Appl Biochem Biotechnol (2011) 163:720–728
DOI 10.1007/s12010-010-9077-y

Pretreatment of Synthetic Dairy Wastewater Using the Sophorolipid-Producing Yeast *Candida bombicola*

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Received: 21 June 2010 / Accepted: 30 August 2010 /
Published online: 6 September 2010

Abstract The presence of high strength fats and oils in dairy industry wastewaters poses serious challenges for biological treatment systems, and, therefore, its pretreatment is necessary in order to remove them. In the present study, synthetic dairy wastewater prepared in the laboratory was pretreated using the sophorolipid-producing yeast *Candida bombicola* in a laboratory-scale bioreactor under batch, fed-batch, and continuous modes of operation. To support the yeast growth, the wastewater was supplemented with sugarcane molasses (1% *w*/*v*) and yeast extract (0.1% *w*/*v*). Results from the batch operated fermentor revealed complete utilization of fats present in the wastewater within 96 h with more than 93% COD removal efficiency. The yeast was, however, able to pretreat the wastewater more quickly and efficiently under fed-batch mode of operation than under batch operated condition in the same fermentor. Continuous experiments were carried out with a wastewater retention time of 28 h in the reactor; results showed very good performance of the system in complete utilization of fats and COD removal efficiency of more than 90%. The study proved the excellent potential of the biosurfactant-producing yeast in pretreating high-fat- and oil-containing dairy industry wastewater.

Keywords Dairy wastewater · Pretreatment · *Candida bombicola* · Fats and oils removal · Sophorolipid

Introduction

Dairy industries have increased in most of the countries of the world including India due to the steady rise in demand of milk and milk products [1, 2]. In dairy industries, water is a key processing medium, and, therefore, a large amount of wastewater is generated from different operations including cleaning, sanitization, floor washing, etc. [3]. In addition to being rich in biodegradable organic molecules and nutrients such as

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ammonia or minerals and phosphates, dairy wastewaters contain high level of fats and oils which are not easily biodegradable and often interfere with the normal biological treatment process [4]. These fats and oils in dairy wastewater are essentially triglycerides consisting of straight-chain fatty acids attached, as esters, to glycerol [5], and the hydrolytic product of these fats and oils are long chain fatty acids that can be toxic to various microorganisms. Therefore, it is essential to pretreat the wastewater in order to remove fats and oils. A large number of pretreatment methods are employed to remove them from dairy wastewater prior to biological treatment, which includes grease-trap, tilted plate separators, dissolved air flotation systems, and other physico-chemical treatment methods. However, the cost of these methods is considered high, and removal efficiency of fats and oils is low [4, 6, 7].

Even though biological pretreatment methods such as use of hydrolytic enzymes to degrade the fats and oils are more effective than physical, chemical, and physico-chemical methods [8–10], production cost is a major limitation. Alternative methods such as thermophilic processes and use of surfactants facilitate the biodegradation of fats and oils primarily by increasing its solubility. In fact, the use of chemical as well as biological surfactants to aid in the emulsification and removal of oils from wastewaters has been explored and used successfully for the treatment of wool-scouring wastewater and high strength pet food wastewater [11, 12]. However, pretreatment of dairy wastewater using biosurfactants and/or its producing microorganism has not been evaluated so far. Therefore, in the present study, synthetic dairy wastewater containing high fats and oils was prepared in laboratory, and its pretreatment by the sophorolipid-producing yeast *Candida bombicola* was evaluated.

Materials and Methods

Microorganism and Maintenance

The yeast *Starmerella bombicola* NRRL Y-17069 (equivalent strain of *C. bombicola* ATCC 22214) was procured from Agricultural Research Service (ARS-Culture collection), USDA, Peoria, USA. The strain was grown, according to the supplier's instructions, for 48 h at 30 °C incubation on agar slants containing (g l^{-1}): glucose, 10; yeast extract, 3; peptone, 5; and agar, 20 (GYP agar). The microorganism was sub-cultured in every 4 weeks and maintained at 4 °C in a refrigerator.

Chemicals and Reagents

All chemicals and solvents used in the study were of analytical grade and supplied by either Hi-Media Pvt. Ltd., India, or Merck India Ltd. Sugarcane molasses, milk powder, and fats (ghee) used in the study were purchased from local market in Guwahati, India.

