ORIGINAL ARTICLE

Batch experiments towards remediation of phenolic syntan using individual as well as co-culture of *Bacillus cereus* and *Pseudomonas aeruginosa*

Sivaranjani Venugopal[1] · Lawrance Irudayarajan[1] · Yasmin Khambhaty[1] · Saravanan Palanivel[1]

Abstract

The presence of very high concentrations of organic pollutants, phenols, tannins and heavy metals mainly chromium in wastewater discharged from leather industries, tags it as one of the most polluting industries. The phenolic syntans discharged from tanning units have an adverse effect on living organisms and cause serious environmental pollution, thereby making it very imperative to remove it. Among various treatment methods available for removal of phenols, biodegradation is environment friendly. The present study aims at the remediation of phenolic syntan used in the leather industry employing individual as well as co-culture of *Bacillus cereus* and *Pseudomonas aeruginosa* at varying syntan concentration in the medium. Parameters such as chemical oxygen demand (COD), total organic carbon (TOC), total phenol content (TPC) and Fourier Transform Infrared Spectroscopy (FTIR) indicating biodegradation were analyzed. Promising results were observed with *P. aeruginosa*, which exhibited a reduction in TPC by 62–72% in all the concentrations of syntan tested just within 12 h of inoculation, whereas about 67 and 83% reduction in COD and TOC respectively was observed for 2000 ppm concentration at the end of 5 days. *B. cereus* also demonstrated very good reduction in the above parameters however; percentage was less as compared to *P. aeruginosa*. In the case of co-culture, the TPC reduction was higher than *B. cereus* but lesser than *P. aeruginosa*. The percentage reduction in TOC and COD was highest for 500 ppm which eventually decreased for subsequent concentrations.

Keywords *Bacillus cereus* · Biodegradation · Bioremediation · Co-culture · Phenolic syntan · *Pseudomonas aeruginosa*

Introduction

The leather industry is one of the largest in India and among the leading foreign exchange earners for the country. However, it has been characterized under the "Red category" due to the enormous amount of waste it generates. Major problems are due to wastewater containing heavy metals, toxic chemicals, chloride, lime, high concentrations of dissolved and suspended salts and other pollutants (Uberoi 2003). Tanneries generate wastewater in the range of 30–35 L kg^{-1} of skin/hide processed with variable pH and high concentrations of suspended solids, BOD, COD, tannins and chromium (Nandy et al. 1999). It is reported that out of 1000 kg of raw hide, nearly 850 kg is generated as solid wastes in leather processing. Only 150 kg of the raw material is converted into leather (Kanagraj et al. 2006).

Syntans; synthetic tannins are high molecular weight organic compounds which are used to make imputrescible leather from hides and skin (Thankappan et al. 2010). Most of the synthetic tanning agents are manufactured by the condensation of aromatic compounds like phenol, phenol sulphonic acid or naphthalene sulphonic acid with formaldehyde (Kleban 2002). Annual production of phenol is around 7 billion kg, and it is used as a precursor to many materials for various uses (Mohite et al. 2010). Since phenol is an essential component used for making synthetic tannin (~40,000 tons of phenolic syntan is used globally), obviously, the leather effluents are known to contain phenolic wastes ranging from 1 to 500 mg L^{-1} which eventually leads to death of aquatic life even at a concentration as low as 15 mg L^{-1} (Senthilvelan et al. 2014). Nevertheless, the permissible limits of direct discharge of phenols to inland waters and sewers range from 1 to

✉ Yasmin Khambhaty
yasmink@clri.res.in

1   Leather Process Technology Department, CSIR-
     Central Leather Research Institute, Adyar, Chennai,
     Tamil Nadu 600 020, India

5 mg L^{-1} (CPCB 2005). Tannery wastewater treatment has been carried out by many conventional processes such as physical, chemical and biological methods (Krishnaswamy et al. 2016). Phenolic polymers are not easily amenable to treatment (Sundarapandiyan et al. 2010) and remain in the effluent due to incomplete absorption by the hides and skin (Thankappan et al. 2010). Removal of phenols can be done by several non-biological methods such as coagulation, solvent extraction, adsorption, chemical oxidation, liquid membrane permeation, etc., but they suffer from drawbacks like high cost and hazardous by-product formation (Agarry et al. 2008; Kanagaraj et al. 2011). A viable option to these could be biodegradation which converts these harmful compounds into harmless products at low cost in an eco-friendly manner (Yamaga et al. 2010; Karthikeyan and Gopalakrishnan 2011).

Several phenol degrading micro-organisms have been isolated from different sources. Among these microbes, the literature reports bacteria as having higher phenol degrading capacity than others (Kafilzadeh et al. 2010). Bacteria such as *Pseudomonas*, *Bacillus*, *Xanthomonas*, *Azotobacter*, *Flavobaterium*, *Acaligenes*; fungi such as *Aspergillus*, *Penicillium*, *Neurospora*, *Trametes*, *Trichosporon*, *Trichoderma* and yeast like *Candida tropicalis* are mainly reported to perform biodegradation (Shen and Wang 1995; Movahedyan et al. 2009; Kumar et al. 2018; Senthilvelan et al. 2018; Lawrance et al. 2018). The objective of the present study was to exploit the potential of bacteria for degradation/accumulation of phenolic syntan (Basyntan DI®), primarily used in leather processing, using individual as well as co-cultures of *B. cereus* and *P. aeruginosa*. This study is unique because it reports the degradation of the actual syntan used in the leather industry, exploiting bacteria.

Materials and methods

Bacterial strains

Bacillus cereus ATCC® 10876™ and *P. aeruginosa* ATCC® 27853™ were obtained from American Type Culture Collection Centre (ATCC). The strains were maintained on Nutrient agar slants by repeated sub-culturing and storage at 4 °C.

