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## Microaerobic Degradation of Melamine Formaldehyde Resin

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### ABSTRACT

#### Keywords

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#### Article Info

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Melamine formaldehyde resin (MFR) has stable aromatic molecular structures used as retanning agent in leather industry. The bacterial strains *Klebsiella pneumoniae* strain CSMB6 and *Enterobacter cloacae* strain CSMB2 as consortium was able to degrade MFR in a microaerobic reactor under optimized conditions. About 83% degradation was obtained with the residual COD concentration of 30 mg/L at 48h of incubation along with the biomass production of 165 mg/L as dry cells. Intermediate metabolites detected by GCMS, indicates the presence of formic acid, methyl butyl alcohol, Methoxymethyl formate and 3-Mercapto-3-methyl butyl formate. The consortium was able to degrade both melamine and formaldehyde simultaneously utilizing them as sole carbon and energy.

### Introduction

Melamine formaldehyde resin (MFR) is a hard, thermosetting plastic material, synthesized by condensation of formaldehyde with melamine. MFR is used in retanning of chrome leathers. It gives fullness to leather and provides a tight and uniform grain surface for leather finishing. To make it a water soluble product, it is further sulphated using naphthalene sulphonic acids. Coatings on day-to-day products like plywood and particleboard adhesives, laminated countertops and tabletops, dishwasher-safe tableware, and automotive surface coatings are also made

from MFR (Lewis, 2001, Bradley *et al.*, 2005; Kandelbauer and Widsten, 2009).

Melamine resin is nitrogen-rich (67% by mass) and hence it is potential nitrogen source during composting (Tian *et al.*, 2012). Melamine is a major component of a colorant (Pigment Yellow 150) and fertilizer (WHO, 2009). Control on melamine contamination in foods (Bhalla *et al.*, 2009) was initiated after an incident of melamine adulteration in infant food (Xin and Stone, 2008), which resulted in renal toxicity. Toxic effects of melamine include

nephrolithiasis, chronic kidney inflammation, and bladder carcinoma, all of which have been studied in animals (Hau, 2009). MFR releases monomers of both melamine and formaldehyde.

Melamine (1, 3, 5-triazine-2, 4, 6-triamine) is a heterocyclic aromatic compound having a six-membered ring of alternating C and N atoms, with three NH<sub>2</sub> groups. It is a member of the s-triazine family and its hydroxyl analogues such as ammeline (4,6-diamino-2-hydroxy-1,3,5-triazine), ammelide (6-amino-2,4-dihydroxy-1,3,5-triazine), and cyanuric acid (1,3,5-triazinane-2,4,6-trione) were detected in vegetable proteins in contaminated pet foods (Dobson *et al.*, 2008). Formaldehyde is a reactive molecule that contains a carbonyl group which reacts with amines, amides, sulphides, and purines of organic compounds (Vorholt *et al.*, 2000). Formaldehyde may cause nasopharyngeal cancer and possibly leukemia (IARC 2006). Thus the biodegradation of MFR in industrial effluents is necessary due to its threats to human health (Xu *et al.*, 2011). To achieve a longer shelf life and an optimum cross linking density, MFR is synthesized with a high degree of methylation, etherified with methanol or butanol. Hence, the processed wastewater using MFR generated contains methanol, butanol, urea and ammonium along with formaldehyde and melamine (Glancer, 2001). Among different methodologies for the degradation of formaldehyde, the biological processes are the most economical methods (Di Maiuta *et al.*, 2009). Several bacteria, yeast, and filamentous fungi have been evaluated for the degradation of formaldehyde. *Mycobacterium gastri*, *Rhodococcus sp.*, *Pseudomonas sp.*, *Acetobacter sp.*, *Paecilomyces sp.*, *Aspergillus sp.*, and *Trichoderma sp.* are few examples. (Mirdamadi *et al.*, 2005, Sawada *et al.*, 2006; Shinagawa *et al.*, 2006 Kondo