Seed Culture Preparation

The medium used for developing the yeast seed culture contained (g l^{-1}): glucose, 100; yeast extract, 10; urea, 1, pH 6.0 [13]. The 250-ml Erlenmeyer flasks containing 50 ml of the seed culture media were autoclaved at 121 °C for 20 min and inoculated with a loop full of the microorganism freshly grown on GYP agar slant. The culture was then incubated for 48 h at 30 °C and 180 rpm in a rotating orbital incubator shaker.

Preparation of Synthetic Dairy Wastewater

Synthetic dairy wastewater based on dairy wastewater composition was prepared in the laboratory as reported by Leal et al. and contained dried milk powder (2 g l^{-1}) and fat (0.2 g l^{-1}) [14]. The major constituents of commercially available milk powder (Amulya™, Amul, India) and fats (Ghee, Britania, India) used to prepare the synthetic dairy wastewater are mentioned in Tables 1 and 2, respectively. The synthetic wastewater had an initial pH 6.0 and an average COD content of 2,800 mg l^{-1}.

Pretreatment of the Synthetic Dairy Wastewater

All experiments to investigate the efficiency of the yeast in pretreating the synthetic dairy wastewater to remove fats and oils were carried out in a 3-L fermentor (Applikon, Holland) with 1-L working volume. For the inoculation, 5% of the yeast seed culture was used. To support the yeast growth, a small amount of sugarcane molasses (1% *w*/*v*) and yeast extract (0.1% *w*/*v*) was added to the synthetic wastewater. The agitation and aeration in the bioreactor were set at 300 rpm and 1.5 l m^{-1}, respectively. The initial pH of the synthetic wastewater was kept the same at 6.0 and was not controlled during the experiments. Initial experiment using the fermentor was carried out in batch mode and later on under fed-batch and continuous modes to evaluate the pretreatment efficiency of the wastewater in the study. Samples were taken during the experiments and analyzed for fats, yeast biomass, sophorolipids, total carbohydrate concentration, and COD.

Analytical Methods

Estimation of Fats Analyses of fats in the samples were performed as per the standard methods for water and wastewater treatment [15]. Briefly, samples collected at different time intervals during the experiments were extracted twice with *n*-hexane, and the organic layer containing the fats was separated and vacuum-dried at 40 °C to remove the *n*-hexane, and the residue obtained after gravimetric analysis was taken as the total fats present.

Estimation of Total Carbohydrate, Sophorolipid, and Yeast Biomass For the estimation of sophorolipids and yeast biomass, samples taken during the experiments were extracted twice with equal volume of ethyl acetate; following separation of the two layers, the aqueous layer was centrifuged at 12,000×*g* for 15 min at 25 °C, and the cell pellets were washed twice with distilled water and dried to constant weight at 80 °C to determine the

Table 1 Composition of the commercially available milk powder (Amulya™, Amul, India) used in the preparation of the synthetic dairy wastewater

Constituents	Amount per 100 g
Total fat	20 g
Saturated fat	12.4 g
Cholesterol	52 mg
Sodium	88 mg
Total carbohydrates	50 g
Sucrose	18 g
Protein	20 g
Calcium	1 g

Table 2 Composition of the commercially available fat (Britania, India; local name: ghee) used in the preparation of the synthetic dairy wastewater

Constituents	Amount per 100 g
Milk fat	99.7 g
Saturated fatty acids	63.0 g
Poly unsaturated fatty acids	1.7 g
Mono unsaturated fatty acids	24.5 g
Trans fatty acids	3.0 g
Cholesterol	0.4 g
Vitamin A	700 μg

yeast biomass concentration [16]. The aqueous layer obtained after centrifugation was analyzed for total carbohydrate content in the sample by the Anthrone method [17].

For the sophorolipids analysis, the previously obtained ethyl acetate extract was vacuum-dried at 40 °C to remove the solvent. The obtained residue was washed twice with *n*-hexane to remove any unutilized fats in the wastewater. Partially purified sophorolipids were thus obtained after vaporizing the residual hexane at 40 °C under vacuum, and its yield were calculated by gravimetric analysis.