Phenolic syntan

The phenolic syntan viz., Basyntan DI® was obtained from M/s BASF India Ltd. This is a phenol formaldehyde condensate used in the leather industry (Fig. 1).
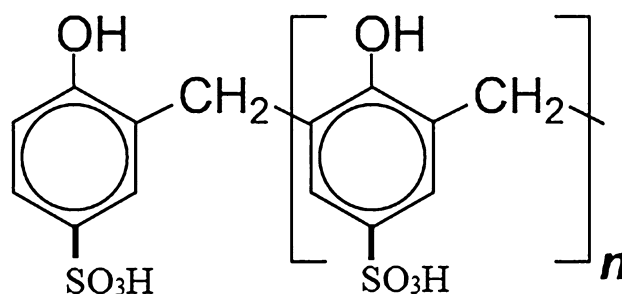
Fig. 1 Structure of phenol formaldehyde condensate

Biodegradation studies

All the biodegradation experiments were carried out in 500 mL Erlenmeyer flasks containing 250 mL of Nutrient broth (consisting of g L^{-1} peptone—5; sodium chloride—5; beef extract—1.5, yeast extract 1.5) with varying concentrations of Basyntan DI® (500–2000 ppm). These flasks were then autoclaved at 121 °C for 15–20 min. For preparation of inoculum, a loopful of cultures of *B. cereus* and *P. aeruginosa* were individually inoculated in Nutrient broth and incubated at 35 ± 2 °C for a period of 16–18 h on a rotary shaker at 150 rpm. Two hundred and fifty μL of over-night grown cultures were inoculated into the flasks with varying syntan concentrations ($\sim$ OD of 1.2 which corresponds to about 1.4–1.5×10^5 Colony Forming Units) and incubated at 35 ± 2 °C at 150 rpm. Similarly, an experiment on co-culture using both the strains added in equal amount was also set up. The samples were withdrawn at regular time intervals and growth pattern of individual as well as when grown as co-culture was observed by measuring the optical density at 540 nm. Positive and negative controls with only bacterial culture and only syntan respectively were also set up. Experiments were conducted in triplicate and the results are represented as their averages.

Determination of TPC

Samples drawn at regular intervals were centrifuged at $7826 \times g$ for 10 min. Supernatant was subjected to phenol estimation by Folin–Ciocalteu method (Slinkard and Singleton 1977). Briefly, 20 μL of supernatant was taken in a test tube. To each, 1.58 mL of distilled water and 100 μL of Folin–Ciocalteu reagent were added and mixed well. After $\sim$ 10 min, 300 μL of 20% sodium carbonate solution was added, shaken well and incubated at 20 °C for 2 h. Absorbance was noted at 765 nm against blank. Gallic acid monohydrate was used as a standard.

Analysis of physico-chemical parameters

COD was estimated as per the procedure of American Public Health Association (APHA-AWWA-WPCF 1989). TOC was analyzed by Elementar TOC analyzer, Germany.

FTIR analysis

The initial and final test and control samples were centrifuged and supernatant was lyophilized. The lyophilized samples were thoroughly mixed with potassium bromide (FT-IR grade) in a mortar while grinding with the pestle. Sample just enough to cover the bottom was placed in the pellet die and pressed at 2000 psi. The pressed sample from die was carefully removed and placed in the FTIR sample holder. The FTIR spectra of the samples were recorded in the range of 400–4000 cm^{-1} using a JASCO FTIR–4200.

Scanning electron microscopy (SEM)

SEM study of cells before and after exposure to syntan was carried out by Phenom-Pro operated at 3 kV on a gold-coated sample, and the images were taken at different magnifications. Briefly, the bacterial broth was centrifuged followed by washing the pellet with phosphate buffer thrice. After this, 0.25% gluteraldehyde (in Na-phosphate buffer, pH 7.2) was added and samples incubated at room temperature for 30 min. This was followed by washing with Na-phosphate buffer twice and the pellet collected by centrifugation. Eventually, the samples were subjected to addition with different ethanol concentrations i.e. 30, 50, 70, 90% (for each ethanol concentration there was an incubation of 10 min) and finally to 100% ethanol for 1 h and allowed to dry. Finally, SEM stub was prepared with adhesive tape and bacterial samples were fixed on the top of the tape.

Results

B. cereus and *P. aeruginosa* were selected for the present study since they are already reported to have good tolerance against phenol and known to degrade higher phenol concentrations (Hasan and Jabeen 2015). Here, the original syntan utilized in leather tanning was used (a formulated product) at varying concentration (500–2000 ppm) in the medium and hence, the detected phenol levels were lower than what was actually added.

The Nutrient broth prepared with different concentrations of syntan (500–2000 ppm) and inoculated with the above mentioned bacteria showed high growth density measured as turbidity after 12 h of incubation. While observing the growth pattern, it was seen that maximum growth for *B. cereus* was recorded at 24 h which remained fairly constant till about 72 h for all the experiments. It was quite surprising to see that although the growth of *B. cereus* in 1000–2000 ppm syntan concentration was very low within the first 12 h after exposure to the syntan, their growth eventually reached to comparable level to that of other concentrations by the end of 24 h indicating the high resistance of this strain to the syntan used in the present study (Fig. 2a). However, for *P. aeruginosa* though the pattern remained same as that of *B. cereus* recording a highest growth after 24 h of inoculation, the major difference was in the growth between positive control and experimental set up, where the optical density recorded was nearly half of that for the positive control (Fig. 2b). Nevertheless, among the experimental samples there seemed to be no significant difference since almost similar growth was recorded for all of them reaching a maximum at 24 h and remaining fairly constant subsequently. When a co-culture of both these organisms was used, the growth pattern was entirely different from both the above. Highest growth was recorded in positive control only by 48 h of inoculation, but the growth in the experimental flasks was highest by about 24 h for 1000–2000 ppm, and 36 h for 500 ppm which gradually decreased by 48 h and remained fairly constant beyond that (Fig. 2c). This could be attributed to the competition for available nutrients between the two organisms; alternatively, it could also be owing to the dominance of one species over the other.

