Ultrasound and enzyme assisted biodegradation of distillery wastewater

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Abstract

Irradiation with ultrasound (US) and use of an enzyme (E) as pretreatment techniques were carried out to treat a complex effluent (distillery wastewater). These two techniques have been used alone as well as in combination and the efficacy of these techniques was tested by subjecting the effluent to subsequent aerobic biological oxidation (AO). When used alone, US exposure for 30 min and 2 h yielded the best COD reduction during the aerobic oxidation step (US + AO). For the enzyme when used alone, a pH value of 4.8 (corresponding to the optimum pH of the enzyme), a dose of 50 U and a pretreatment time of 24 h yielded better COD removal efficiency as compared to untreated effluent (aerobic oxidation alone). When used in combination, ultrasound followed by enzymatic pretreatment (US + E + AO) yielded the best COD removal efficiencies during aerobic oxidation as compared to the other combinations tested for the treatment of the distillery wastewater. A 4-fold increase in the initial oxidation rate was observed over the untreated batch for the integrated technique (US + E + AO). On the basis of the variation in the values of the biokinetic parameters it can be concluded that the type of pretreatment scheme affects the subsequent rate of the aerobic oxidation significantly.

Keywords: Distillery wastewater; Ultrasound; Enzyme; Pretreatment; Biodegradation

1. Introduction

Distilleries are amongst the most highly polluting industries with reference to water pollution. The quantity of wastewater generated from distilleries is large and is characterized by a high pollution load. Molasses (the by-product of the sugar industry) containing roughly 50% of fermentable sugars (sucrose, glucose and fructose) is one of the most important raw materials used in the production of ethanol (by fermentation) owing to its cheapness and ready availability. After distillation of the alcohol from the fermentation broth containing 8–12% of ethanol, large amounts of a highly colored spent wash remain in the distillation still and require disposal. In a typical batch process, 11–13 L of spent wash is generated per liter of alcohol distilled, whereas in a continuous process, this reduces to about 8–10 L of spent wash per liter of distilled alcohol (Lele et al., 2000).

The characteristics of the distillery wastewater vary considerably according to the fermentation feed stock, location and the fermentation process adopted. The wastewater is characterized by a high concentration of dissolved solids (of which 50% may be present as reducing sugars), high ash content, high temperature ($\cong$104 °C) and low pH ($\cong$3.9–4.0).

Cane molasses distillery stillage contains all the dissolved impurities present in the cane juice, nutrients added during the molasses fermentation, by-products of fermentation and decomposition products. The suspended impurities like dust, cellulosic fibers, etc. are usually removed before the concentration of the juice. However, water soluble hemicelluloses, proteins, gums, organic non-sugars and minerals present in the cane juice are present in the stillage in the original or converted forms exerting an oxygen demand during its treatment.

Typical BOD and COD values for a batch distillery spent wash are 35,000–50,000 and 80,000–100,000 mg/L, respectively, whereas for a continuous process, they are in the range of 60,000–100,000 and 160,000–200,000 mg/L, respectively (Lele et al., 2000).

Primarily, as a result of the difficulties of treating stillage and due to high concentrations of dissolved organic and inorganic matter, a host of treatment schemes have been proposed (Sheehan and Greenfield, 1980). Thermal methods, like thermal pre-treatment, thermochemical liquefaction, wet air oxidation and anaerobic digestion are also employed, the latter being extensively used (Lele et al., 2000). Some of

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Notations

S/S_0	residual substrate concentration, in terms of COD
X/X_0	increased biomass concentration, in terms of MLSS
X_0	biomass concentration, mg/L or mg
$[-dS/dt]_0$	initial rate of oxidation, mg of COD per h
BOD	biochemical oxygen demand, mg/L
COD	chemical oxygen demand, mg/L or mg
DO	dissolved oxygen, mg/L
MLSS	mixed liquor suspended solids, mg/L
TDS	total dissolved solids, mg/L
ASN6	culture isolated from aeration tank of the effluent treatment plant of Asian paints, Mumbai
ETP	effluent treatment plant
FPU	filter paper unit
IU	international unit
PTFE	polytetrafluoroethylene
AO	batch subjected to aerobic oxidation
US	ultrasonically pretreated sample
E	enzyme pretreated sample
US + E	effluent sample subjected to ultrasound and then enzyme pretreatments

these methods have been operated on an industrial scale, whereas some have been tried out only on bench or pilot plant scale. Each of these methods does have some technical or techno-economic problems and it appears that no single method could be suggested as a complete economic solution to the distillery spent wash disposal problem (Lele et al., 2000). Hybrid methods combining two or more oxidation processes generally used for wastewater treatment are also becoming popular for distillery wastewater treatment. These processes generate complimentary conditions of oxidation and also help in eliminating the drawbacks associated with the individual methods. Two altogether different strategies have been tried out to enhance the biological aerobic degradation of the distillery-spent wash in this work. The effects of pretreatment using ultrasound and enzyme on the biodegradation rate of the distillery-spent wash have been discussed in this work.

Ultrasound is increasingly being seen as having potential for use in the treatment of the water, wastewater and sewage sludge. Sonochemical oxidation employs the use of ultrasound resulting in cavitation phenomena, which is defined as the phenomena of the formation, growth and subsequent collapse of microbubbles or cavities. It occurs in an extremely small interval of time (microseconds) releasing large magnitudes of energy simultaneously at millions of such locations in water with contaminants. Because of the highly localized concentrations of oxidizing species generated as a result of cavitation phenomena, such as hydroxyl radicals and hydrogen peroxide, and high localized temperatures and pressures, the contaminants get completely or partially oxidized instantly. The combination of the physical and the chemical effects of cavitation, thus are responsible during the application of ultrasound in water and effluent treatment (Shah et al., 1999).

The biological effects of ultrasound have been studied since the 1920 s. Ultrasound has been shown to disrupt the microbial cells (Mason et al., 1994) and cause water disinfection (Jyoti and Pandit, 2000; Clasen and Sobotta, 1994). Ultrasound has also been used in combination with technologies such as ozone to improve water and effluent treatment (Chendke and Fogler, 1975; Dahi, 1976) Ultrasound combined with ozone has been used to enhance the degradation of natural organic matter (Olson and Barbier, 1994) and cyclohexene (Weavers and Hoffmann, 1998).