Estimation of COD Samples were centrifuged at 8,000 rpm for 15 min to separate any yeast biomass and sophorolipids. The obtained supernatant was used for the COD analyses as per the standard methods for water and wastewater treatment [15].

Results and Discussion

Pretreatment of dairy wastewater to remove fats and oils using physical and/or chemical methods are largely limited due to poor efficiency of the methods, high cost, or both. Although biological methods using microorganisms and/or their products such as hydrolytic enzymes are usually preferred [4, 10], the major problem with such methods is that the hydrolysis products of fats and oils present in wastewater are mainly long chain fatty acids which are potentially toxic to several microorganisms commonly employed in secondary treatment of such high strength wastewaters [18, 19]. As an alternative approach to pretreat dairy wastewater, the present study investigated the potential of biosurfactant-producing yeast in utilizing the fats and oils present in the wastewater.

Batch Operation

Results of initial batch experiment carried out using the synthetic dairy wastewater prepared in the laboratory showed that the yeast could remove only a maximum of 50% of initial COD even after 120 h and also utilized only a small amount of the fats present in the wastewater (Fig. 1). The produced sophorolipid was found to be below detectable limit which could be attributed to the poor growth of the yeast. Hence, in order to improve the pretreatment efficiency and to support the yeast growth during the process, small amounts of sugarcane molasses (1%) and yeast extract (0.1%) were added to the wastewater.

Figure 2 shows the results of pretreating the synthetic wastewater supplemented with sugarcane molasses (1%) and yeast extract (0.1%) in the batch operated fermentor

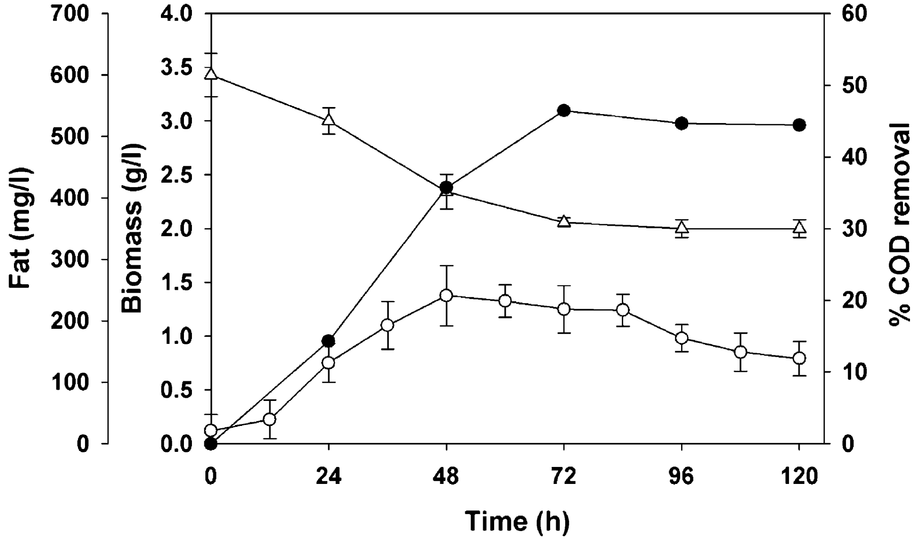

Fig. 1 COD reduction, fat utilization, and yeast biomass growth by *C. bombicola* using the synthetic dairy wastewater in the fermentor operated under batch mode (*empty circles* yeast biomass, *empty triangles* fat, *filled circles* % COD removal)

which reveals complete utilization of fats in the wastewater within 96 h; also, the total carbohydrate remaining to be utilized was negligible with a maximum biomass concentration obtained at 72 h. Further, the yeast showed very high COD removal efficiency of more than 93% in the system (Fig. 2). The amount of sophorolipid produced by the yeast was 1–2 g l^{-1}, which was sufficient enough to completely solubilize the