TPC determination

As observed from the results of TPC, *B. cereus* could degrade 500 and 1000 ppm of syntan to nearly 50% within just 12 h which could correspond to its high growth in this period. However, in the case of other concentrations, 50% degradation was observed only after 36 h and 48 h for 1500 and 2000 ppm respectively. Reduction in TPC reached to 100% for 500–1500 ppm by 96 h, however, for 2000 ppm only about 80% reduction was achieved by 96 h (Fig. 3a). Very promising results were obtained for TPC when treated with *P. aeruginosa* where about 62–72% of reduction of TPC in all the concentrations of syntan tested was recorded just within 12 h which eventually resulted in ∼100% reduction after 24 h (Fig. 3b). Nevertheless, when both of these were co-cultured, the reduction in TPC was slower as compared to *P. aeruginosa* alone but faster than *B. cereus* (Fig. 3c). Also, for higher concentrations like 1500 and 2000 ppm, even though the degradation reached to about 80%, complete reduction was not observed even after 96 h.

COD and TOC reduction

The COD and TOC treating efficiency of these organisms in terms of percentage reduction is presented in Fig. 4 which was calculated for initial and final samples after 5 days of

A

B

C
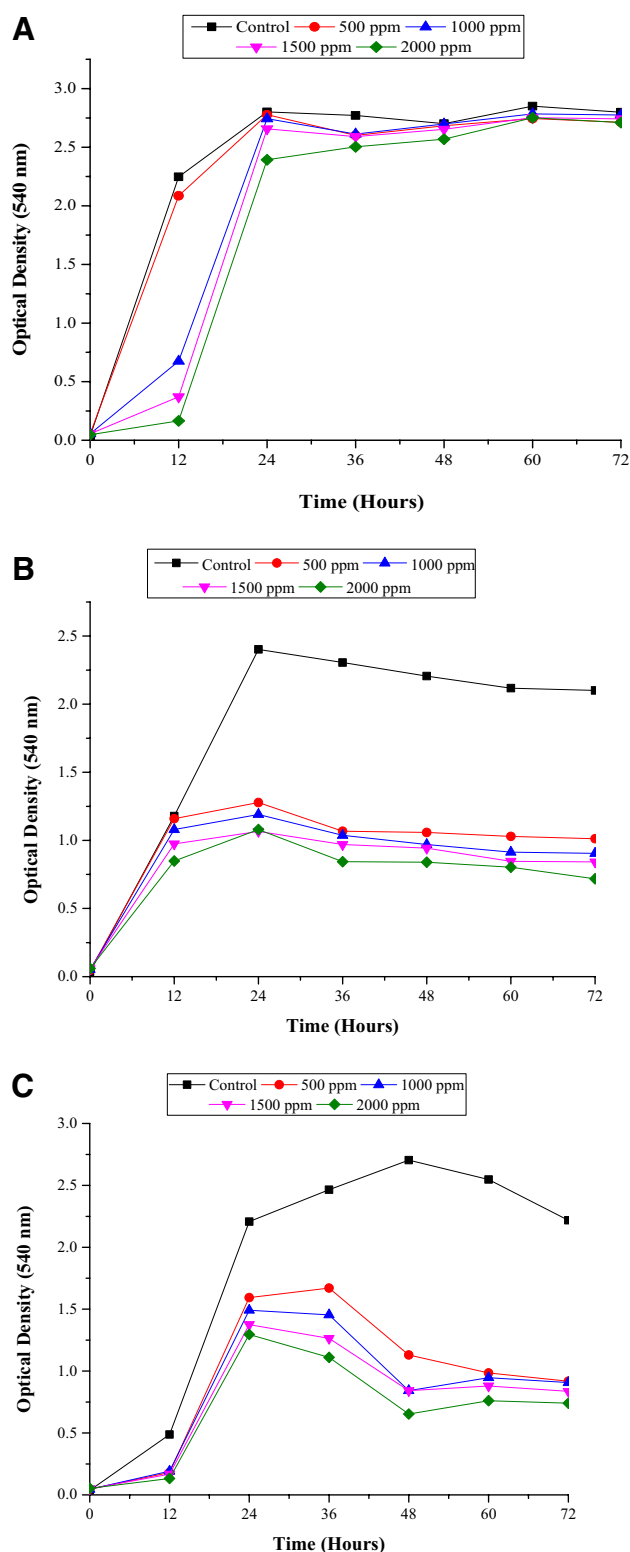
Fig. 2 **a** Growth pattern of *Bacillus cereus* with varying concentration of Basyntan. **b** Growth pattern of *Pseudomonas aeruginosa* with varying concentration of Basyntan. **c** Growth pattern of co-culture of *Bacillus cereus* and *Pseudomonas aeruginosa* with varying concentration of Basyntan

incubation. It is evident from the figure that COD of the treated sample is substantially reduced by the treatment with *B. cereus* and *P. aeruginosa*. As observed in the figure *B. cereus* showed an increase in percentage reduction of COD till 1000 ppm (~54%) however; it was less for subsequent concentrations. Similar trend was observed for percentage reduction in TOC which was about 64% for 1000 ppm. Whereas in case of *P. aeruginosa,* the trend was quite different where the percent reduction gradually rose with an increase in concentration, recording about 67% for COD and 83% for TOC at 2000 ppm. In contrast to both of these, the percent reduction when a co-culture of these organisms was used was highest for 500 ppm followed by reduced reduction in subsequent concentrations both for COD and TOC. The analyzed results illustrated that COD values were higher than those of prescribed standards fixed according to the ISI (ISI 2000) which is 250 mg L^{-1}.

