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Improvement of fungal lipids esterification process by bacterial lipase for biodiesel synthesis

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HIGHLIGHTS

- Lipase producing isolates were tested for their application in FAME synthesis.
- The highest FAME synthesis from fungal lipids was obtained by *B. firmus* lipases.
- *B. firmus* displayed a higher thermal stability and methanol tolerance.
- The immobilized *B. firmus* cells have been proven to be important in enhancing FAMEs.
- Separation of the hydrolysis from esterification process improves the FAME synthesis.

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ABSTRACT

Lipase-catalyzed biotransformation of fungal lipids into biodiesel via bacterial enzymes or whole cell catalysts has been considered as one of the most promising methods to produce applicable, renewable and environmentally friendly alternative liquid fuels. Four highly lipolytic bacterial isolates were isolated from seeds and grains of some plant species and screened for their potentiality to synthesis of fatty acid methyl esters (FAME) by lipase esterification process for the production of cost-competitive biodiesel. The four isolates were identified based on phenotypic and gene encoding 16S rRNA as *Bacillus vallismortis* ASU 3 (KP777551), *Bacillus tequilensis* ASU 11 (KP777550), *Bacillus amyloliquefaciens* ASU 16 (KP777549) and *Bacillus firmus* ASU 32 (KP777552). Among the four tested bacterial lipases, extracellular lipase of *B firmus* ASU 32 (KP777552) showed the highest activity toward the transesterification of fungal lipids as 71.2% of total fatty acid methyl eseters (FAMEs). *B. firmus* ASU 32 lipases displayed a higher thermal stability and methanol tolerance ensuring their application as a promising biocatalyst for FAME synthesis. The results proved that, the most active acyl acceptors for biodiesel production from fungal lipids by *B. firmus* lipase were methanol and ethyl acetate. *B. firmus* has applicable future as a whole cell biocatalyst for FAME synthesis from fungal lipids. Alleviation the inhibitory effect of methanol in the transesterification process of fungal lipids by lipases might be performed through separation of hydrolysis step from esterification process by methanol for FAME synthesis. This paper is expected to provide a competitive economic outcome for industrial FAME synthesis.

1. Introduction

During the few next decades, the world's energy system is likely to radically change, with energy consumption expected to double. A depletion in ancient energy stock reserves, additionally to political, economic, health and environmental issues enhances scientists to go looking for alternative sustainable fuel from available sources are coming in vogue [1–5]. Biodiesel is one of the most potential renewable fuels, environmental friendliness, biodegradable, non-toxic, a low air pollutant emitting fuel than the petroleum diesel and has a similar engine performance [4]. Oleaginous microorganisms have the capability to accumulate significant amounts of storage triacylglycerols, considered to be a viable and attractive substitute for vegetable oils and animal fats for biodiesel production [6,7]. Biodiesel production using microbial lipids as single cell oils (SCO), has attracted great attention in the whole world.

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Although there are all kinds of microorganisms accumulating oils, such as algae, bacteria, fungi and yeasts that produce more than 20% (g/g) biomass lipid are known as oleaginous microbes [8–11].

Conventionally, biodiesel is produced by transesterification of triacylglycerides and short chain alcohols (methyl, ethyl, propyl) in the presence of chemical catalysts (acids or alkali) [12]. Although, chemical transesterification proved to be the most economic process because it has high fatty acid esters yield, low catalyst cost and high process productivity [13]. There are serious drawbacks that limit the utilization of chemical transesterification technology for FAME synthesis, such as glycerol and catalyst recovery from biodiesel, soap formation, removal of inorganic salt, high temperature, the corrosive effect of acids with very slow reaction rate, environmental pollution, waste water treatment and intensive energy needed. So, the acids and alkali transesterification processes require extensive downstream processing and multi-step purification of end products during FAME production [14]. These obstacles led to search for different strategies for FAME production. Enzymatic transesterification by lipases may become an attractive alternative solution for FAME synthesis over chemical methods because of the reduced feedstock limitations, downstream processing and environmental impact, enzymes do not form soaps, are not severely inhibited by water, so there is little concern about water production [15]. Lipases are capable of completely converting free fatty acids to fatty acid alkyl esters (FAAEs), while the free fatty acids contained in waste oils and fats can be completely converted to alkyl esters [16]. The enzymes are most often immobilized when used, which simplify the separation of products, produce a high quality glycerol, can also be reused for multiple reactions and able to tolerate enzyme inhibitory effect of methanol and glycerol [17].