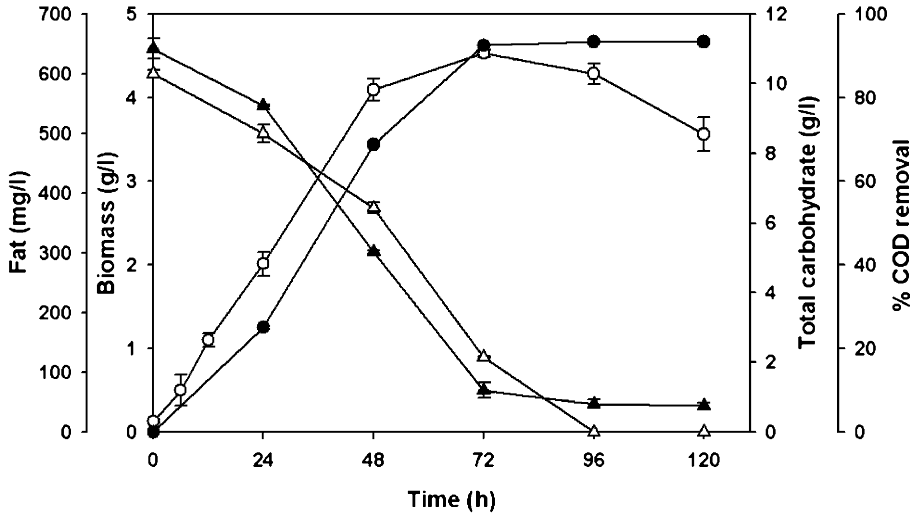

Fig. 2 COD reduction, fat utilization, carbohydrate utilization, and yeast biomass growth by *C. bombicola* using the synthetic dairy wastewater supplemented with sugarcane molasses (1%) and yeast extract (0.1%) in the fermentor operated under batch mode (*empty circles* yeast biomass, *filled triangles* total carbohydrate, *empty triangles* fat, *filled circles* % COD removal)

fats in the wastewater. Loperena et al. isolated fat/protein degrading microorganisms from different locations of a dairy wastewater treatment plant and tested potential of the isolates in treating synthetic dairy wastewater having an initial COD of 3,000 mg l^{-1} [20]. An inoculum of the best isolates (*Bacillus pumillus* strain VII, *Pseudomonas* strain 23, and *Acinetobacter* strain 38) in the study was able to remove only 75% of fats and 57% of COD from the synthetic dairy wastewater. Compared with this result, the present study showed excellent results in terms of COD removal and fats utilization. Recently, Jacobucci et al. studied the utilization and COD reduction of oily soap industry effluent by two bacterial strains capable of producing biosurfactants [21]. The authors reported that both the strains *Planococcus citreus* and *Pantoea agglomerans* were able to remove 76% and 70% COD of oily effluent, respectively. It was also suggested that in situ biosurfactant production by the microorganisms can be a potential approach for pretreatment of such oily wastewaters.

Following batch experiments in the fermentor, fed-batch and continuous modes of operation were further evaluated to investigate the efficiency of the biosurfactant-producing yeast in pretreating the wastewater.

Fed-Batch Operation

Fed-batch operation to treat wastewaters is a relatively new concept and only a few reports are available. Using this mode of operation, while Kargi and Dincer successfully treated saline wastewater [22], Bali and Sengul achieved treatment of wastewater containing 4-chlorophenol [23].

Fed-batch operation in a bioreactor usually involves, at the end of batch operation, slow addition of a feed containing highly concentrated wastewater or nutrients into the bioreactor with no effluent removal until the tank is full. During the operation, due to sufficiently large volume of highly active and dense organisms in the reactor, dilution of the feed followed by high COD/BOD removal rates are effected in the system.

To improve the pretreatment efficiency of the synthetic wastewater in the present study, fed-batch experiment was carried out in a 3-L fermentor with 1-L initial working volume. For the operation of the fermentor under fed-batch mode, the following equation was used to calculate the feed rate of the wastewater [24]:

$$F = \frac{\mu X_0 V_0 e^{\mu t}}{Y_{X/s} S_0} \tag{1}$$

where F is the feed rate of the wastewater (h^{-1}); X_0 is the yeast biomass concentration at the end of batch operation (g l^{-1}); V_0 is the volume of wastewater in the fermentor at the end of batch operation; S_0 is total carbohydrate concentration taken as input to the wastewater.