FTIR studies

The conversion of phenol into other simpler forms of aromatic compounds and their presence were analysed by FTIR spectroscopy. The FTIR results are presented in Fig. 5a–c. The peaks identified at the wavenumbers 3414 cm^{-1} and 1313 cm^{-1} correspond to intermolecular hydrogen bonded O–H and the peak at 3045 cm^{-1} represents aromatic C–H stretch. The bands observed at the wavenumbers such as 1593, 1499, and 1472 cm^{-1} represent C=C stretching. C=O stretching is indicated by the peak at 1238 cm^{-1}.

FTIR analyses were done for the initial and final samples (after degradation) of syntan treated with *B. cereus* using syntan concentrations of 500 ppm and 2000 ppm (Fig. 5a). The control samples of both the concentrations showed similar peaks. The peaks at 3412 and 3402 cm^{-1} display the intermolecular hydrogen bonding aromatic –OH and O–H stretching while the band extending from 2100–3300 cm^{-1} with peaks at 2967, and 2354 cm^{-1} may be contributed by O–H and N–H stretching and CH_2 groups. The band at 1637/1636 cm^{-1} is due to the stretching of carbon–oxygen double bond and the C=O stretching vibrations are observed at 1343/1349 cm^{-1}. Peaks at 1343/1345, 2967 cm^{-1} and 1125 cm^{-1} reflect the alkanes C–H deformation and asymmetric stretching, C=O stretch, respectively. Degradation metabolites of syntan with *B. cereus* showed a shift in peak from 3409 to 3400 cm^{-1} and 1454 to 1448 cm^{-1} indicating the involvement of –OH stretching of the benzene ring and are blue shifted with increase in frequency. The red shifted peaks with decrease in frequency from 1636 to 1651 cm^{-1} and blue shifted peaks from 1637 to 1611 cm^{-1} are indicative of amide I groups while aliphatic O–H bending seems to be the dominant contributor at 1345/1331 cm^{-1}. FTIR spectrum of *P. aeruginosa* (Fig. 5b) reveals the presence of wave numbers 2360 cm^{-1}, 2339 cm^{-1}, 1641 cm^{-1},

