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Gaschromatography/mass spectrometry analysis of degradation of ethylacetoacetate achieved in shake flask culture using a previously characterized yeast strain *Tichosporon dermatis.*

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Abstract

Public and regulatory interest regarding the presence of pharmaceutically active compounds in the environment its increasing adverse impact has increased in the recent years. Detection of a wide variety of pharmaceutical compounds in water environment has been a serious and growing concern in the last few decades. Understanding the biological degradation of pharmaceutical compounds is essential for accurately determining their ultimate environmental fate, conducting accurate risk assessments, and improving removal of such micro pollutants. Present investigation was designed to accomplish biodegradation of ethylacetoacetate in shake flask culture using whole cells of previously isolated and identified yeast strain *Trichosporon dermatis,* from pharmaceutical effluents using enrichment culture technique. The strain was cultivated for two generations on an orbital shaker at 120 rpm at 28 ± 2^0C and the biomass was separated by centrifugation at 10,000 rpm for 20 mts. Normal saline washed cells were used in degradation carried out in Erlenmeyer flasks containing 500 ml of mineral medium containing ethylacetoacetate at standard conditions; wet cell weight= 20g/l; ethylacetoacetate concentration = 0.5% in mineral medium (w/v); time of biodegradation= 72 hrs; temperature= 28 ± 2^0C. Gas chromatography/mass spectrometry (GC-MS) analysis of microbially degraded product revealed that complete degradation of ethylacetoacetate in mineral medium was achieved in 72 hours using whole cells of *Trichosporon dermatis* yeast strain. Degradation of ethylacetoacetate by this yeast strain has not been reported before the present investigation.

Keywords: ethyl acetoacetate, biodegradation, Gass chromatography/mass spectrometry and effluents,

Introduction:

In the recent decades detection of a wide variety of organic pharmaceutical compounds in surface, ground and drinking waters, and their fate in water bodies is raising growing concern as regards the adverse consequences of these pollutants and future reuse of treated water **(Gusseme *et al.*, 2011)**. High quality groundwater resources are becoming less available with the rising demand of the drinking water day by day (**(Aguilar *et al.*, 2011**). plenty of waste water, from chemical syntheses and fermentation processes is generated by growing pharmaceutical industries worldwide and the microbial pretreatment is required **(Recek *et al.*, 2002).** Ethyl acetoacetate (EAA) is an organic compound. Its large scale application as a chemical intermediate in the production of a wide range of pharmaceutical and chemical products such as amino acids, various analgesics, antibiotics, antimalarial agents, antipyrine and aminopyrine, and vitamin B_1 has increased, and moreover its application in the manufacture of dyes, inks, lacquers, perfumes, plastics, and yellow paint pigments has also grown enormously **(Cary and Francis, 2006).** Pharmaceutical industries releasing waste water containing residual ethylacetoacetate in bulk which gain access to the water cycle. There has been a continuous threat in the last years for both surface and ground water owing to significant increase in generation of pharmaceutical pollutants. Wet hydrogen peroxide oxidation was used to deal with wasted water containing ethyl acetoacetate **(Ding *et al.*, 2005).** The use of pharmaceuticals continues to grow worldwide on par with many agrochemicals. The pharmaceuticals and personal care products (PCPs) enter our environment and act as a trigger on organisms regularly exposed to them (Sharma *et al.,* 2010). Steady release of pharmaceutical compounds into environment has been shown to have detrimental environmental consequence and could potentially impact public health safety **(Kagle *et al.*, 2009).** Detection of pharmaceutical compounds in trace concentrations both in groundwater **(Ikehata *et al.*, 2006)** and surface waters **(Jasim *et al.*, 2006)** are reported