Specific growth rate, μ (h^{-1}), and yield of yeast biomass, $Y_{X/S}$, were the kinetic constants estimated from the previously run batch experiment, and for which the following equations were used:

$$\mu = \frac{1}{X}\frac{dx}{dt} \tag{2}$$

$$Y_{X/S} = \frac{X_m - X_0}{S_0 - S_m} \tag{3}$$

In Eq. 3, X_m represents maximum cell concentration (g l^{-1}) at time (t), X_0 is the initial cell concentration (g l^{-1}) at initial time (t=0), S_m is the total substrate concentration (g l^{-1}) at time (t), and S_0 is the total substrate concentration (g l^{-1}) at initial time (t=0).

Based on data obtained (μ, 0.072 h^{-1} and $Y_{X/S}$, 0.675) from the batch experiment, fed-batch experiment was carried out at the end of 48 h of batch.

Figure 3 shows the results of COD reduction, fats utilization, yeast biomass, and carbohydrate utilization in the wastewater in the fermentor operated under fed-batch mode, which indicate complete utilization of fats in the wastewater within 10 h of fed-batch operation and 93% COD removal efficiency at the end of 84 h. Similar to the batch experiments, approximately 2 g/l of sophorolipids was obtained in the fed-batch experiments with the fermentor. However, the wastewater volume (2 l) treated under fed-batch mode of operation was twice the volume treated under batch mode. Also, under fed-batch mode the time required for the yeast to completely utilize the fats present in the wastewater was less (60 h) compared with the time (96 h) required in the batch experiments.

All these results clearly established that under fed-batch mode of operation, the yeast could utilize the fats and pretreat the wastewater more quickly and efficiently than under batch-operated condition. However, since continuous mode of operation is most often the norm for real-time wastewater treatment, the same was evaluated further in the study.

Continuous Operation

For improved productivity of the treatment systems, continuous operations are always preferred. In this study, for the operation of the fermentor under continuous mode, the reactor was initially run under batch mode up to 72 h and then shifted to continuous mode with a wastewater retention time (HRT) of 28.5 h, which corresponded to a dilution rate of 0.035 h^{-1} in the reactor. Figure 4 depicts the results obtained from the continuous

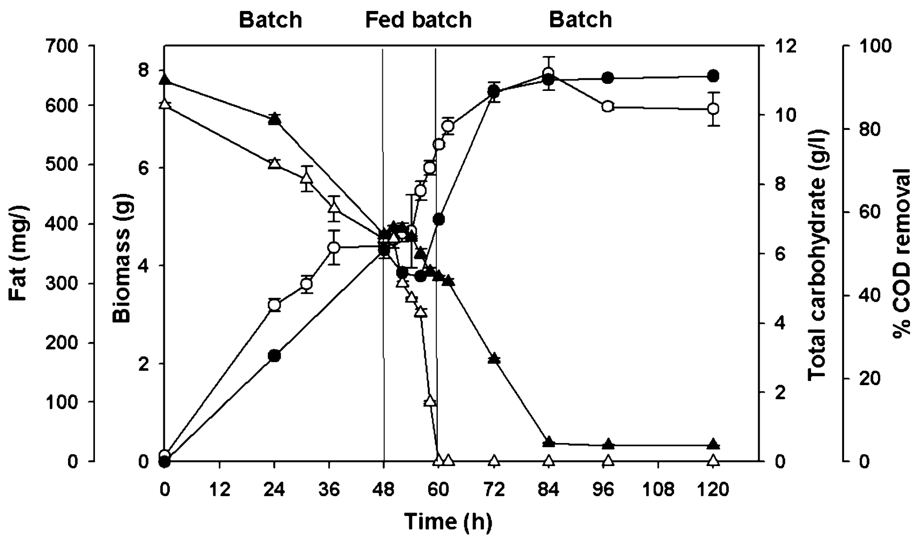

Fig. 3 COD reduction, fat utilization, carbohydrate utilization, and yeast biomass growth by *C. bombicola* using the synthetic dairy wastewater supplemented with sugarcane molasses (1%) and yeast extract (0.1%) in the fermentor operated under fed-batch mode (*empty circles* yeast biomass, *filled triangles* total carbohydrate, *empty triangles* fat, *filled circles* % COD removal)

experiment. It could be seen from the figure that throughout the experiments, fats present in the wastewater were completely utilized by the yeast and the yeast biomass concentration remained constant. Similar to the previous batch and fed-batch experiments, the yeast showed 93% COD removal from the wastewater. Also, the yeast produced 2–3 g l^{-1} sophorolipids after 72 h of operation, which remained constant later throughout the experiment.