Fig. 3 **a** Reduction in TPC by *Bacillus cereus*. **b** Reduction in TPC by *Pseudomonas aeruginosa*. **c** Reduction in TPC by co-culture of bacteria
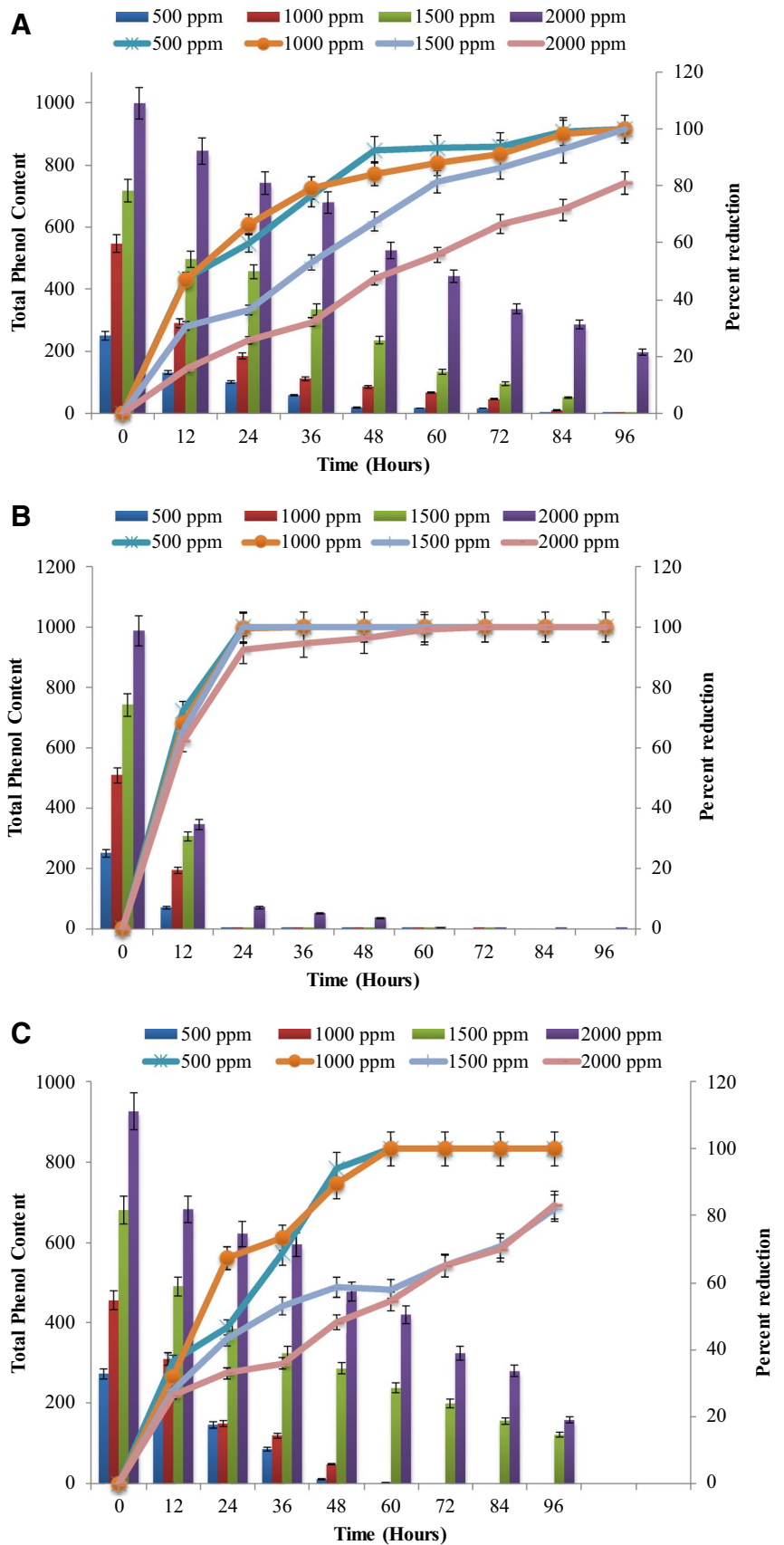
Fig. 4 Percent reduction in TOC and COD as a result of biodegradation after 5 days of incubation
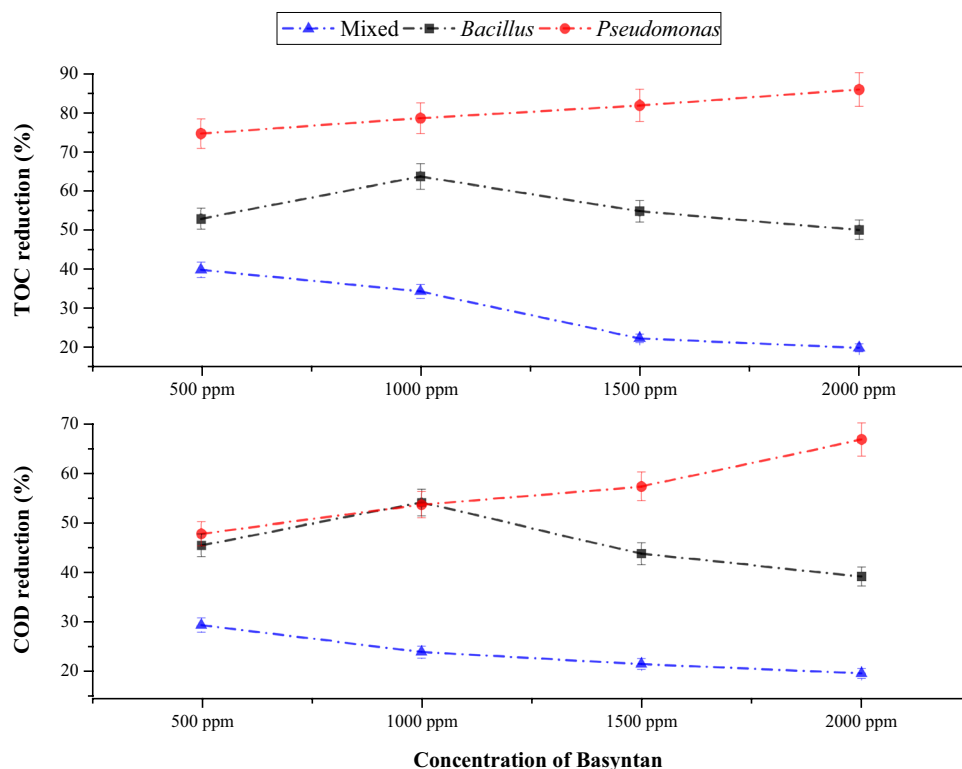
1406 cm^{-1}, 1352 cm^{-1} and 1119 cm^{-1}, corresponding to C=O, CH$_2$ bend, C=C stretch, CH$_3$ symmetric deformation, O–H bending and C–O–C, respectively. After syntan degradation, the spectrum showed wave numbers 1453 cm^{-1}, 1328 cm^{-1}, 1236 cm^{-1} and 1109 cm^{-1} indicating involvement of hydroxyl and C–O–C groups.

The control spectrum of mixed culture (Fig. 5c) displayed peaks at 2368, 2127, 1244, and 1123 cm^{-1} signifying the presence of C=O, –OH, C–H deforming and C–O–C, respectively. After syntan degradation, the frequency of the absorption decreased from 1337 to 1329, 1244 to 1237 and 1123 to 1109 cm^{-1}, due to modification of the hydroxyl groups to C=O. The new peaks at wavenumbers 1178 and 1039 cm^{-1} showed the existence of OH vibrational mode and −CO stretching of –COOH after degradation (Serna et al. 1986).

SEM studies

Morphological deviation between the bacterial isolates during the bioremediation process was studied by scanning electron microscopy. The SEM images reveal that the isolated strains are rod-shaped cells which would be the case for both *Bacillus* and *Pseudomonas*, with approximate 1 µm in width and 2–4 µm in length (Fig. 6). The cells growing in Nutrient broth were observed to be scattered. However, cells growing in Nutrient broth added with syntan tend to form clusters for avoiding damages due to higher syntan concentration and the

metabolites that are formed intermediately. This behaviour due to the occurrence of toxic compounds can be attributed to the defence mechanism in several microbial strains (Hasan and Jabeen 2015). Substrate mineralization can be achieved by proteins mainly expressed under normal growth conditions. However, several other proteins (i.e., chaperonin, superoxide dismutase, synthetase-b-subunit, thio redoxin reductase and succinyl-CoA) are also expressed, that help the cells growing under stress (i.e., high substrate concentration in this study) (Cabiscol et al. 2000).

