Defence Science Journal, Vol. 60, No. 3, May 2010, pp. 251-254

Emulsification and Hydrolysis of Oil by *Syncephalastrum racemosum*

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ABSTRACT

A fungal strain, *Syncephalastrum racemosum,* was isolated from oil-leak contaminated soils from Kanpur, India. The strain was examined for potential to emulsify soybean oil using a 18 per cent oil supplement as carbon source in minimal salt medium. On 72 h growth of the fungus in oil and salt medium, the cell-free supernatant (CFS) showed presence of mono- and di-glycerides indicating degradation of oils to free fatty acids (FFAs). Increasing concentration of oil resulted in enhanced formation of FFAs. The degradation process was observed to be related to the emulsification activity in CFS which was observed to increase with time. The study reports the emulsification and hydrolytic activity of *S. racemosum,* an activity that can be exploited for increasing the accessibility and treatment of hazardous substance including hydrophobic explosives.

Keywords: Fungus, *Syncephalastrum racemosum,* emulsification, esterification, vegetable oil, biotransformation, lipolysis

1. INTRODUCTION

Biotransformation of water-immiscible substances, such as hydrocarbons and oils, is limited due to the low availability of these compounds to microbial cells[1]. In natural or induced adapted conditions, many microbial systems, viz., fungi and bacteria, are able to produce excellent emulsifying agents which facilitate enhancing hydrophobic substrate utilisation[2]. Kosaric[3] indicated the use of biosurfactants in facilitating the bioaccessibility of hazardous substances such as explosives, metals, waxes, petroleum products, and processes. Associated with the generation of surface active substances, cell-bound esterase synthesis has been recently reported indicating the coupled function of emulsification with lipolytic activity[4,5]. Paraszkiewicz[6], *et al*. reported that filamentous fungus, *Curvularia lunata,* released emulsifying agent from the exponential phase to the middle of the stationary phase. The surfactant concentration in the case of *C. lunata* was from 0.3 g/l (the emulsifier introduced with inoculum at 0 h of growth) up to 2.6 g/l for 47 h of incubation and then decreased. Bioemulsifier production by yeast *Candida utilis* was dependent upon process and carbon source conditions[7]. However, such reports have been limited to bacterial species only with scarce observations with fungi. The present study is the preliminary report discussing the emulsification activity and lipolysis of soybean oil, used as carbon source during growth of *Syncephalastrum racemosum.*

2. MATERIALS AND METHODS

The heterothallic fungus, *S. racemosum,* has been isolated from oil-contaminated soil surrounding petrol/diesel storage underground bins in Kanpur, India. The oil contamination of the soil was observed to be due to spillovers during the filling of bins. The test strain was isolated using standard methods and repeated sub-culturing on potato-dextrose agar (PDA). The organism was characterised using classical morphological and biochemical techniques by the microbial type culture collection and gene bank (MTCC). Earlier report by Gopinath[8], *et al*. on *S. racemosum,* isolated from oil-rich environment indicated extracellular enzyme activity in this strain.

For these studies, the isolate was grown in potato-dextrose broth (PDB-Himedia) with 1 per cent soybean oil at 28 °C on orbital shaker set at 120 rpm to get organism acclimatised to oil as carbon source. After 48 h of incubation, the biomass devoid of PDB was transferred to 150 ml Czepak Dox medium supplemented with increasing concentration (4–18 %) of soybean oil as sole carbon source in 250 ml Erlenmeyer flask and maintained in the above-mentioned conditions. The biomass was filtered and the CFS was collected from all the flasks after 72 h to examine the surfactant activity and products of oil degradation. The CFS was allowed to settle in separating funnels for approx. 3 h to separate oil and spent medium.The test culture was found to be viable after 72 h exposure to oil.

Received 08 July 2009, Revised 31 August 2009

The cell free supernatant separated after exposure to 4 per cent oil was subjected to xylene emulsification method[5] to assay emulsification activity. 35 µl of the cell-free supernatant or Tris buffer (2.0 mM, pH 8.0) were used as control, plus an equal volume of xylol (SD Fine) was added to the surface of 5 ml of 20 mM Tris buffer, in a glass test tube. The mixture was vigorously shaken for 45 s and allowed to stand for 20 min in a cuvette before taking reading for the optical density (660 nm) against control blank using visible spectrophotometer (Hitachi U2800). The increase in optical density (Table 1) was taken as indicator of the presence of surface-active substances causing dispersion of oil in the buffer.