Another method under focus is the use of enzymes in waste treatment applications. A large number of enzymes from a variety of different plants and microorganisms have been reported to play an important role in an array of waste treatment applications. The potential advantages of enzyme treatment as compared with the conventional treatment include: its application to biorefractory compounds; operability over a wide range of pH, temperature and salinity; immunity from shock loading effects, absence of delay associated with the acclimatization of the biomass, reduction in the sludge volume and the ease or simplicity of the process control (Karam and Nicell, 1997). A large number of enzymes (for e.g. peroxidases, oxidoreductases, cellulolytic enzymes, cyanidase, proteases, amylases, etc.) from a variety of different sources have been reported to play an important role in various waste treatment applications. In recognition of these potential advantages, recent research has focused on the development of enzyme-based processes for the treatment of wastewaters, solid wastes, hazardous wastes and soils. Karam and Nicell (1997) have summarized the enzymes utilized in the field of wastewater treatment according to the categories of specific waste types and origins in their review. In another review, Aitken (1993) has presented different waste treatment situations that may be appropriate for enzyme technology along with the selection criteria for enzymes that may have a near-term applicability.

In our earlier work, application of ultrasound as a pretreatment step has been found to enhance the biodegradability of the effluent for the subsequent aerobic oxidation step (Sangave and Pandit, 2004), while another study (Sangave and Pandit, in press) demonstrated that pretreatment of distillery effluent with the enzyme cellulase increased the rate of COD removal. This work investigates the feasibility of combining these two techniques (ultrasound and enzyme) in treating the distillery effluent.

2. Experimental

2.1. Materials

2.1.1. Distillery wastewater

The distillery wastewater was procured from Terna Shetkari Sahakari Sakhar Karkhana, Osmanabad Maharashtra, India. The waste was filtered to remove the suspended solids, and this was used for further study. Table 1 shows the characteristics of the raw distillery spent wash after filtration.

2.1.2. Culture for aerobic oxidation

The aerobic oxidation was carried out using a single strain (named *ASN*6). The organism was isolated from the activated sludge of the effluent treatment plant (Asian Paints, Mumbai, India). The strain was identified as a potential degrader of this effluent after it underwent differential screening and enrichment techniques utilizing the effluent as the sole carbon source in the medium. The staining procedures and biochemical tests revealed that the strain was a facultative Gram-positive bacterium belonging to the genus *Bacillus*. The pure culture was used rather that the mixed culture (as is the usual practice) to specifically maintain a similar microbial environment in the aerobic biodegradation studies.

2.1.3. Cellulase enzyme

The enzyme cellulase (form: powder) was procured from M/s Advanced Biochemicals, (Mumbai, India). The enzyme used included a blend of endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) and had an activity of 100 FPU/g of powder.

2.1.4. Bioreactor

All the biodegradability studies were performed in a bioreactor made of glass having a capacity of 1 L. The setup used for this study is similar to that described and reported by Sangave and Pandit (in press).

2.1.5. Ultrasound setup-ultrasonic bath

In the present study, the cavitation experiments (ultrasonic irradiation treatment) were carried out using an ultrasonic bath. The setup used for this study is similar to that described elsewhere (Sangave and Pandit, 2004).

Table 1
Characteristics of the raw distillery spent wash

Parameter	Values
Color	Brown
PH	3.8–4.0
COD (mg/L)	100,000–110,000
TDS (mg/L)	98,000–110,000
TSS (mg/L)	3000–5000
TOC (mg/L)	55,000–56,000
Ammoniacal N (mg/L)	12,000–14,000
Reducing Sugars (mg/L)	9000–10,000
Chloride[a] (mg/L)	4000–6000
Sulphate[a] (mg/L)	4500–6000
BOD_5[a] (mg/L)	30,000–45,000

[a] Data obtained from the factory report.

2.1.6. Enzymatic pretreatment setup

The enzymatic pretreatment step was carried out in a constant temperature bath maintained at 50 °C. The contents were continuously stirred during the treatment with the help of a magnetic stirrer.

2.2. Experimental protocol

2.2.1. Preparation of the effluent sample

The raw undiluted effluent sample to be sonicated was centrifuged in order to remove the suspended solids from the effluent sample. The sample was centrifuged at 8000 rpm for 20 min (Research Centrifuge-REMI Equipments, India; around 15,000 *g* where *g* is the gravitational acceleration) to remove the suspended particulate matter. The sample was then diluted using distilled water to bring down the initial COD to the range of 10,000–12,000 mg/L for each experiment. The sample was then ready for the pretreatment experiment.

2.2.2. Ultrasonication as a pretreatment step (only US)

The experiments were carried out in an ultrasonic bath. The volume of liquid was kept constant at 0.7 L. No pH adjustment was done during the sonication step; hence the pH of the effluent was the same as the initial pH (pH ≅ 3.8–3.9) for the treatment. The effluent sample was subjected to ultrasonic irradiation for different time intervals. A preliminary study of irradiation of the distillery effluent sample with ultrasound for 2 h had shown encouraging results (discussed later) hence; lower and higher sonication periods (less than 2 h and more than 2 h) were also tried. Sonication periods of 30 min, 1, 2 and 3 h were studied. One-milliliter sample aliquots were withdrawn after every 10 min of the treatment. A 10 min break was given after every sampling during the US-run to cool down the spent wash to the room temperature. The samples were analyzed for COD and sugars. At the end of the sonication runs, the pH of the effluent was measured. The temperature conditions were not controlled in any of the sonication experiments studied in this investigation. Hence, the rise in temperature was monitored during the ultrasonic pretreatment and the volume variation due to the evaporative losses was also accounted for. The effluent pre-treated with ultrasound were then subjected to aerobic oxidation to study its effect on the biodegradability.

