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Aromatic hydrocarbon biodegradation activates neutral lipid biosynthesis in oleaginous yeast

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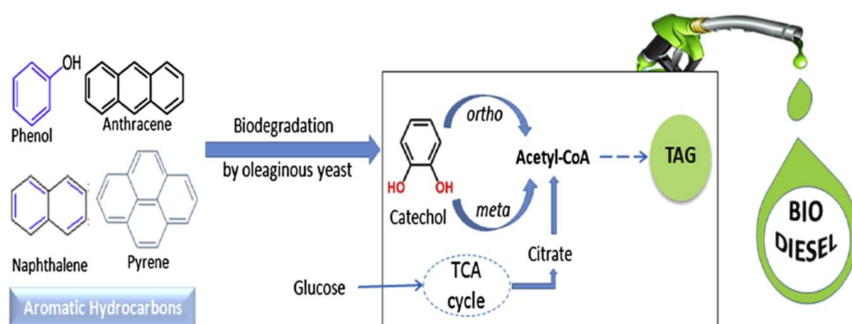
GRAPHICAL ABSTRACT



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ABSTRACT

In this study, the biodegradation ability of oleaginous yeast *Cryptococcus psychrotolerans* IITRFD for aromatic hydrocarbons (AHs) was investigated. It was found to completely degrade range of AHs such as 1 g/L phenol, 0.75 g/L naphthalene, 0.50 g/L anthracene and 0.50 g/L pyrene with lipid productivity (g/L/h) of 0.0444, 0.0441, 0.0394 and 0.0383, respectively. This work demonstrated the ring cleavage pathways of AHs by this yeast which follow *ortho* route for phenol and naphthalene while *meta* route for anthracene and pyrene degradation. The end products generated during biodegradation of AHs are feed as precursors for *de novo* triacylglycerols (TAG) biosynthesis pathway of oleaginous yeast. A high quantity of lipid content (46.54%) was observed on phenol as compared to lipid content on naphthalene (46.38%), anthracene (44.97%) and pyrene (44.16%). The lipid profile revealed by GC–MS analysis shows elevated monounsaturated fatty acid (MUFA) content with improved biodiesel quality.

1. Introduction

Aromatic hydrocarbons (AHs) are ubiquitous environmental pollutants produced during the incomplete combustion of wood, petrol, oil and coal (Abdel-Shafy and Mansour, 2016; Igwe and Ukaogo, 2015). AHs possess genotoxic, carcinogenic, mutagenic and toxic properties due to which their fate in nature is of huge environmental concern (Mrozik et al., 2003). Thus, the elimination of these environmental contaminants from wastewaters such as industrial effluent is essential. Among different remediation technologies, AHs degradation via microorganisms is the most sustainable way to remove these contaminants in a cost-effective and safe means with less input of time, energy and chemicals (Balachandran et al., 2012). Yeasts are found to be the most important AHs bio-degraders which can utilize AHs as a sole carbon and

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energy source (Pan et al., 2004). They can adapt the extreme conditions of waste streams quite rapidly using toxic compounds as energy source for their growth (Abdel-Shafy and Mansour, 2016). Yeast species such as *Candida* spps., *Rhodotorula* spps., *Saccharomyces rosinii*, *Cryptococcus* spps., *Rhodosporidium* spps. and *Trichosporon* spps. have been found to degrade wide variety of AHs (Patel et al., 2017a; Gargouri et al., 2015; El-Naggar et al., 2012; Margesin et al., 2003). The *Cryptococcus* sps are often utilized for metabolic pathway studies evaluating the biodegradation of many aromatic compounds like phenol, anthracene and phenanthrene (Margesin et al., 2003). The aerobic biodegradation pathway of aromatic compounds generally involve three stages (i) aromatic ring activation, (ii) dearomatization, (iii) conversion of the ring-cleavage products to the intermediary metabolites of tricarboxylic acid (TCA) cycle (Mahiudddin et al., 2012; Patel et al., 2017a). The typical pathway for the metabolic cleavage of AH is to dehydroxylate the benzene ring to produce a catechol derivative by hydroxylase enzyme followed by ring cleavage through *ortho* (intradiol) or *meta* (extradiol) oxidation pathway. Catechol get oxidized to 2-hydroxymuconic semialdehyde (HMSA) by catechol 2,3-dioxygenase (C2,3-D) cleavage enzyme via *meta*- pathway or to cis, *cis*-muconic acid by catechol 1, 2-dioxygenase (C1,2-D) cleavage enzyme via *ortho*- pathway (Cao et al., 2008). HMSA is then degraded either via the 4-oxalocrotonate (OC) route or the hydrolytic route (Comte et al., 2013). The end products generated from both the pathways enter the TCA cycle and subsequently degradation takes place (Balachandran et al., 2012).

The *ortho* and *meta* routes for degradation of AHs depends on several factors such as microbial species, structure and nature of the substrate to be degraded as well as on environmental conditions (Abdel-Shafy and Mansour, 2016; Cao et al., 2008). For example, *Rhodotorula* sps and *Arthrobacter* sps by activating both the routes can degrade phenol up to 5 mM and 10 mM, respectively at 10 °C while the *Cryptococcus* sps follows the *ortho* route at same conditions with 12.5 mM of phenol degradation (Margesin et al., 2003). *Rhodotorula, Candida and Cryptococcus* strains of yeast exhibits C1,2-D activity in cells grown on phenol, indicating that catechol ring fission occurs by *ortho* cleavage pathway (Margesin et al., 2003). While *Rhodosporidium* follow *meta* cleavage pathway by C2,3-D for the degradation of phenol (Patel et al., 2017a). However, there is a need to understand the biodegradation of different types of AHs in oleaginous yeast *Cryptococcus psychrotolerans* IITRFD.