Table 1. Emulsification activity (optical density) of CFS from growth medium of *S. racemosum* after 48 h of incubation in Czepak Dox plus 4 % soybean oil (average of three replicates)

Condition	With xylene	With soybean oil
Czepak Dox broth	0	0
CFS after 24 h	0.360 ± 0.05^{b}	0.190 ± 0.03^{a}
CFS after 48 h	0.404 ± 0.1^{b}	0.267 ± 0.05^{b}

Note: The significant difference in observations from one another at 5 % confidence limits is indicated by 'a' and 'b'.

Breakdown of soybean oil into free fatty acid (FFA) was also analysed by thin layer chromatography (TLC). For TLC plate preparation, slurry of silica-H (TLC grade) was prepared in hexane. Glass plates were immersed in the slurry and air-dried. Solvent system of hexane: diethyl ether: acetic acid was used in the ratio of 77:22:1. The spots were developed in iodine chamber and compared with those reported in the literature[9]. The observation was further extended to 10 days by allowing the growth of organism with oil in shaking condition as outlined earlier.

3. RESULTS AND DISCUSSION

In the present study, soil fungus *S. racemosum,* hitherto unknown to produce emulsification activity is observed to not only produce surface-active substances but also facilitate hydrophobic substrate utilisation through simultaneous breakdown of the vegetable oil. The strain is from the order *Mucorales* and family *Syncephalastraceae*. It has two species. This strain is mainly found in dunk and soil in tropical, subtropical regions, and sometimes from leaf litter too. *S. racemosum* forms light to dark-grey, and very fast growing colonies. The sporangiophores are 10-15 µm wide, merosporangia with spores are smooth-walled, globose-to-ovoid, 3-5 µm in diameter. The fungal strain has been designated code MTCC 9623 by microbial type culture collection facility at Institute of Microbial Technology, Chandigarh. Hitherto, many fungi have been reported to facilitate emulsification of water immiscible hydrocarbons, this property is being reported in *S. racemosum* only to a limited extent.

Emulsifying surface-active substances are a subclass of surfactants that stabilise dispersion of one liquid in oil in water emulsion[10]. Bioemulsifiers effectively enhance growth on bound substrates by desorbing and mobilising them from surfaces or by increasing their apparent water solubility.[11] The emulsification activity of cell-free supernatant noted in the study (Table 1), using xylene emulsification method, showed notable and comparable stability of emulsion layer with reference to other reports on bacteria such as *Bacillus stearothermophilus*[12] and *Bacillus* sp[13]. However, in contrast to majority of the bacterial emulsifiers[14,15] and comparable to emulsifiers from fungal sources[6,16], the bioemulsifying substance produced by *S. racemosum* does not seem to be solely dependent on hydrocarbons for stabilising the emulsifying activity. Xylene is known to emulsify hydrophobic substances better than other substances due to the presence of water-miscible fraction, and is therefore generally used as strandard for examining emulsification activity[17].

In the present study, the yield of crude emulsifying substance was 4 g/l which significantly increased in exponential phase with gradual stabilisation at early stationary phase. The process of purification is in progress and is expected to yield 1.5 g/l to 2 g/l of pure substance. Smut fungi *Ustilago maydis* DSM 4500 and ATCC 14826, when grown on vegetable oils produced glycolipid biosurfactant on a level comparable with *C. lunata* IM 2901 (2 g/l)[16]. Similar kinetics of biosurfactant production (mixed growth- and non-growth-associated) was described for bacterial cell-free emulsan produced by *Acinetobacter calcoaceticus*[18].

Observably, the natural role of emulsifying substances is to facilitate bioavailability of hydrophobic water insoluble substrates. Such substances that lower interfacial tension are particularly effective in mobilising bound hydrophobic molecules and making them available for biodegradation and subsequent assimilation[10]. In the current study, exposure of *S. racemosum* to soybean oil as sole carbon source resulted in generation of emulsifying substances, thus facilitating the mobilisation of oil into water layer. Examination using TLC with emulsified oil separated after 72 h of exposure to soybean oil as carbon source indicated its mobilisation and degradation into glycerides and free fatty acids (Fig. 1). However, better activity was observed in the presence of xylene when compared to soybean oil as the former is observed to facilitate accelerated production of emulsifiers by microbial systems[17].

