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Cleaner production of vanillin through biotransformation of ferulic acid esters from agroresidue by *Streptomyces sannanensis*

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ABSTRACT

Vanillin (4-hydroxy-3-methoxybenzaldehyde; $C_8H_8O_3$) is the major organoleptic compound of vanilla flavor originally obtained from *Vanilla planifolia*. Attempt has been made for production of vanillin from ferulic acid esters present in wheat bran, the agro-residue. *Streptomyces sannanensis* MTCC 6637 was used for biotransformation of ferulic acid esters. The key enzymes involving vanillin production were assayed and the products were estimated using thin layer chromatography and high pressure liquid chromatography. Vanillin production was optimized through response surface methodology. Optimum vanillin production (708 mg L^{-1}) was achieved with de-starched wheat bran (10% w/v), sucrose (0.2% w/v), peptone (1% w/v) at pH 7.5, agitation 220 rpm, and temperature 28 °C and fermentation continued for a period of 5 days. The experimental strain converts ferulic acid ester into ferulic acid with the help of ferulic acid esterase. Ferulic acid was catabolized through Coenzyme-A dependent non−β-oxidation (retro-aldol reaction) of ferulic acid. Feruloyal Coenzyme-A synthetase and Enoyl-Coenzyme-A hydratase/aldolase were involved in conversion of ferulic acid into vanillin. Transient formation of vanillic acid from vanillin was found due to steady state expression of vanillin dehydrogenase. To the best of authors' knowledge this is the first report of vanillin production directly from agroresidue by *S. sannanensis*.

1. Introduction

Vanillin is the major organoleptic compound of natural vanilla obtained from beans of *Vanilla planifolia*, an orchid. Vanillin is one of the most demanding food additives also used in beverages, perfumes and pharmaceuticals (Priefert et al., 2001). The natural production from *Vanilla* bean, beside its high cost, cannot satisfy the total vanillin demand of the world. Annually, more than 12,000 tons of vanillin is produced out of which only 1% originates from *V. planifolia* (Sindhwani et al., 2017). Considering the increased interest in natural products, the production of flavors via biotransformation process offers a viable alternative to the natural and chemical sources (Sindhwani et al., 2017). According to European commission (EC) legislation, production of nature identical vanillin can be achieved either by microbial or enzymatic transformation of a precursor or by microbial *de novo* synthesis, that may well be referred as biovanillin (Zamzuri and Abd-Aziz, 2013).

Biotechnologically vanillin production may be based on microbial fermentation processes or on bioconversion of natural precursor using tailored microbial cells or enzymes. Ferulic acid [4-hydroxy-3-methoxycinnamate (FA)] is a phenolic compound, linked to the plant cell wall through ester bonds and can be released by enzymatic hydrolysis of the ester bonds (di Gioia et al., 2007). The cell wall of agroresidue like wheat bran contains approximately 95% (w/w) phenolic compounds (Parker et al., 2005). The major drawback of microbial production of vanillin is it's the high price of its raw material i.e. FA. Green productions of vanillin from wood chips (Modahl et al., 2015) and bamboo (Harshvardhan et al., 2017) through bacterial consortium are also attempted that could produce vanillin through lignin biodegradation. The search for possible eco-friendly single-step vanillin production system through different mechanism (biotransformation/

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bioconversions/*de novo* synthesis) using various biological agents (microorganisms/plant cells/isolated enzymes) is still going on. Agroresidues like wheat bran are cheap and contains ferulic acid esters (FAE) that may directly be used as substrate for cleaner production of vanillin. Employing agroresidue as the raw material for vanillin production represents an interesting way of disposing the wastes and at the same time value addition to them. To achieve this, the qualified strain has to be survived well in presence of phenolic acids by hydrolyzing ester linkage present in agroresidue through ferulic acid esterase (FAEase), and to convert FA into vanillin. Present study describes biotransformation of FAE of de-starched wheat bran (DSWB) into vanillin by *Streptomyces sannanensis* (MTCC 6637). The present paper also deals with the process optimization to check the potentiality of this system for commercial vanillin production in future.

2. Materials and methods

2.1. Strain used

Streptomyces sannanensis MTCC 6637 was procured from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. The strain was maintained in Arginine Glycerol Salt (AGS) slants at 4 °C.

2.2. Agroresidue used

Wheat bran is a reach source of FAE (Kim et al., 2006). Wheat (*Triticum aestivum*) bran samples were collected from local market at Santiniketan, India. Collected wheat bran was de-starched following the method of Sarangi and Sahoo (2010). Physical properties of wheat bran (particle size, intrinsic toughness, intrinsic strength) vary with the cell wall composition and milling quality (Liu, 2013). However, physical properties become identical in de-starched wheat bran (DSWB). DSWB was used in all the experiments as primary source of carbohydrate unless otherwise stated.