Nakhla et al. studied the effect of commercial biosurfactant (BOD-Balance™) on the treatment of pet food wastewater by anaerobic digestion system and found that the biosurfactant at doses in the rage 130–200 mg/l decreased oil and grease concentrations from 66,300 to 10,200 mg/l, besides reducing the initial COD to 40.86% for an operation period of over 2 months [12]. The effect of different surfactants including saponin biosurfactant on the continuous treatment of salad oil-containing synthetic wastewater by an activated sludge was studied by Matsui et al. [25]. They found that the outlet oil concentration in the wastewater remained quite low at 30 mg/l in presence of surfactants compared with more than 100 mg/l without any added surfactant [25]. Recently, Banu et al. studied the pretreatment of dairy wastewater by anaerobic hybrid upflow anaerobic sludge blanket reactor followed by final treatment using solar photocatalytic method [26]. The anaerobic pretreatment method was able to remove 84% of COD with the secondary solar photocatalytic treatment increasing the efficiency up to 95%. However, all these methods were reported to suffer from drawbacks such as requirement of ex situ addition of biosurfactant/coagulant/adsorbent, which enhance the process cost, low COD removal efficiency, and longer pretreatment time.

Compared with these literature reports, the present study revealed not only complete utilization of fats and oils present in the synthetic wastewater by the yeast but also very high COD removal efficiency, both for a very low HRT of 28.5 h. These aspects clearly demonstrate very good potential of the biosurfactant-producing yeast in removing fats and oils, and biological load from real dairy wastewater.

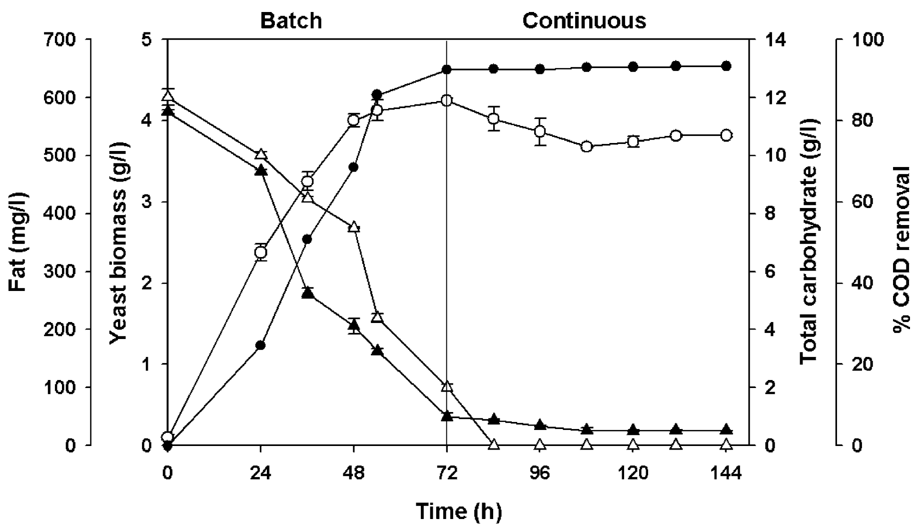

Fig. 4 COD reduction, fat utilization, carbohydrate utilization, and yeast biomass growth by *C. bombicola* using the synthetic dairy wastewater supplemented with sugarcane molasses (1%) and yeast extract (0.1%) in the fermentor operated under continuous mode (*empty circles* yeast biomass, *filled triangles* total carbohydrate, *empty triangles* fat, *filled circles* % COD removal)

Conclusion

Synthetic dairy wastewater containing high fats and oils was prepared in the laboratory, and its pretreatment was successfully carried out by the sophorolipid-producing yeast *C. bombicola* in a fermentor under three different modes of operation. The results showed that when supplemented with very low amount of sugarcane molasses and yeast extract, the yeast could completely utilize all the fats and oils present in the wastewater besides reducing the wastewater COD to a significant degree. Overall, the present study revealed very good potential of the system in pretreating real dairy industry wastewater.

Acknowledgement This study was funded by the Department of Science and Technology, India, vide sanction no SR/FT/LS-072/2007.

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