Oleaginous yeast can accumulate neutral lipid mainly in triacylglycerols (TAG) form inside their lipid droplets (LDs) and may utilize large number of low-cost renewable substrates for their growth (Deeba et al., 2016). The *C. psychrotolerans* IITRFD can convert the carbon source present in the medium into TAG in its intracellular compartment (Deeba et al., 2017a). The present study deals with the pioneering investigation to know the mechanism of degradation of different AHs in *C. psychrotolerans* IITRFD and proposing the hypothesis for utilization of their final products as precursors for *de-novo* TAG biosynthesis pathway. The data reveals that in this oleaginous yeast, anthracene and pyrene is degraded via *meta* cleavage pathway which give rise to acetaldehyde and pyruvate as final products. Moreover, naphthalene and pyrene are degraded by *ortho* cleavage pathway which produces succinyl-CoA and acetyl CoA as end products. Pyruvate and succinyl-CoA produced can be directly metabolized by TCA cycle while acetaldehyde by the action of acetaldehyde dehydrogenase (AcDH) gets converted to acetyl-CoA (Boubekeur et al., 2001; Patel et al., 2017a). The acetyl-CoA form serve as the precursor for *de novo* TAG biosynthesis pathway also called Kennedy Pathway (Qin et al., 2017). The neutral lipid accumulation in this yeast was confirmed by Nile red fluorescence cell imaging technique and degradation pathway was identified via enzyme assay. The enhanced TAG obtained was further transesterified to generate fatty acid methyl esters (FAMEs) i.e. biodiesel. This study predicts the metabolic route of aromatic compounds degradation leading to build up of acetyl-CoA which is a central building block for fatty acid (FA) synthesis. The end products of the *meta* and *ortho* cleavage pathway of AHs degradation; pyruvate, succinate, acetyl-CoA and acetaldehyde feed as precursors for TAG biosynthesis which causes higher accumulation of TAG *C. psychrotolerans* IITRFD. Patel et al. (2017a) have shown enhanced lipid accumulation with only phenol degradation by oleaginous yeast (Patel et al., 2017a) while in this study we have utilized different types of AHs.

This is the first novel study to illustrate *C. psychrotolerans* IITRFD ability of utilizing wide range of MAH (phenol) and PAHs (naphthalene, anthracene and pyrene) by *ortho* or *meta* degradation pathway with improved lipid agglomeration. The main objective of this work is to enhance lipid accumulation and to assess the mechanism of AH biodegradation in *C. psychrotolerans* IITRFD with simultaneous production of biodiesel.

2. Materials and methods

2.1. Chemicals and media

Yeast nitrogen base (YNB), phenol, naphthalene, anthracene, pyrene, glucose, yeast extract peptone dextrose (YPD) and agar were purchased from Himedia (India) for growth and batch cultivation of oleaginous yeast. Tris-HCl buffer, 2-mercaptoethanol, catechol, ammonium hydroxide, potassium ferricyanide [$K_3Fe(CN)_6$] and 4-amino antipyrine were procured from Sigma Aldrich (USA) for determining residual phenol concentration and AHs degradation pathway. For lipid extraction solvents such as chloroform, methanol and hexane were obtained from Merk (India). Neutral lipid staining red fluorescent dye, Nile red was obtained from Invitrogen (Life Technology, USA). All the solvents and chemicals used in this study were of the highest purity available.

2.2. Microorganism and growth condition

C. psychrotolerans IITRFD, oleaginous yeast used in this study was isolated from the water sample collected from Hooghly River, West Bengal (India) (Deeba et al., 2017a). Inoculums were prepared in 50 ml YPD medium, supplemented with AHs (0.25%, w/v) by inoculating the activated culture broth of this strain having 0.063 optical density (OD) at 600 nm and incubated for 48 h at 25 °C on a rotary shaker at 200 rpm (Patel et al., 2017a, 2017b). Further, the 100 ml YNB medium was inoculated with the yeast culture (2%) for batch cultivation experiments (25 °C, 144 h).

2.3. Biodegradation experiment of AHs

Different aromatic compounds were supplemented as carbon source to the medium. The aromatic compounds used in this experiment were: phenol, naphthalene, anthracene and pyrene. The YNB medium (100 ml) containing glucose (4%) was supplemented with one of these aromatic compounds (g/L) having 0.25, 0.50, 0.75, 1 and 1.25 concentrations in different flasks. Further, the medium was inoculated with the yeast culture and incubated at 25 °C (pH 7) at 200 rpm for 144 h in an orbital shaker. Thus for each aromatic compound taken five experiments were carried out. The YNB medium with no supplementation of aromatic compound was taken as control. Sterilization of AHs solution was done by 0.22 μm filter with the help of a syringe. The growth of the culture was detected by measuring the cell density at OD_{600nm}. Samples were withdrawn from the growing culture at every 12 h of interval to evaluate the cell growth, lipid accumulation and degradation of the AH. After 144 h the culture broth was centrifuged at 9000*g* for 10 min to harvest the biomass.

2.4. Estimation of residual AH

The residual phenol concentrations in the sample collected were evaluated by following the standard 4-aminoantipyrine colorimetric

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