2.2.3. Enzyme pretreatment step (only E)

Before commencing the enzymatic pretreatment studies, the optimization of the pH and the temperature for the maximum enzyme activity was carried out. The enzyme was assayed at different pH ranging from 3.0–6.0 using 0.1 M citrate buffer. The enzyme was also assayed at different temperatures of 30, 40, 50 and 60 °C. These studies indicated that a pH of 4.8 and temperature of 50 °C were optimum for the maximum enzyme activity. These optimized parameters were then used during the actual treatment studies.

The effluent sample (700 mL, untreated and diluted) was contacted with enzyme under different conditions. The pH was adjusted with the help of a sodium hydroxide solution (2 N) to a value of 4.8. Twenty-five millilitre sample aliquots were withdrawn at regular time intervals. The samples were analyzed for the enzyme activity, COD and sugars. At the end of the pretreatment step the pH of the effluent was again measured. The pre-treated effluent was then subjected to the aerobic oxidation to study the effect of the enzymatic pretreatment.

2.2.4. Ultrasound followed by enzyme as a pretreatment step (US+E)

During this step, the ultrasonically pretreated effluent was subjected to subsequent enzymatic treatment, which was carried out under optimized parameters as stated earlier. After the combined pretreatment the effluent was then subjected to aerobic oxidation and its biodegradability was studied.

2.2.5. Preparation of the inoculum (culture acclimatization)

Before subjecting the sample to the biodegradability tests, the inoculum for the biodegradability test was prepared. The isolate *ASN*6 was stored on nutrient agar (NA) slants (7 mL) at 4 °C. The culture from two such slants was aseptically transferred to the sterile diluted waste and it was incubated on a rotary shaker (180 rpm) at ambient temperature (30–34 °C) for 12 h before its inoculation into the bioreactor. The inoculum volume was kept constant at 50 mL for every batch experiment.

2.2.6. Aerobic oxidation of the pretreated samples (biodegradability tests)

Biodegradability as a parameter can be used for evaluating the possibility of an aerobic oxidation step as a part of the overall biological treatment. The biological parameter measurement allows the evaluation of the biological treatability of the wastewater before and after the pretreatment. The initial volume of the effluent in the reactor was kept at 650 mL for every batch. The enzyme-pretreated effluent was sterilized. Inoculum was aseptically transferred to the reactor and the degradation was carried out under aerobic conditions.

Preliminary experiments for optimization of the operating conditions for the aerobic oxidation process indicated an optimum operating speed of rotation of the impeller of 700 rpm, optimum operating initial pH of 7.5 and requirement of a DO level above 2 ppm. Hence, in all the subsequent experiments, the contents were maintained well mixed with the help of the impeller rotating at 700 rpm, and the filtered air was sparged at the bottom of the impeller by using a single point sparger to maintain a DO level above 2 ppm. The initial pH of the effluent was maintained at 7.5. Four-milliliter aliquots were withdrawn at regular time intervals and every sample was analyzed for COD, MLSS and the reducing sugars. During the run, pH measurements were also carried out periodically.

The effective biodegradation of the pre-treated effluent was compared with the results obtained for the untreated effluent. The untreated effluent was directly taken for the aerobic oxidation step by prior centrifugation and dilution to the required concentration. It was observed that autoclaving of the effluent sample (for sterilization) leads to a decrease in its pH value by approximately one unit. Hence, for all the biodegradability studies the pH of the effluent was adjusted to 8.5 so that the pH of the sterilized sample dropped to ~7.5 (which was then the initial pH for the subsequent aerobic oxidation).

The effect of different pretreatment conditions like pH during the pretreatment step, and the effect of the enzyme concentration and the pretreatment time on the rates of subsequent biological oxidation steps have been studied for the different pretreatment schemes.

2.3. Analysis

COD determination was done according to the standard methods of analysis of wastewater and water, (APHA, 1998). For the MLSS measurements, 4 mL of sample was centrifuged at 5000 rpm (around 6000 g where g is the gravitational acceleration) and the cell weight was found out by dry cell weight method. The pH was monitored during the aerobic oxidation step with the help of a digital pH meter (Equiptronics, India) under aseptic conditions. Cellulase assay was carried out according to the procedure mentioned by Ghose (1987). One IU is defined as 1 μmol min^{-1} of substrate converted to 1 μmol min^{-1} of glucose (reducing sugars as glucose) formed during the hydrolysis reaction, which in turn would be 0.18 mg min^{-1} when the product is glucose. The enzyme activity was then expressed in filter paper units (FPU) (Sangave and Pandit, in press). Online measurement of oxygen uptake rate or respiration rate was done by stopping the aeration and agitation and then following the decrease in the DO with time.

3. Results and discussion

3.1. Effect of ultrasound pre-treatment on the effluent (only US)

The effect of cavitation was studied as a pre-oxidation step. Cavitation of the effluent was carried out at an operational ultrasound frequency of 22.5 kHz. The effect of this low frequency ultrasound for different exposure periods was studied in order to determine the optimum pre-treatment conditions. The samples withdrawn were analyzed for the changes in the COD. Within the range explored for the ultrasound exposure periods between 30 min and 3 h, a negligible reduction (less than 10%) in the COD of the effluent sample due to the passage of ultrasound was observed in all the cases. The effect of the ultrasound on the effluent COD is shown in Fig. 1. Fig. 1 seems to demonstrate the role of ultrasonic waves and the resultant cavitation in restructuring the molecules present in the waste. This observation holds true

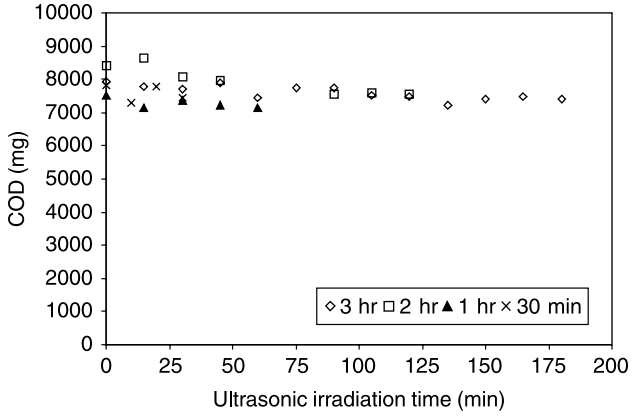

Fig. 1. Effect of ultrasound pretreatment on the effluent (Effluent type: Raw diluted; pH: 3.98; Volume: 1 L).

since higher ultrasound exposure periods (1, 2 and 3 h) also had very little effect on the net COD change of the effluent.