Discussion

It is well known that syntans are frequently used for retanning leather. The residual tannins discharged during the process have adverse effects on living organisms and cause serious environmental pollution, however, some microorganisms are resistant to tannins and have the ability to degrade them (Krishnaswamy et al. 2016). The present study reports the remediation of phenolic syntan using individual as well as co-culture of bacteria. Among the microbes, bacteria are reported to have comparatively higher degrading capacity (Kafilzadeh et al. 2010) which may be further improved by the addition of supplements like glucose at certain level (Armenante et al. 1995). In an earlier study, an anaerobic digester fed with shea cake rich in tannins and phenolic compounds and previously inoculated with anaerobic

Fig. 5 **a** FTIR spectroscopy of phenolic syntan before and after treatment with *Bacillus cereus* (where I = initial sample and F = final sample). **b** FTIR spectroscopy of phenolic syntan before and after treatment with *Pseudomonas aeruginosa* (where I = initial sample and F = final sample). **c** FTIR spectroscopy of phenolic syntan before and after treatment with co-culture of *B. cereus* and *P. aeruginosa* (where I = initial sample and F = final sample)
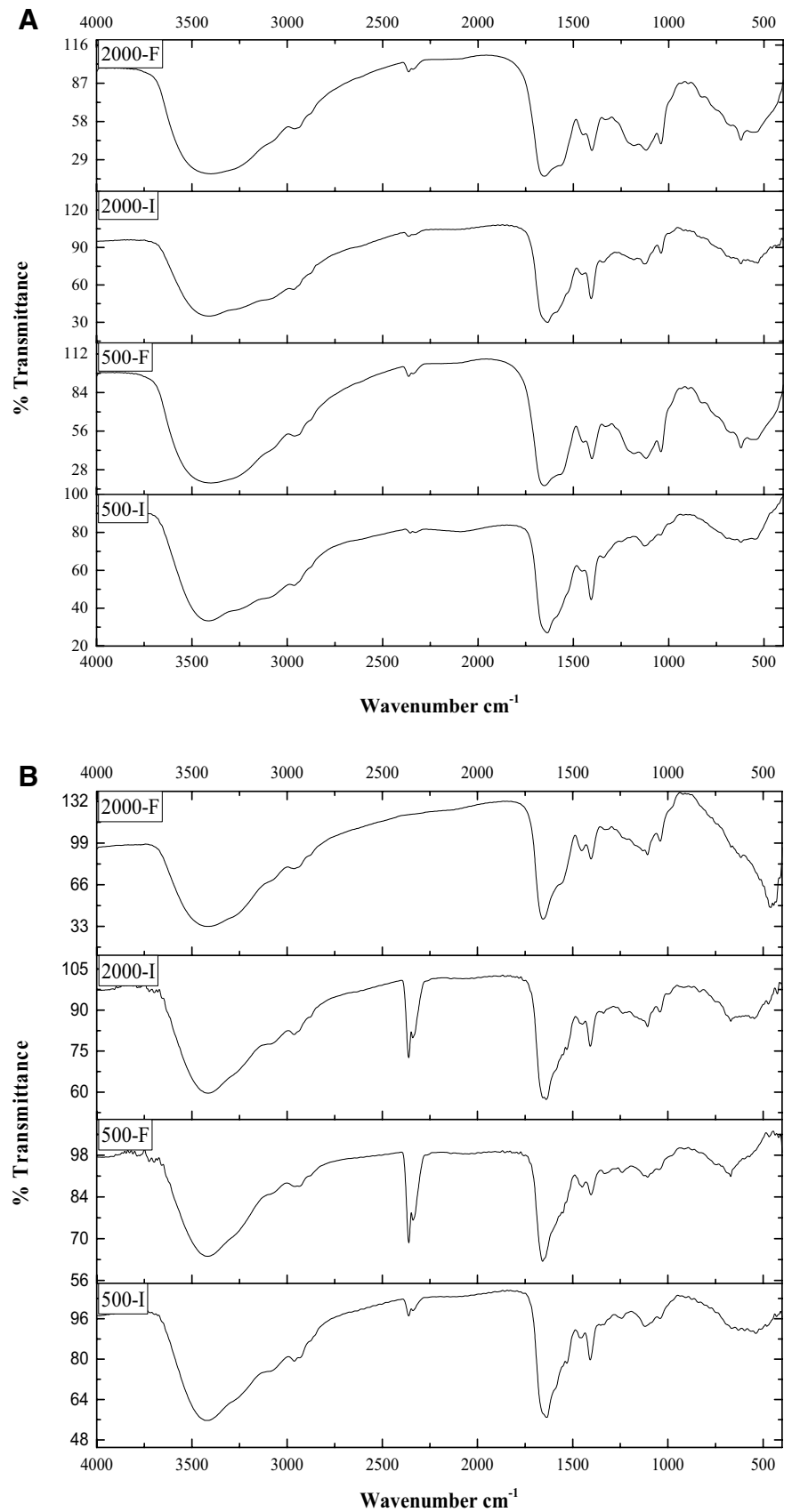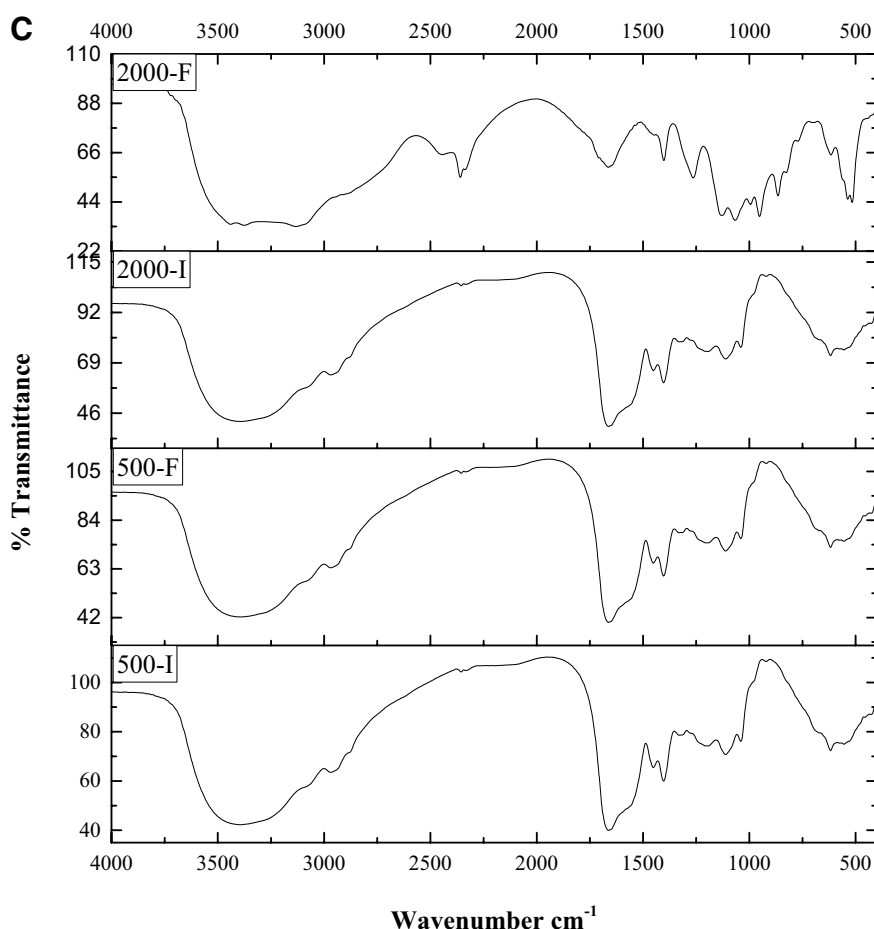
Fig. 5 (continued)

