Bioresource Technology 144 (2013) 684–688

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Short Communication

Sustainable bio-production of styrene from forest waste

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HIGHLIGHTS

- A new way of utilizing abundantly available tree bark.
- Styrene production using forest waste biomass.
- A strain of *Penicillium expansum* produced styrene when grown on tree bark media.
- 52.5 μg/h styrene production rate from fungus grown on 10 g bark media.

ARTICLE INFO

Article history:
Received 28 April 2013
Received in revised form 3 July 2013
Accepted 9 July 2013
Available online 15 July 2013

Keywords:
Forest biomass
Penicillium expansum
Styrene
Bark
Fungal volatiles

ABSTRACT

A strain of *Penicillium expansum* was studied for the production of styrene using forest waste biomass as a feeding substrate. The fungal strain was cultivated on bark of various trees supplemented with yeast extract and the volatiles produced were collected on Tenax TA and analyzed by gas chromatography–mass spectrometry. Fungus cultured on grated soft bark of pine (*Pinus sylvestris*) stems (GPB) and mature bark of oak (*Quercus robur*) supplemented with yeast extract produced relatively the highest amounts of styrene. The maximum styrene production rate was 52.5 μg/h, 41 μg/h and 27 μg/h from fungus cultivated on 50 mL liquid media with 10 g GPB or mature bark of oak and potato dextrose broth respectively. These promising results suggest that the fungal strain could be used to produce "green" styrene plastics using renewable forest waste biomass.

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1. Introduction

Today, plastic products are very important in our daily life with anything from pens, packing materials, pipes, helmets and computers are made from plastics. Styrene is an organic hydrocarbon and a precursor to polystyrene and other copolymers. It is used as a building block for the manufacturing of a broad range of materials like rubber and plastic components used in hundreds of products throughout the world. In addition, fiberglass products used for boats are also made from polyester resins dissolved in styrene. In 2008, annual production of styrene in the U.S was more than 6 million kg (Rosemond et al., 2010). More than 65% of styrene produced is used in the production of polystyrene plastics and resins (James and Castor, 2005).

Styrene is produced from petroleum products on a commercial basis. It is mainly (90%) produced by the catalytic dehydrogenation of ethylbenzene that is a catalytic alkylation product of benzene and ethylene. Both raw materials of synthetic styrene are derived from fossil fuel (James and Castor, 2005). There are also many environmental and health issues related to styrene production using benzene (Yardley-Jones et al., 1991). Increasing petroleum prices, diminishing world petroleum resources and cost of raw materials are major concerns and alternative ways to produce energy and important industrial chemicals like styrene using environmentally friendly and sustainable biomass are needed (Cherubini, 2010; McKenna and Nielsen, 2011).

There are a number of reports in literature describing the production of styrene by microorganisms cultivated on various synthetic or semi synthetic substrates. For example, styrene was produced by the decomposition of trans-cinnamic acid by *Pichia carsonii* (Shimada et al., 1992); two strains of *P. expansum* cultured on malt extract agar (Fiedler et al., 2001); strains of *P. citrinum*, *P. oxalicum*, *Aspergillus niger* cultivated on cinnamon related compounds supplemented with buffered peptone water (Lafeuille et al., 2009). Beck et al. (2008) reported styrene production from *Fusarium oxysporum* by cultivating it on potato dextrose broth (PDB) and proposed glucose as the biosynthetic precursor of

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http://dx.doi.org/10.1016/j.biortech.2013.07.033

styrene. In 2011 McKenna and Nielsen used an engineered *Escherichia coli* strain to convert phenylalanine and cinnamic acid to styrene. Recently, Du et al. (2013) reported the production of styrene and other aromatics by catalytic pyrolysis of a microalgae *Chlorella vulgaris*.

Over the last few decades there has been an emphasis on the use of renewable resources for energy production and as raw materials for industrial products. Agriculture and forestry wastes are the biggest sources of biomass (Ahmed et al., 2012; Clark and Deswarte, 2008) that could increase the economic growth of industries without compromising on environmental issues. Plant based biomass can for example be converted to liquid fuel or industrial chemicals by using microorganisms (Huang et al., 2009; Kim et al., 2013; Miura et al., 2004). However, there are no reports on styrene production using agricultural or forest waste materials.

The present study focuses on the production of styrene from *Penicillium expansum* cultivated on forest waste biomass such as fresh leaves, wood, soft bark and also mature bark of Scots pine (*Pinus sylvestris* L.), Pedunculate Oak (*Quercus robur* L.), Norway spruce (*Picea abies* (L.) H. Karst), and Silver Birch (*Betula pendula* Roth) in the presence of yeast extract. Optimum conditions and potential biomass for the maximum production of styrene are investigated and reported.

2. Methods

2.1. Fungus seed culture

Yeast extract broth (YEB) was prepared by adding 5 g yeast extract (purchased from Sigma–Aldrich, Sweden) to 1 L distilled water and autoclaved at 121 °C for 20 min. A loop of *P. expansum* strain was inoculated (previously isolated from *Hylobius abietis* frass (Azeem et al., 2013)) to 200 mL YEB in a flask and incubated under static conditions at room temperature (22 ± 2 °C) for two days and then incubated in an orbital shaker with 80 RPM and 25 °C for another two days. The culture broth was mixed thoroughly by vigorous shaking and, after removing larger particles, the homogenized mixture was used as seed culture to inoculate feed stock broths.