worldwide. The incidence and the environmental fate of pharmaceutical drugs, illicit or recreational drugs, veterinarian medicines, over-the-counter medications, nutraceuticals and Personal care products (PCPs) reflect in literatures (**Thomas 2002; Kim et al. 2007; Liu et al. 2010).** Diverse and extensive studies have revealed the presence of ibuprofen, acetaminophen, aspirin, diclofenac, metoprolol, propranolol, nadolol andcarbamazepine in different water systems **(Jones *et al.,* 2002; Huber *et al*., 2003; Löeffler *et al.,* 2005; Hebere *et al.,* 1998; Ternes 1998; Hirsch *et al.,* 1996).** Very little is known about the ecotoxic effects of pharmaceutical pollutants which could pose enormous threat due to their regular discharge into the environment (**Daughton and Ternes, 1999**). Highly toxic pollutants such as disinfectants and pesticides and their residues are dangerous for human health and the environment, and are not easily degradable (**Moctezuma *et al,* 2003**). The environmental sciences have an enormous progress in recent years **(Oliveria *et al.,* 2011)** and there is a need to develop new technologies, new processes, and new materials for the prevention and control of such pollution **(Bertazzoli *et al.,* 1998).** Interest in the microbial transformation and or degradation of *pollutants* has intensified in recent years as humanity strives to find sustainable ways to clean up contaminated environments **(Diaz, 2008; Koukkou, 2011).** The major applications of enzymatic catalysis are associated with biodegradation and biotransformation **(Parales *et al.,* 2002).** The recent advances in bioremediation techniques for the treatment of toxic waste are of high significance (Fulekar, 2005). The process of bioremediation is an evolving method for the removal and degradation of many environmental pollutants (Das and Chandaran, 2010). In the present study degradation of ethyl acetoacetate using microbial strain previously isolated from pharmaceutical effluent was investigated in order to contribute to knowledge of biodegradation and transformation.

Materials and methods:

2.1 Maintenance of previously characterized pure yeast strain: Previously isolated yeast strain from pharmaceutical effluents by enrichment culture technique using mineral medium prepared in double distilled water (g L^{-1}, KNO_3-0.5, NaCl-2.0, K_2HPO_4-2.0, KH_2PO_4-2.0, $MgSO_4.7H_2O$-0.5, $CaCO_3$-0.02, and FeSO4.7H2O-0.01) supplemented with an extra pure ethylacetoacetate and paracetamol (SDFCL Chemical Manufacturer) as sole carbon and energy source was phenotypically and genotypically characterized as *Tichosporon dermatis* was used in the present study of microbial degradation. The pure strain was maintained by the periodic sub-culturing on agar slopes prepared by using malt yeast extract agar supplemented with few drops of 10% lactic acid in 28 ml screw-capped McCartney bottles. Slants were stored at 4^0C.

2.2 Preservation of the pure yeast strain: 812 µl of the broth culture grown at 28 ± 2^0C for 48 hours was dispensed into a 2 ml cryo-vial. 188 µl of 80% sterile glycerol was added and subjected to the vortex. Vial was placed in -20^0C freezer (**Sherman *et al.*, 1986).**

2.3 Optimization of concentration of ethylacetoacetate for efficient microbial degradation: 45 ml of the mineral medium containing different concentrations ranging from 0.1%, 0.5%, 1.0% and 1.5% of extra pure ethylacetoacetate were inoculated with 05 ml of 48 hours old broth culture of the yeast strain and incubated at 28 ± 2^0C for seven days under shaking conditions at 120 rpm. Growth was observed spectrophotometrically using a spectrophotometer (UV1 Thermo Electron Corporation, model no. 702602) at 600 nm at every two days interval. The same experiment was also carried out in distilled water in place of mineral medium at the same various concentrations of ethyl acetoacetate to compare the growth in both the conditions.

2.4 Biodegradation of ethylacetoacetate using whole yeast cells: Degradation of ethylacetoacetate was accomplished using whole yeast cells according to **Popa *et al*. (2008).** The active biomass for degradation was obtained by cultivating cultures in 500 ml erlenmayer flasks containing 100 ml Malt extract broth. The strain was cultivated for two generations on an orbital shaker at 120 rpm at 28 ± 2^0C. The biomass was separated by centrifugation at 10,000 rpm for 20 mts. Cells were washed with normal saline. The degradation was carried out in 20 erlenmayer flasks containing 500ml of mineral medium containing ethylacetoacetate at optimal concentrations. Standard conditions for the biodegradation process were as follows:

Wet cell weight= 20g/l;

Ethylacetoacetate concentration = 0.5% in mineral medium (w/v).

Time of biodegradation= 72 hrs.

Temperature= 28 ± 2^0C

2.5 Gas chromatography Mass spectrometry (GC/MS) analysis of degraded products.

Gas chromatography–mass spectrometry (GC-MS) is a method that combines the features of gas-liquid chromatography *and* mass spectrometry *to identify different substances within a test sam*ple. GC/MS analysis of standard and microbially degraded ethylacetoacetate was carried out using Clarus 500 gas chromatograph coupled with Clarus 500 mass spectrometer. Elite 5 MS and methanol were used as capillary column and solvent respectively.

