mL/min. The temperatures of the column and detector were 60 °C and 50 °C, respectively. The mobile phase was 5 mM H_2SO_4 filtered through 0.2 μm filter and degassed.

The biomass represented as dry cell weight (DCW − (g/L)) was determined by the gravimetric method. The total carbohydrate content of the samples (cell-free supernatant) was determined according to the phenol sulphuric acid method described by Dubois et al. [13]. The amount of carbohydrates was determined by using Varian Cary Bio 100 UV–Visible spectrophotometer at 490 nm against the blank. The total reducing sugar amount was determined according to the assay given by Somogyi [14]. The absorbance was read on Varian Cary Bio 100 UV–Visible spectrophotometer at 500 nm against water.