sludge from the pit of a slaughterhouse, enabled six months acclimatization of the bacteria to aromatic compounds. The isolated strain converted tannic acid to gallic acid and was found to belong to *Streptococcus* sp., (Nitiema et al. 2010). Optimizing the condition for the biodegradation was reported to be very important for the efficient degradation of the compound. Umesh et al. (2011) reported that the addition of glucose (1%) and ammonium sulphate in particular produced maximum tannin degradation upto 98%. Karthikeyan and Gurunathan (2011) reported that among the nitrogen sources tested in their study, inclusion of ammonium nitrate resulted in the highest enzyme production which plays an important role in degradation of tannin by *Klebsiella pneumonia*. Degradation capability of phenol by different species of *Pseudomonas* and *Bacillus* has been reported by various researchers. Chung et al. (2003) demonstrated that due to the substrate inhibition effect, free cells could degrade phenol up to about 600 mg L^{-1}, while immobilized cells could tolerate levels as high as 1000 mg L^{-1}, although the degradation rate was slower. In another study, Arutchelvan et al. (2006) reported degradation of phenol to a maximum concentration of 1750 mg L^{-1} in about 144 h by *B. brevis*. Ravikumar et al. (2011) have reported that *P. putida* showed

the maximum phenol degradation up to 100% within 72 h at pH 7 followed by *Bacillus* sp., and *P. horikoshii* within 80 h. *P. putida* also demonstrated further degradation of phenol up to 2000 mg L^{-1} within 144 h. A single paper pertaining to degradation of wattle, a polyphenolic material, used as a tanning and retanning agent, by a mixed culture of *P. aeruginosa* and *B. subtilis* is available. The mixed culture was able to grow at 500 mg L^{-1} nevertheless, time taken for complete degradation of 250 mg L^{-1} wattle was 48 h (Aravindhan et al. 2014). Whereas Senthilvelan et al. (2014) studied the degradation of phenol by strains of *P. putida* Tan-1 and *Staphylococcus aureus* Tan-2 employing single as well as mixed cultures. The biodegradation rates were found to be 94.2 and 88.1% for the phenol concentrations of 600 and 800 mg L^{-1} respectively. In yet another study the phenol degrading capacity of *Pseudomonas* and *Bacillus* to a level of 700 and 500 mg L^{-1} respectively was also exhibited (Hasan and Jabeen 2015). However, it was important to mention that most of the studies on degradation of phenol using bacteria were conducted using pure phenol. Nevertheless, the present study reports degradation of actual syntan, a phenol formaldehyde condensate used in the leather industry, to a concentration of 2000 ppm. Hence, biological treatment of
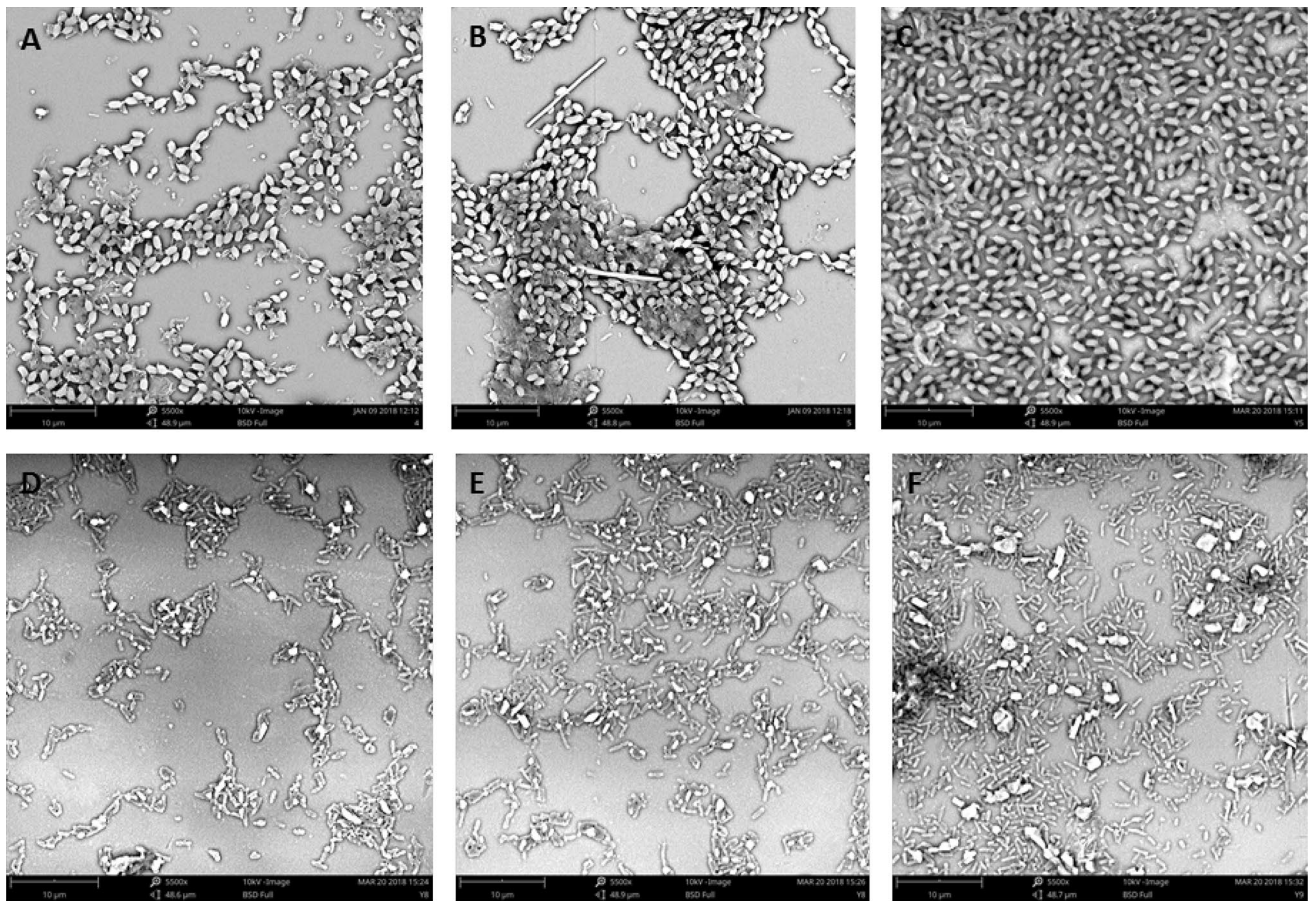
Fig. 6 **a** Control *Bacillus cereus*, **b** *Bacillus cereus* in 500 ppm syntan, **c** *Bacillus cereus* in 2000 ppm syntan, **d** Control *Pseudomonas aeruginosa*, **e** *Pseudomonas aeruginosa* in 500 ppm syntan, **f** *Pseudomonas aeruginosa* in 2000 ppm syntan