The results indicated a well-defined breaking of oil into different components such as mono- and di-glycerides along with fatty acids (Fig. 2). The breaking of oil was more prominent in growth medium with 18 per cent of oil as carbon source when compared to other oil level additions. There was no significant variation in the degradation between fresh oil and supplementation of 8 per cent oil in the growth medium, which is indicative that a sufficient substitution of oil is required to facilitate its use as carbon source and subsequent degradation. The present study, however, could not make a comparison with standard bioemulsifiers.

Extracellular emulsifying agents, broadly produced

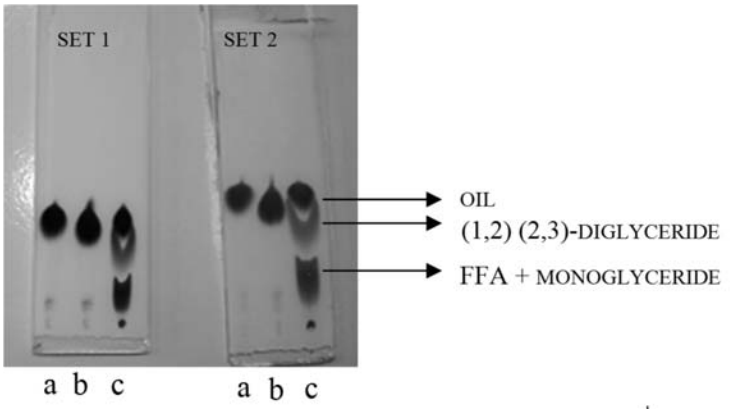

Figure 1. Thin layer chromatogram (in duplicate) showing breaking of oil (lane *c*) in comparison to pure oil (lane *a*) and abiotic growth medium with pure oil (lane *b*) maintained in the same conditions as that of experimental flasks.

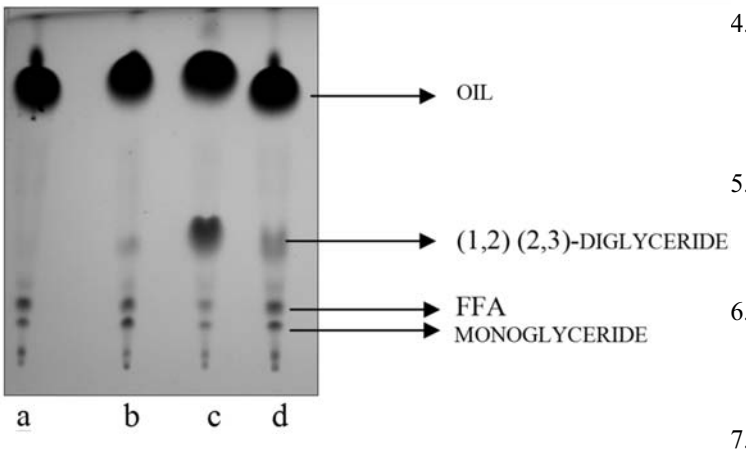

Figure 2. Thin layer chromatogram showing breaking of oil with increasing concentration of oil (lane *a* – fresh oil [control]; lane *b* – 8 %; lane *c* – 16 %; lane *d* – 18 %) as carbon source during growth of the organisms after 10 day exposure.

by bacterial species, constitute high-molecular-mass polysaccharides associated with proteins[4]. Some examples include emulsan from *A. calcoaceticus* and alasan from *A. radioresistens* K53. The studies carried out by earlier researchers with bacterial emulsifiers show that the proteins associated with the emulsifying agents are possibly involved in activities similar to esterases significantly increasing the biodegradation of hydrophobic chemical moieties such as polyaromatic hydrocarbons,[14,19] organo-chlorines[20], and petroleum hydrocarbons[21]. The results obtained in the present study are also indicative that along with the production of the emulsifying substance, the enzymatic substances are also released which are observed to catalyse the breaking of oil, the action of which presumably is facilitated through extracellular emulsification. The association between emulsification and lipolysis indicate that the emulsifier is presumably a lipoprotein[21].

4. CONCLUSIONS

The study thus presents the preliminary but significant findings of the emulsification and lipolytic activity of extracellular substances produced by *S. racemosum*. Further work is being done on (i) isolation and purification of emulsifying agent in the extracellular medium and (ii) exploitation of emulsification nature of the extracellular medium from *S. racemosum* for facilitation of the bioaccessibility of hydrophobic substances.

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