A lot of studies have investigated the process feasibility with synthetic aqueous solutions containing model pollutants. In the case of a single compound, the concentration of the target pollutant and the first intermediates can be measured and their reaction kinetics can be modeled. But, in the case of a real wastewater system, ultrasonic irradiation leads to the formation of multitudes of products due to the inherent complexity of the effluent, which in turn are difficult to identify (Gonze et al., 2003).

Here, the application of low frequency ultrasound caused disintegration of the pollutant molecules into smaller fractions rather than their complete oxidation. It is a well-known fact that the passage of ultrasound waves through a liquid causes periodical compression and rarefaction of the medium. Cavitation occurs above a certain ultrasound intensity threshold, when gas/vapor bubbles are created, which first grow in size before violently collapsing within a few microseconds. The violent collapse produces very powerful hydro-mechanical shear forces in the bulk liquid surrounding the bubble. The mechanical forces are most effective at frequencies below 100 kHz (Tiehm and Neis, 2004). The extreme temperature and pressure generated due to cavitation conditions can lead to the thermal destruction of compounds present in the cavitation bubbles and can also result in the generation of very reactive hydroxyl radicals (Mason, 1991). In this way sonochemical reactions can degrade volatile pollutants by pyrolytic processes inside the cavitation bubbles and the oxidation of the non-volatile pollutants by hydroxyl radical reactions in the bulk liquid. Hua and Hoffmann (1997) have found that sonochemical degradation processes can occur in a broad ultrasound frequency range from 20 kHz up to about 1 MHz. The sonochemical effects are more pronounced at frequencies above 100 kHz, whereas sono-physical effects are more pronounced at frequencies below 100 kHz.

The presence of dissolved solids in the distillery spent wash to the tune of 8000–9000 ppm, acting as nuclei for cavitation, certainly affected the number and size distribution of the cavitation bubbles. In the pretreatment conditions studied (low frequency), the cavitation phenomena generated powerful hydro-mechanical shear forces, which dominated over the sonochemical effects (oxidation due to hydroxyl radicals) of ultrasound in the medium.

In the present study, the bulk solution temperature in the US-bath was not controlled with any external cooling mechanism. Due to this, the temperature rose gradually as the US-irradiation time increased. A break of 10 min was provided after each ultrasound exposure for 10 min. The maximum temperature attained for 30 min of treatment was 40 °C from an initial value of 31 °C. The maximum temperatures attained for 1, 2 and 3 h batches were 42.5, 44 and 50 °C, respectively. Temperature has been reported to have both positive and negative effects on the degradation rates of various compounds. Sonophotochemical destruction of 2, 4, 6-trichlorophenol was higher at higher temperature (Shirgaonkar and Pandit, 1998) while higher bulk solution temperature had a negative effect on the kinetics of *p*-nitrophenol degradation (Sivakumar and Pandit, 2002). For COD estimation, the volume change due to the increased temperature and evaporative losses has been accounted for.

The pH was also monitored before and after the ultrasonic irradiation. It was found that cavitation did not result in any change in the pH of the effluent sample. The yields of sonochemical degradation of contaminants in water are enhanced at higher frequencies and the rate of degradation is linked with the physical and chemical properties of the target molecules. Jiang et al. (2002) have carried out a detailed study of the effect of pH on the degradation of ionic compounds. Tauber et al. (2000) have reported that for molecules with ionisable functional groups, the rate of degradation during ultrasonic irradiation is expected to be affected by the solution pH in view of the fact that negative charges exist near the periphery of cavitational bubbles. In addition, the gas-bubble interfaces have been found to be highly hydrophobic and the ionic state of the contaminant molecules may affect their tendency to partition into this hydrophobic region.

Thus, the bulk solution temperature and the effluent pH also must have governed the formation of different intermediate molecular rearrangement products (not analyzed in the present work) owing to the complexity of the wastewater coupled with the acoustic effects of the ultrasound.

3.2. Effect of enzyme pretreatment on the effluent (only E)

The distillery spent wash was contacted with enzyme during the pretreatment step as described earlier. It was found that the enzyme pretreatment step did not change the COD value of the effluent. The initial COD values matched with those obtained at the end of the pretreatment step. It was also observed that there was a marginal increase in the pH value of the effluent sample, an increase of 0.5–0.8 pH units at the end of the pretreatment. This observation was as expected since the enzyme pretreatment was intended to breakdown the cellulosic material present in the distillery-spent wash and not to mineralize the pollutants.

3.3. Effect of ultrasound on the biodegradability of the effluent (US+AO)

Ultrasound pretreatment of the distillery-spent wash alone did not result in the mineralization of its pollutants (as there was only a small change in the COD). The US-pretreated effluent was then subjected to the aerobic oxidation step to test its effect on the biodegradability. The aerobic oxidation of the effluent was carried out by inoculating the bioreactor with the seed culture as described earlier. The batches were run for 3 days and sampling was done at regular intervals. The samples were analyzed for COD, MLSS, pH, and sugar levels.

The biodegradability of the effluent is defined as the initial rate of aerobic oxidation of the US pretreated effluent and was studied during the aerobic oxidation step, in terms of its percentage COD reduction. The rates of oxidation for the pretreated effluent were compared with that of the control, which was subjected to aerobic oxidation without any US-pretreatment.

Fig. 2 shows the rate of degradation of the US pretreated samples and that of the control. The US pretreatment step affected the degradation rates tremendously. The initial rates of aerobic oxidation were higher as compared to the untreated sample, in all the cases. At the end of 12 h of aerobic oxidation the percentage COD reduction attained was 17.56, 18.3, 17.36 and 16.4% for 30 min, 1, 2, and 3 h-pretreated samples as compared to a mere 5.87% for the untreated sample indicating on average a 3-fold increase in the initial biodegradation rates. The rates of oxidation increased gradually in all the cases, but the 2 h pretreated batch yielded the maximum and faster percentage COD reduction at the end of 48 h. In the pretreated samples, a maximum of 50–60% of reduction was achieved at the end of the 72 h period, indicating the inability of the organisms to degrade the effluent further. Though it is difficult to characterize the effluent fully, the results suggest that the chemical composition of the effluent was affected by the passage of ultrasound. Another observation to be noted was that, though the ultrasonic irradiation time was doubled or tripled, the extent of COD reduction in the subsequent aerobic oxidation step did not show a similar increase. 30 min and 2 h-US pretreated batches yielded comparatively the best results in terms of the enhanced rate and the extent of COD reduction. Hence, these US pretreatment conditions were chosen during the study of the effect of the combined pretreatment step on the biodegradability of the effluent.