wastewaters was found to be the most suitable and attractive way to reduce phenol concentration because of its potential to degrade phenol at higher levels yielding end products with minimum secondary waste generation.

Reports state that mixed cultures possess higher capability to degrade phenolic compounds having differences in structure and toxicity. Although individual isolates are competent in degrading phenol at various concentrations, in most of the cases, the degradation rate of mixed microbial culture was significantly higher than that for any of the individual strains. The reason was individual organisms might be able to metabolize only a limited range of substrates; therefore, it is likely that mixed cultures with broader enzymatic capabilities have a greater capacity to degrade complex phenolic compounds (Puhakka et al. 1995; Hamitouche et al. 2010). However, the results of the present study were completely opposite to what was reported earlier. Here, the co-culture resulted in a much lower degree of degradation as compared to individual ones. This might be attributed to use of pure phenol or its compounds, nevertheless, in the present study the actual phenolic resin used in leather industry was used,

yet, the degradation efficiency was quite comparable to earlier studies.

Conclusions

In the present study, the bacterial consortium and the individual strains of *B. cereus* and *P. aeruginosa* resulted in an effective biodegradation process under different phenolic syntan concentrations up to 2000 ppm. It confirmed that *Pseudomonas* is promising in degrading phenolic syntan up to 2000 ppm within 72 h when compared to *Bacillus* and could be used effectively in the treatment of tannery wastewater. However, it should be noted that *P. aeruginosa* needs proper care while using it for the studies and hence, precautions should be taken to avoid exposure to aerosols from bacterial cultures.

The growth of society will continue to be hampered by the production of hazardous waste materials, and hence it is imperative to develop efficient strategies for waste management. The use of biotechnology may help in the development

of biological systems that initiate the detoxification, degradation or decontamination of environmental pollutants. Microbial degradation of phenol and its derivatives using various microbes has been the focus of scientific interest for a number of decades and is currently practiced for the treatment of industrial wastewaters. This can be done by employing both pure and mixed cultures of microorganisms and enzymes. The findings of this study suggest that aerobic method of degradation can be healthy and promising for effective wastewater treatment containing phenol and its derivatives. The further work includes the development of technology for the treatment of syntan-containing wastewater from the knowledge generated as a result of this work.

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