2.2. Pine tree (Pinus sylvestris) waste biomass media

We tested three different parts of pine tree including fresh pine leaves (PN), grated soft bark of actively growing pine stems (GPB) with particle size 2–4 mm, and pine wood (PW) pieces with size (10 mm × 15 mm × 2 mm). 10 g of each substrate were autoclaved at 121 °C for 20 min along with 40 ml YEB in 1 L quick fit Erlenmeyer flasks. Potato dextrose broth (PDB) was prepared (40 mL) and autoclaved separately in 1 L flasks (20 g potato extract powder and 20 g glucose in 1 L distilled water). Four replicates of each treatment were prepared as described above. Two replicates of each treatment were inoculated with 10 mL seed culture (Section 2.1) of *P. expansum* and 10 mL of YEB was added to the control replicates. All the flasks were covered with aluminum foil and incubated at room temperature without shaking.

2.3. Mature bark media

Oven dried mature bark (taken from the main trunk of about 30 year old tree) pieces (10 mm × 15 mm × 4 mm) of pine (*P. sylvestris*), spruce (*Piceae abies*), oak (*Quercus roubur*) and birch (*Betula pendula*) were used as feeding substrate for the fungus under two different conditions. 10 g of each tree bark were supplemented with either 40 mL (liquid fermentation) or 10 mL (solid state fermentation) YEB in 1 L E-flask. Four replicates of each tree bark and each culturing condition were prepared and inoculated with fungus seed culture as described in Section 2.2.

2.4. Volatile collection

Volatiles produced by fungus grown in different biomass media were collected on days 5, 9, 13 and 18 using a dynamic volatiles collection setup (Fäldt et al., 2000). Prior to collection of volatiles, volatiles that had accumulated in the flasks were removed by pumping charcoal filtered clean air into the flasks for 5 min at rate of 200 mL/min using a pump (NMP 830 KNDC B, KNF Germany) and flow meter (GPE Scientific, UK). After that volatiles from the flasks were collected on Tenax TA adsorbent (60/80 mesh, Supelco, Sweden), packed in glass tubes, for 60 min. Tenax adsorbed compounds were desorbed in hexane and the first 15 or 25 drops (approx. 330 μL or 550 μL) were collected in glass vials and analyzed by GC–MS without further concentrating. Tenax TA tubes were cleaned by using hexane and dried at 180 °C for 5 min before each volatile collection occasion.

2.5. Chemical analysis

All the extracted volatiles were analyzed by gas chromatography–mass spectrometry (GC–MS) using a Varian 3400 GC connected to a Finnigan SSQ 7000 quadrupole MS. The GC was equipped with a split/split less injector (split less mode 30 s) and a DB-WAX capillary column (30 m, 0.25 mm ID and 25 μm film, J & W Agilent, USA). The temperature program was: 45 °C for 0.5 min followed by 8 °C/min up to 235 °C and then isothermal at 235 °C for 5.75 min. The injector temperature was 230 °C whereas the interface between the GC and MS was isothermally set at 235 °C. The temperature of the ion source was 150 °C. Mass spectra were obtained at 70 eV with a mass range of 30 m/z to 400 m/z. 1 μL of the samples were injected. Separated compounds were identified by comparing their mass spectra to NIST-08 MS library data base and confirmed by analyzing pure standards (purchased from Sigma–Aldrich, Sweden) at the same parameters.

Styrene quantification was carried out by making a standard curve using five different concentrations (0.00056 μg/μL, 0.0028 μg/μL, 0.014 μg/μL, 0.07 μg/μL, and 0.35 μg/μL) prepared from pure styrene standard solutions. Plotting the amount of styrene versus the area of the peak gave rise to a straight line passing through origin with the line equation $y = 4E + 9x$ and $R^2 = 0.992$.

3. Results and discussion

3.1. Styrene production from fungus cultivated on pine tree biomass

The major compound produced by *P. expansum* cultivated on all the tested media was styrene. The fungus cultivated on media containing different parts of pine tree started producing styrene by day 5, whereas fungus cultured on PDB started emitting styrene by day 9 (Fig. 1). On the 13th day of incubation the maximum styrene production rate was observed from *P. expansum* cultivated on GPB medium (52.5 μg/h), PN medium (14.8 μg/h) and on PDB medium (27 μg/h) whereas fungus grown on PW medium produced only a minute amount of styrene (Fig. 1).

Fungus cultured on GPB and PDB produced almost pure styrene (Table 1). Volatiles collected from the headspace of fungus inoculated on PN and PW media were contaminated with large amounts of 3-carene whereas volatiles from fungus cultured on GPB medium contained more than 80% of styrene with some contaminants from the grated soft bark itself (Table 1). Styrene production using GPB was better than PDB media, which is normally considered

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