3.4. Effect of enzyme on the biodegradability of the effluent (E+AO)

It was important to study how the COD removal efficiency depended on the conditions of the enzymatic pretreatment step. The effluent was contacted with the enzyme under two pH conditions, one represented the optimum pH (pH 4.8) of the enzyme (maximum activity) and the other represented the pH of the untreated effluent (pH 3.98) which was approximately one unit less than the optimum pH of the enzyme. 50 U of the enzyme was loaded for the pretreatment step and the run was carried out for 24 h. The pretreated samples were then subjected to aerobic oxidation.

Fig. 3 shows the percentage COD reduction obtained during the biodegradability studies for the enzymatically pretreated effluent at two different pH values and this is compared with the untreated effluent. Fig. 3 indicates that the enzymatic pretreatment step enhanced the biodegradability of the effluent. This figure shows the dependence of the aerobic oxidation step as a function of pH. It was found that the maintenance of optimum pH of the enzyme (pH 4.8) favored the maximum removal of COD. At the end of 12 h of aerobic oxidation the percentage COD reduction attained was 20.5, and 15.3% for enzymatically-pretreated samples at pH 4.8 and 3.98, respectively, as compared to 12.4% for the untreated effluent (control). At the end of 72 h of aerobic oxidation, the pretreatment condition of pH 4.8 yielded a maximum COD reduction of 54.3% as compared to 44.6 and 47.3% for the batch treated with an enzyme at pH 3.98 and the untreated effluent, respectively. Hence, the initial pH during the enzyme pretreatment was kept at a value of 4.8 for further studies.

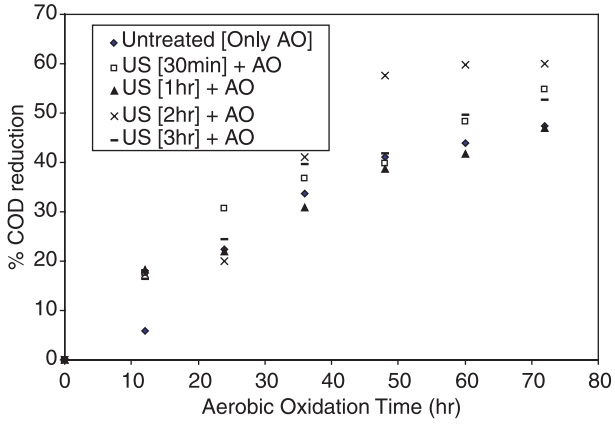

Fig. 2. Percentage COD reduction for the different pretreatment conditions as compared to control.

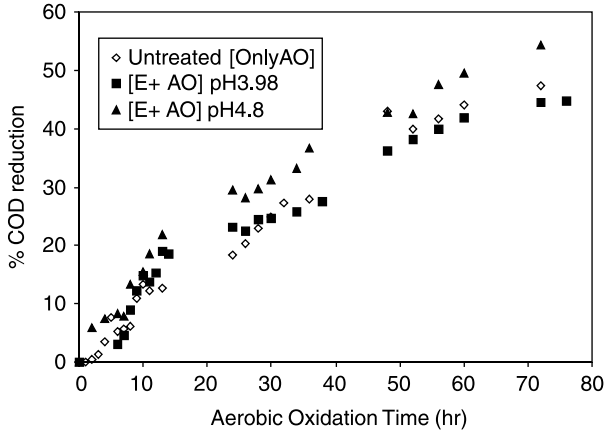

Fig. 3. Percentage COD reduction for the enzymatically pretreated samples as compared to the control (Enzyme dose: 50 U and pretreatment time: 24 h).

3.5. Effect of combined pretreatment step on the effluent (only US+E)

The effect of the combined pretreatment step was similar to that observed when they were used as stand alone pretreatments. It was observed that the enzyme treatment did not bring about any change in the COD value of the effluent at the end of the treatment step. These results indicate that the combined pretreatment technique was only responsible for the molecular restructuring. The pH was also monitored during the combined pretreatment step and no change in its value was observed.

3.6. Effect of combined pretreatment step on the biodegradability of the effluent (US+E+AO)

The effluent samples subjected to the combined treatment (US+E) were later treated biologically. The rates of oxidation for the pretreated effluent were compared with that of the control, and also with the rates obtained when these techniques were used individually. Fig. 4 shows the rates of degradation obtained under different conditions. From Fig. 4 it is clear that the combined treatment technique yielded the best results; the initial rates of aerobic oxidation were significantly enhanced. At the end of 36 h of aerobic oxidation, the combined pretreatment technique (US+E+AO [1]) yielded the highest COD reduction of 62.25% as compared to 34.9, 36.61, 42.26 and 52.4% COD reduction for the untreated, enzymatically pretreated (only E+AO), US pretreated (only US+AO) and for the other combined technique (US+E+AO [2]) employed, respectively.