2.3. Fermentation of DSWB

S. sannanensis MTCC 6637 was grown in liquid minimal medium (Muheim and Lerch, 1999) containing wheat bran (1%, w/v) as a sole carbon source in 250 mL flasks containing 50 mL medium. The initial pH of the medium was adjusted to 7.0 and sterilized by autoclaving for 15 min at 15 lbs. Fermentation was carried at 35 °C and analyzed daily up to 14 days.

2.4. Growth curve determination

To obtain the dry weight the biomass was separated from fermented broth by centrifugation (1000xg for 20 min at 4 °C) and dried in a hot air oven (45 °C) unless the weight reaches a constant value. To determine the growth, biomass concentration (dry weight) was monitored throughout the fermentation period at every 3 h interval.

2.5. Extraction and analysis of biotransformed products

The fermented broth was separated from biomass by centrifugation (1000xg for 20 min at 4 °C). The supernatant was acidified (pH 1.0–2.0) with 1 M hydrochloric acid (HCl), and extracted with equal volume of ethyl acetate. The ethyl acetate was evaporated in a vacuum evaporator and the residue was dissolved in 50% methanol. In this process the fermented broth was subjected to thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC) (Sachan et al., 2004). HPLC data were analyzed and quantified based on custom software.

2.6. Preparation of crude cell extracts

For preparation of cell extract *S. sannanensis* MTCC 6637 was grown as described with on DSWB as sole carbon source. Cell pellet was collected (by centrifugation at 12,000 rpm for 20 min), washed (in cold Tris–HCl buffer, 50 mM, pH 7.8), and sonicated (in short bursts of 30 s with a total exposure time of 5 min). Cell extract was again centrifuged and the supernatant was collected, concentrated (using rotary vacuum evaporator), sterilized (through 0.45-μm membrane filter) and was used as crude extract for enzyme assay and *in vitro* conversion study.

2.7. Assay of key enzymes of DSWB biotransformation

To monitor the biotransform process, four key enzymes of the FAE biotransformation catabolic route (Gallage and Moller, 2015) were estimated throughout the fermentation process. For assay of extracellular FAEase, the centrifuged fermented broth was extracted with equal volume of ethyl acetate and FA was measured spectrophotometrically at 310 nm against the standard curve (Kaur et al., 2013). To estimate enoyl-Coenzyme-A hydratase/aldolase (ECH), crude cell extract (0.5 mL) was mixed with equal volume reaction mixture [90 mM sodium phosphate buffer (pH 7.0), 3 mM Magnesium chloride ($MgCl_2$), and 0.2 mM 4-hydroxy-3-methoxyphenyl-bhydroxypropionyl-Coenzyme-A], and incubated at 30 °C (Overhage et al., 1999). The resultant vanillin was estimated through HPLC. For feruloyl-Coenzyme-A synthetase (FCS) assay crude cell extract (0.5 mL) was mixed with equal volume reaction mixture [100 mM potassium phosphate buffer (pH 7.0), 2.5 mM $MgCl_2$, 0.7 mM ferulic acid, 2 mM Adenosine three phosphate (ATP), 0.4 mM Coenzyme-A (CoA)], and incubated at 30 °C. The resultant feruloyl-CoA was measures spectrophotometrically at 345 nm against the standard curve (Overhage et al., 1999). For measuring vanillin dehydrogenase (VDH) activity, cell free fermented broth was mixed with equal volume reaction mixture [vanillin 1 gL^{-1}, triammonium citrate 2 gL^{-1}, sodium acetate 2 gL^{-1}, magnesium sulphate 50.1 gL^{-1}, manganous sulphate 0.05 gL^{-1}, di-potassium hydrogen phosphate 2 gL^{-1}; pH 5.6] and incubated at 37 °C for 24 h (Kaur et al., 2013). Thereafter 1 mL of reaction mixture carefully mixed with 5 mL of HCl (24% v/v) and 2 mL of thiobarbituric acid (1% w/v) and heated in a 55 °C for 10 min and allowed to cool at room temperature for 20 min. The absorbance was recorded at 434 nm. The specific activity in each case was expressed in term of product produced min^{-1} mg^{-1} protein (U mg^{-1} protein).

2.8. Determining the metabolic route of DSWB biotransformation

2.8.1. In vivo conversion study

In order to explore the metabolic route of DSWB biotransformation by *S. sannanensis*, DSWB two separate set of experiments were carried out. In first set, ferulic acid, vanillin and vanillic acid were independently supplied to the medium as a sole carbon source. The fermented broth was analyzed up to eight days for the detection of transformed product(s). To explore the CoA thioesters-dependent route of biotransformation, in second set, *S. sannanensis* was grown on minimal medium supplemented with a metabolic inhibitor [3,4 (Methylenedioxy)-cinnamic acid (MDCA)], and changes in the accumulation of products were compared to control as previously described by Ghosh et al. (2007).