Also, lipase transesterification process has many advantages; there are some major drawbacks that effect on feasibility and commercialization of FAME production, e.g. longer reaction time, higher catalyst concentration is required to completion of reaction and high cost of production [18]. The cost of lipase makes up 90% of the total cost of enzymatic FAME production [19]. To avoid these drawbacks, the system outlined in the present paper aimed to detect the potentiality of different bacterial lipases for the production of cost-competitive biodiesel by lipase transesterification process of immobilized potent bacterial cells and free-cell enzyme. In addition to, the present paper outlined FAME synthesis by direct bacterial growth as biocatalysts on fungal biomass. Such system may improve the efficiency and commercialization of FAME yield by reducing the cost involved in the purification or immobilization steps.

2. Materials and methods

2.1. Culture medium and cultivation method for fungal lipid production

The flask culture medium contained (g l^{-1}) sugarcane molasses, 79; peptone bacteriological (Oxoid), 4 and chloramphenicol 0.25. Sugarcane molasses was obtained from Sugar Institute Technology, Assiut University, Assiut and kept in refrigerator until used. Some physical and chemical characteristics of sugarcane molasses were described previously [4]. The initial pH of the culture medium was adjusted to 7. After sterilization the medium was inoculated with a spore suspension (1×10^6 spores ml^{-1}) of oleaginous fungus *Cunninghamella echinulata* [5]. Flask cultures of *C. echinulata* were carried out in 250-ml Erlenmeyer flasks containing 100 ml of sterilized medium on a rotary shaker (Environ 3597-1, Labline Instruments, USA) at 120 rpm at 30 °C for 15 days. After 15 days mycelial mats were harvested and filtered by filter paper (Whatman No. 1). Morphological characteristics of *C. echinulata* were studied using light and electron microscope techniques. Free-hand sections of fresh fungal mycelia and the lipid droplets in the hyphae were examined using an Olympus CX 41 microscope equipped with a digital camera (S30 Olympus Digital Camera, Japan). The fungal morphology and lipid accumulation were visualized by scanning and transmission electron microscopes. The sample preparation and fixation were performed as described by Abd-Alla et al. [20,21].

2.2. Preparation of fungal dry biomass

Fresh fungal mycelia containing lipid were washed with sterilized distilled water several times to remove the residual sugars from fungal mycelium and dried at 50 °C up to constant weight. The dried fungal biomass was ground to fine powder by mortar and pistil and kept for further experiments as a substrate for FAME synthesis.

2.3. Isolation of lipase producing bacteria

Bacterial isolates were recovered from seeds and grains on lipase detection medium containing (g l^{-1}): peptone, 15.0; NaCl, 5.0; $CaCl_2$, 1.0; tween 80, 10.0 ml and agar, 15.0 [22]. Five surface sterilized seeds or grains with 1% sodium hypochlorite were dispersed in Petri dishes containing lipase detection medium and incubated at 37 °C for 3 days. Formation of white precipitate of calcium monolaurate around the grown colonies indicates a positive lipase activity [22]. Bacterial isolates were selected and purified by sub-culturing single colonies on nutrient agar medium (NA). One colony of the purified bacterial isolates was selected and retained on nutrient agar slants at 4 °C for further experiments.

2.4. Screening for extracellular lipase production

2.4.1. Culture medium and cultivation conditions

Bacterial isolates were grown aerobically in an Erlenmeyer flask containing 25 ml of liquid medium containing (g l^{-1}): bacteriological peptone, 15.0; yeast extract, 5.0; NaCl, 2.0; $MgSO_4$, 0.4; K_2HPO_4, 0.3; KH_2PO_4, 0.3 and tween 80, 10.0 ml for lipase induction [23]. Cultures were incubated in an orbital shaking incubator for 36 h at 120 rpm and 37 °C. The culture broth was then centrifuged at 8000 rpm to remove cells. The clear supernatant was collected for lipase assay.

2.4.2. Assay of lipase enzyme activity

Lipase activity was determined with p-nitrophenylpalmitate (pNPP) as described by Pera et al. [24]. The standard curve was prepared using para-nitrophenol (0.4–4 μmoles). One lipase unit is defined as the amount of enzyme that liberated 1 μM p-nitrophenol per min. under the assay conditions as described by Parra et al. [25]. Each experiment was conducted with three replicates.

2.4.3. Lipase specific activity

Lipase specific activity is defined as the number of lipase units per mg extracellular protein. The total extracellular protein was measured in extracellular crude enzyme as described by Lowry et al. [26] a standard curve prepared using bovine serum albumin.

2.5. Phenotypic and genotypic characterization of highly lipase producing bacterial isolates

2.5.1. Phenotypic characterization of bacterial isolates

Bacteriological characteristics (Gram stain, oxidase reaction, arginine dihydroxylase activity, nitrate reduction, utilization of carbohydrates, catalase test, gelatin hydrolysis, starch hydrolysis and esculine hydrolyzation) of the four highly lipase producing