The experimental results of the study in the form of first order degradation plots are shown in Fig. 5. For the two combined treatment techniques employed, US+E+AO [1] yielded the best degradation rates. The oxidation rate constant was almost double ($0.0310\ h^{-1}$) that obtained for US+E+AO [2] ($0.0157\ h^{-1}$) and almost 4 times higher than the untreated waste ($0.0078\ h^{-1}$). The initial rate constant for the ultrasonically

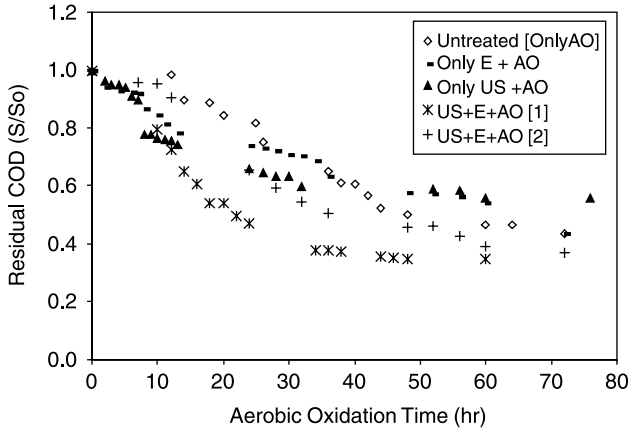

Fig. 4. Variation in residual COD values for different treatment conditions (E+AO: E [50 U, 24 h]+AO [72 h]; US+AO: US [30 min]+AO [72 h]; US+E+AO [1]: US [2 h]+E [50 U, 24 h]+AO [72 h] US+E+AO [2]: US [30 min]+E [50 U, 12 h]+AO [72 h]).

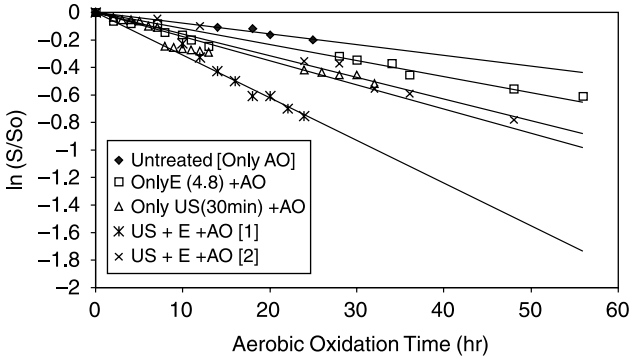

Fig. 5. First order degradation plots for the different treatment conditions (E+AO: E [50 U, 24 h]+AO [72 h]; US+AO: US [30 min]+AO [72 h]; US+E+AO [1]: US [2 h]+E [50 U, 24 h]+AO [72 h]; US+E+AO [2]: US [30 min]+E [50 U, 12 h]+AO [72 h]).

pretreated sample (US+AO) was $0.0175\ h^{-1}$ while that for the enzyme pretreated sample (E+AO) was $0.0115\ h^{-1}$.

The difference in the rates of oxidation due to the difference in the treatment conditions employed can be attributed to the formation of different kinds of products during the US pretreatment, which were further acted upon by the enzyme cellulase during the combined pre-treatment step. This difference in the rates again confirms the observation of the formation of different transformation products as a result of different US exposure periods (30 min and 2 h in this study). The bioavailability of the environmental pollutants often is limited by their water solubility, adsorption tendency and the dissolution rate. Biodegradation depends on the diffusion of the pollutants across the microbial membrane. Bacterial degradation of large molecular weight compounds is initially very different from the degradation of small molecular weight compounds. Many small compounds can be transported intact across the outer cell membrane, whereas larger molecules must be hydrolyzed by one or more enzymes (usually extra-cellular) to sub-units small enough to be transported into the cell (Haldane and Logan, 1994).

The combination of the two techniques enhanced the substrate (pollutant) metabolic value by altering the molecular size of the parent pollutant molecules and hence their properties, in turn leading to a better degradation rate. The intermediate transformation products seem to dictate the response of the biomass and hence, their degradation in the subsequent biodegradation studies.

3.7. Evolution of biomass

Grady (1985) has stated that the bacteria can only do those things for which they have a genetic capability. Furthermore, not all bacteria can do all things, and the environment is an important determinant of whether a reaction can be carried out by an organism genetically capable of performing it. Consequently, even for a biogenic compound, there is no guarantee that biodegradation of a given compound can occur in a particular environment. First, a capable organism must be present; second, an opportunity must exist for the requisite

enzymes to be synthesized; and third, environmental conditions must be sufficient for the enzymatically-catalyzed reactions to proceed at a significant rate. Thus, the process of biodegradation is highly system-specific.

In the present study, the MLSS levels were also analyzed during the aerobic oxidation of the different batches. In these studies, the initial biomass level was maintained in the range of 60–100 ppm. The response of the biomass during the biological degradation of the pollutants is shown in Fig. 6.

The inoculum responded differently to each of the pretreated. The growth curve followed different patterns of lag–log–stationary–decay. These differences in the growth patterns were probably as a result of different 'intermediate transformation products' generated during the pretreatment steps.

Metabolism of macromolecular substrates is different from that of small-molecular-weight compounds. Diffusivities of macromolecules are much lower than the diffusivities of small molecules. For example, the diffusivity of protein bovine serum albumin (BSA; 65,000 amu) is $6.8\times10^{-7}\,cm^2\,s^{-1}$, more than an order of magnitude lower than the diffusivity of the amino acid leucine (131 amu, $D=8.74\times10^{-6}\,cm^2\,s^{-1}$) (Confer and Logan, 1997). Mass transfer in mixed fluid regimes is more complex, but is also affected by the substrate diffusivity. The combination of the pretreatment techniques must have led to the alternation of the molecular composition of the effluent, in turn resulting in altered molecular size of the components, as reflected in the enhanced rates of aerobic degradation.