Results and discussion

In the early phase of the present study optimization of concentration of ethyl acetoacetate as sole carbon and energy source before going for an efficient microbial degradation process was carried out in two reaction media, a) mineral medium (same composition that was used at the time of the isolation of the strain *Trichosporon dermatis* using enrichment culture technique) and, b) distilled water. Survival and growth of the yeast strain was found to be proportionate throughout the incubation period on mineral medium containing ethylacetoacetate at 0.5% & 0.1% while that of the strain on distilled water containing with ethyl acetoacetate as carbon and energy source, was found higher in the initial phase of incubation, but a lagging in the last phase at almost every concentration was spectrophotometrically recorded which clearly indicated that mineral medium supported better growth and survival of the yeast strain than pure distilled water (Fig.1 & 2). Linear increase in the absorbance (optical density) was noticed better at concentration of 0.5% of ethylacetoacetate in mineral medium. However, at other concentrations initial increase in absorbance was recorded but a drop was noticed in the later phase of the incubation (Fig. 1), thus biodegration was carried out at the most suitable concentration (0.5%) ethylacetoacetate in mineral medium. Chromatogram and spectrograph obtained from gas chromatography/mass spectrophotometry analysis of the product of microbial degradation and that of standard ethyl acetoacetate were compared. Two peaks in the chromatogram of standard ethylacetoacetate (Fig.3) and their corresponding peaks in full-scan mass spectra at m/z 43 and m/z were observed. The base peak at m/z 43 and molecular ion peak at m/z 130 (Fig. 4) revealed the presence of ethylacetoacetate in the standard sample while both the peaks in the chromatogram of the microbially degraded sample were completely absent (Fig. 5) and, hence the corresponding mass spectra was not obtained. The disappearance of the peak in the microbially degraded sample revealed the complete degradation of ethyl acetoacetate in the mineral medium in 72 hours of incubation with the yeast *Trichosporon dermatis*.

Conclusion:

Consequently to our investigations, it could be noticed that the novel yeast strain previously isolated from pharmaceutical effluent and identified as *Trichosporon dermatis* has catabolic properties to degrade ethylacetoacetate in mineral medium at 0.5% (w/v) concentration using shake flask culture technique in 72 hours. The outcome of the present investigation would make a real contribution in the knowledge of biodegradation of organic compounds and thus may help coping up the challenges to minimize pharmaceutical pollution.

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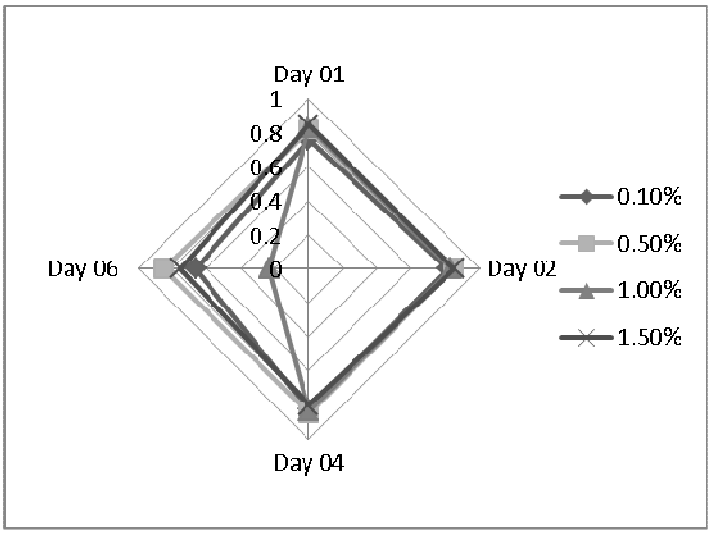

Figure.1 Growth and Survival of the yeast strain on Different Concentrations of Ethyl Acetoacetates in Mineral Medium.

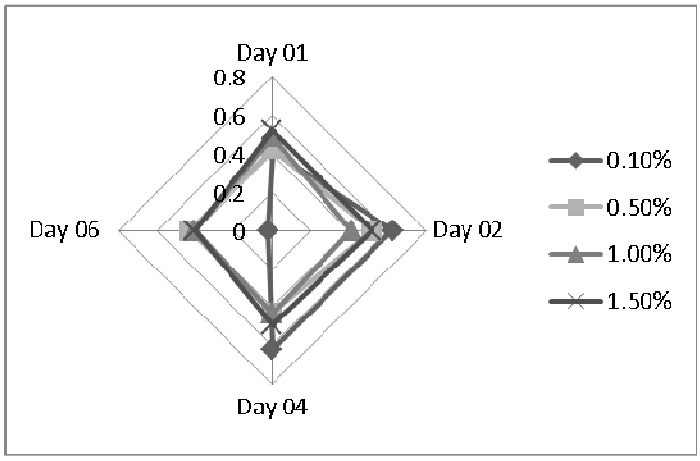

Figure.2 Growth and Survival of the yeast strain on Different Concentrations of Ethyl Acetoacetate in Distilled Water