The experimental data of the variation in the cell mass as a function of the available substrate was fitted with the kinetic expressions reported in the literature (Bailey and Ollis, 1977; Vavilin, 1982; Contois, 1959). The widely accepted model for aerobic processes, Monod's model (Monod, 1949) however, could not be employed to describe the relationships between the substrate utilization and the biological growth. The aerobic treatment of the distillery wastewaters of different origins showed that all these experimental findings could be very well

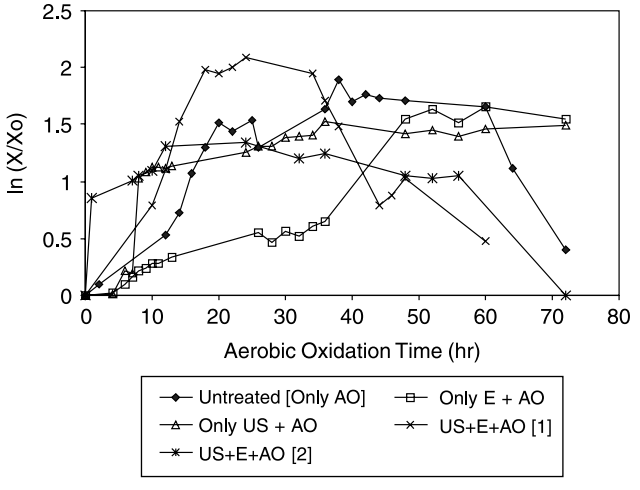

Fig. 6. Evolution of biomass (E+AO: E [50 U, 24 h]+AO [72 h]; US+AO: US [30 min]+AO [72 h]; US+E+AO [1]: US [2 h]+E [50 U, 24 h]+AO [72 h] US+E+AO [2]: US [30 min]+E [50 U, 12 h]+AO [72 h]).

correlated with the Contois model (Beltran et al., 2001, Beltran et al., 1999). According to the Contois model, the relationship between the specific substrate based growth rate of microorganisms (μ) and the substrate concentration in a batch process, limited by the amount of the available substrate and can be expressed as:

$$\mu = \frac{1}{t}\frac{dX}{dt} = \mu_{max}\frac{S}{\alpha X + S} \tag{1}$$

where μ_{max} is the maximum specific growth rate of microorganisms, S is the rate-limiting substrate concentration, and α is a dimensionless kinetic parameter related to the inhibition of the process. For the purpose of kinetic study, COD was considered to represent the multi-component substrate (S) while the cell concentration (X) was evaluated by estimating the concentration of MLSS as described before.

Since the substrate degradation resulted in the biomass growth, the yield coefficient (Y) can be defined using Eq. (2), to explain the exponential cell growth phase as,

$$(X - X_0) = Y(S_0 - S) \tag{2}$$

where, X_0 and S_0 are the MLSS and COD at time $t=0$, respectively, and X and S are the corresponding values at any given time, 't'. The evaluation of Y for each batch study was carried out.

Thus, combining and rearranging the Eqs. (1) and (2) gave the following differential form (Eq. 3);

$$\mu = \frac{1}{t}\frac{dX}{dt} = \mu_{max}\frac{\beta + X}{\beta + (\alpha Y - S)X} \tag{3}$$

where, β is a constant defined as:

$$\beta = YS_0 + X_0 \tag{4}$$

Eq. (3) can be integrated to give:

$$\frac{1}{t}\ln\left(\frac{\beta - YS}{X_0}\right) = \mu_{max} + \frac{\alpha Y}{t}\ln\left(\frac{S}{S_0}\right) \tag{5}$$

According to Eq. (5) a plot of the left-hand side against $(1/t)$ $\ln(S/S_0)$ should lead to a straight line having a slope αY with an intercept of μ_{max}. Table 2 presents the values of Y, μ_{max} and α determined by fitting Eq. (5) to the data from various batches.

The level of toxicity during the chemical pre-treatment is known to be a strong function of the treatment conditions (Mantzavinos and Psillakis, 2004). In several cases, toxicity of the original effluent was found to increase steeply and reach a maximum during the early stages of the pretreatment, presumably due to the formation of toxic intermediates (Gonze et al., 2003; Wang et al., 2003; Shang and Yu, 2002, Tiehm and Neis, 2004).

In the present study, from Table 2 it is clear that the pretreatment steps did affect the values of the inhibition factor, 'α' but, the rate of aerobic oxidation was found to vary independent of this inhibition factor. In other words, the rate of aerobic oxidation strongly depended on the pretreatment steps carried out but did not show any relation with the inhibition factor α.

Table 2
Calculated values for the growth yield (Y), the maximum specific growth rate of microorganisms (μ_{max}), the inhibition parameter (α) and oxidation rate constant

Conditions	Yield (mg MLSS per mg COD)	μ_{max} (h^{-1})	α (mg COD per mg MLSS)	Oxidation rate constant (h^{-1})
Untreated [Only AO]	0.0802	1.24	0.624	0.0078
Only US [30 min]+AO	0.11	3.72	0.75	0.175
Only E [pH 4.8], 50 U, 24 h]+AO	0.132	13.6	3.2	0.0115
US [2 h]+E [24 h]+AO…[1]	0.121	1.75	1.4	0.031
US [30 min]+E[12 h]+AO…[2]	0.177	8.62	1.4	0.0151

In order to confirm this observation, a few experiments were conducted to study the effect of higher levels of biomass on the rate of aerobic oxidation. Also to support this observation, experiments were conducted to study whether the addition of new cells into the system at a later time affected the performance of the system. In this respect, dosing of the biomass (Fed-batch mode: Set-1 and Set-2, Table 3) was carried out during the aerobic oxidation run at different time intervals as a measure of biodegradability. Two experiments were conducted at different levels of initial biomass concentrations. The results have been summarized in Table 3.

In the present study, the initial rate of biodegradation was found to be linearly dependent on the initial biomass concentration as shown in Fig. 7. The inoculum biomass was acclimatized to the untreated effluent sample. Fig. 7 indicates that during the acclimatization step, the biomass produced or secreted consortia of enzymes which governed the subsequent biological oxidation rates. Thus, higher biomass levels produced larger enzyme quantities which subsequently led to the enhanced initial oxidation rates.

Table 3 indicates that the rate of oxidation was independent of the new biomass added during the aerobic oxidation for this system but was dependent on the initial load of the biomass inoculated at the beginning of the aerobic oxidation. These results also indicate that at higher levels of initial biomass concentration, the rate of aerobic oxidation was also higher but could be attributed to the initial higher concentration of the secreted enzymes.

Another suggested biodegradability measure is the oxygen uptake rate (Scott and Ollis, 1995). Hence, the oxygen uptake was measured at different time intervals and corresponding respiration rates were calculated during the course of the

Table 3
Effect of biomass dosing on the rate of aerobic oxidation

Fed-Batch	Time (h)	Rate of oxidation, (mg of COD/h)	Biomass level, X (mg)
Set-1	0 h	44.19	68.18
	24 h-1st dose	41.27	165.0
	48 h-2nd dose	40.08	201.24
Set-2	0 h	110.35	129.25
	24 h-1st dose	97.58	638.05

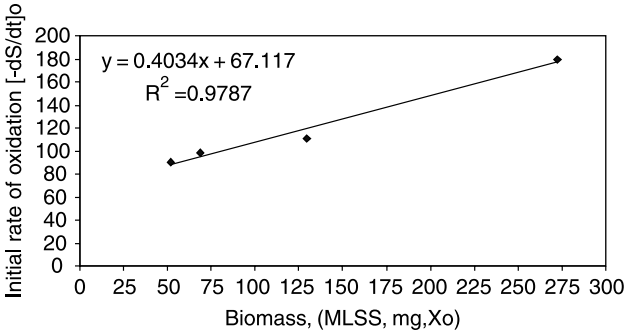

Fig. 7. Plot showing dependence of the initial rate of degradation on the initial biomass concentration.

reaction. A typical pattern of the oxygen uptake observed during the transient reaction is shown in Fig. 8. Fig. 8 indicates that the respiration rate increased drastically in the initial period of aerobic oxidation. It attained a peak owing to very high metabolic activities by the biomass and later the rate dropped drastically and attained a plateau during the course of biological reactions indicating the transition of biomass from the log phase to the stationary phase.

Thus, various experiments (Fig. 7), the initial respiratory activity by the biomass (Fig. 8) and the values of the inhibition factor α (Table 2), indicate that the rate of the aerobic oxidation process was a strong function of the initial biomass inoculum. This observation indicates that an increase in the toxicity following an oxidation or a pretreatment step is not necessarily accompanied by a decrease in its biodegradability. Wang et al. (2003), who studied ozonation of azo-dye Remazol Black, have reported that samples subjected to ozonation for up to 150 min were more biodegradable (in terms of increased BOD_5 and BOD_{28} values) but were also more toxic to *Vibrio fischeri* and rat hepatoma cells than the original effluent; this supports this observation.

3.8. Evolution of pH

The process by which microorganisms grow and obtain energy is complex and intricate; there are many pathways and cycles. Vital to the reactions involved in these pathways and cycles is the action of enzymes. The optimum pH and

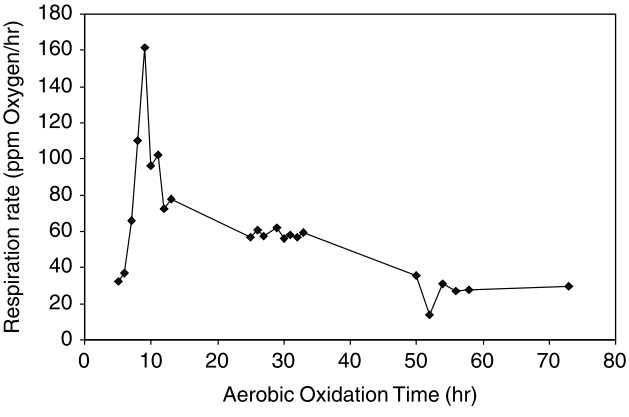

Fig. 8. Typical oxygen uptake pattern during the aerobic oxidation.

temperature for the action of the key enzymes in the cell are reflected in the overall temperature and pH preferences of the cell (Metcalf and Eddy, 1979). An effective pH range reported for cotton kiering liquor and sulfite waste is a pH of 5–11. For spent yeast broth and slaughterhouse wastes, the optimum pH was 5–9 and for antibiotics a pH of 5–7 has been reported (Eckenfelder and O'Connor, 1961).

Thus, the evolution of pH was observed during the biological oxidation step for the enzymatically pretreated effluent. A preliminary study of the aerobic oxidation at a constant pH value maintained between 7.5 and 7.7 indicated that the performance was independent of the pH. Hence, all the aerobic oxidation studies were carried out under uncontrolled-pH conditions.

The various runs conducted indicated a specific variation in the pH during the aerobic oxidation step. The pH value gradually decreased from the initial value of 7.5–7.6 in the first 8 h of oxidation to a value of 6.7–6.9 and then the pH gradually rose till the end of the run (72 h) attaining a value of 9.0–9.1. This observation can be possibly attributed to the conversion of the parent molecules present in the effluent sample, initially into acidic intermediates, which were further consumed to carry out various metabolic processes of the cell.

Eckenfelder and O'Connor (1961) have suggested that the effect of pH on the overall oxidation process is normally associated with the specific processes occurring during the growth cycle of the microorganism. Over some pH range, for each particular enzyme, it approaches a maximum and falls off above or below this range of pH. Values below 6.4 and above 9.4 were found to be detrimental to the organism, when the organism ceased to grow. The pH limits for the growth of any organism reflect the pH limits for the activity of the enzymes of that organism. If the substrate pH falls outside the higher and lower limits, death of the organisms usually occurs. The specific variation in the pH observed during the oxidation was noted in all the batches studied (untreated and pretreated distillery waste).

4. Conclusions

The effects of acoustic cavitation (US treatment) and the enzyme cellulase in the treatment of raw distillery wastewater have been investigated. From the experiments, it has been established that the low intensity ultrasound (radical formation and shear forces) brings about transformations of the effluent constituents at a molecular level. Rather than the sonochemical effects of the ultrasound, the powerful hydrodynamic shear forces governed these transformations. Hence, the acoustic cavitation alone did not result in the mineralization of the effluent contents. The enzymatic pretreatment also brought about changes in the composition of the effluent, thus altering the metabolic value of the distillery spent wash for subsequent biodegradation. The combination of the ultrasound and enzyme yielded the best COD removal efficiencies as compared to the processes when they were used as stand-alone treatment techniques. A 4-fold increase in the initial biological oxidation rate was observed over the untreated batch for the integrated technique (US + E + AO). From the biokinetic parameter analysis, the rate of the aerobic oxidation showed a strong dependence on the type of the pretreatment schemes carried out.

Though these techniques are not absolute replacements for the existing treatment technologies adopted for the treatment of distillery wastewater, they can definitely be incorporated into the existing treatment facilities as 'add ons', resulting in improved process performance of the biological treatments.

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