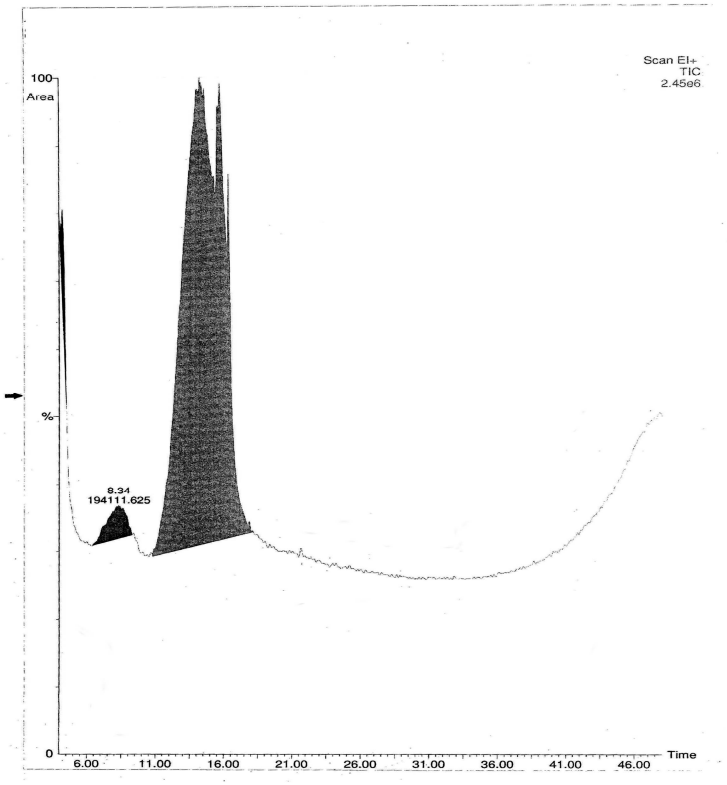

Figure 3. Exhibiting the GC/MS chromatogram of the standard ethyl acetoacetate sample.

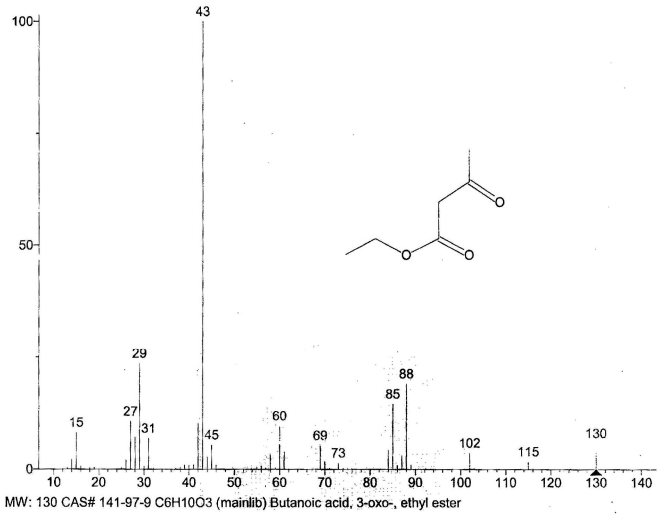

Figure 4. Mass spectrograph of the standard ethyl acetoacetate sample.

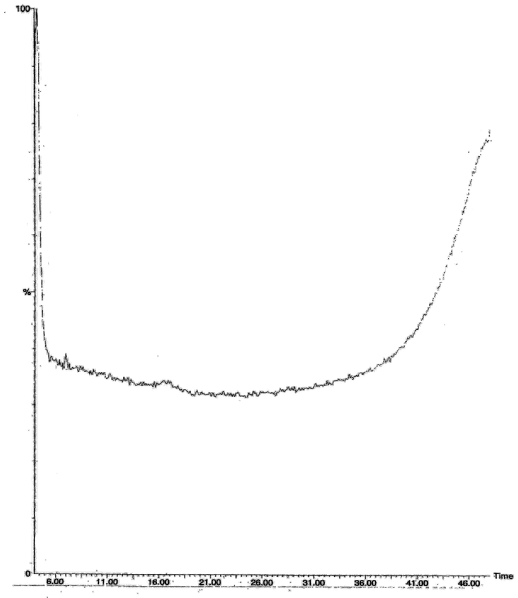

Figure 5. Showing the GC/MS chromatogram of the degraded ethyl acetoacetate sample.

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