*et al.*, 2008; Yurimoto *et al.*, 2009; Morikawa *et al.*, 2013; Luo, 2013; Bo.X 2015). The possible products of formaldehyde biodegradation are methanol and formic acid (Eiroa 2005). In plants and animals, formaldehyde is metabolized to formate and oxidised to CO<sub>2</sub> by dehydrogenase (Kornbrust, 1983) and catalase enzymes (Halliwell 1974). It has been reported that melamine is recalcitrant to activated sludge, as the induction of enzymes responsible for hydrolytic deamination is poor even after a long period of sludge adaptation (Xu *et al.*, 2013). While many bacteria and fungi are known to degrade melamine and formaldehyde independently, biodegradation studies of MFR are scarce. Although *Klebsiella pneumoniae* (Ping, 2014) and *Enterobacter cloacae* (Saadoun, 2002) is known for their ability to biodegrade polycyclic aromatic hydrocarbons (PAH), degradation of MFR has not yet been reported by these bacterial strains. *Klebsiella terrigena* strain DRS-1, isolated from sewage sludge enrichments (Shelton *et al.*, 1997) and *Micrococcus sp.* strain MF-1 isolated from wastewater from an amino plastic plant. (El-Sayed *et al.*, 2006) were reported to have mineralized melamine.

The present investigation was carried out to study the simultaneous removal of formaldehyde and melamine in a lab-scale microaerobic reactor. A consortium that consisted of two isolated bacterial strains was evaluated for the degradation of MFR, as well for finding out if MFR is used as the substrate source for growth by the consortium. The pure chemicals which are intermediate products of MFR were also tested independently for degradation. UV-Visible spectrum and GCMS studies were used to identify the intermediate metabolites to construct a degradative pathway for Melamine formaldehyde resin (MFR)

## Materials and Methods

### Chemicals

The Melamine formaldehyde resin (MFR) was provided by a tannery. Formaldehyde, Melamine, Ammeline, ammelide, cyanuric acid and biuret were purchased from Sigma-Aldrich, India. The culture medium used for the study is a mineral salt medium containing (g/l):  $K_2HPO_4$ , 1.2;  $KH_2PO_4$ , 0.3;  $MgCl_2 \cdot 7H_2O$ , 0.5; NaCl 1.0;  $CaCl_2 \cdot 2H_2O$ , 0.2;  $FeSO_4 \cdot 7H_2O$ , 0.02; and 10 ml of trace elements. All medium components of analytical grade were procured from E.Merck Mumbai (India).

MFR (ChemSpider ID: 84298) is a hard, thermosetting plastic material made from melamine and formaldehyde by polymerization. Formaldehyde and Melamine (1,3,5-triazine-2,4,6-triamine) are in equal proportion (1:1) in MFR, with molecular formula  $C_4H_8N_6O$  and average mass of 156.146 Da;. Structure of MFR is shown in Fig.1.

### Microorganism

The bacterial strains used in this study were isolated from a phenol degrading microaerobic reactor. The *Klebsiella pneumonia strain CSMB6* and the *Enterobacter cloacae strain CSMB2* were selected based on their ability to degrade formaldehyde and melamine respectively and also due to their sustainability even after repeated cycles. They are capable of degrading a mixture of heterocyclic compounds in a consortium along with other four isolated strains. For enrichment of MFR and for the degradation studies, a specially designed laboratory scale bioreactor (3.5 l) with the automatic control biosensors to maintain microaerobic condition was used. The working mechanism has been explained elsewhere (Umamaheswari and Rama 2014).

### Degradation of MFR

The concentration of MFR used in the study was 100 mg/L (COD = 320mg/L), corresponding to the concentration present tannery effluents. The isolated strains *Klebsiella pneumoniae strain CSMB6* and *Enterobacter cloacae strain CSMB2* were added in equal proportions. MFR degradation was monitored by following COD and biomass growth. The degradation of MFR was also monitored by measuring spectral changes in a UV-Visible spectrophotometer (ShimadzuUV2450). Studies were carried out in mineral medium with the MFR as the sole carbon and nitrogen under optimized conditions in a microaerobic reactor (Umamaheswari and Rama 2014). To estimate biomass growth, the centrifuged pellets of the culture were dried at 60°C overnight for 24 h, until a constant weight was obtained. Chemical Oxygen Demand (COD) (5220-B) and Nitrogen Ammonia (4500-NH3-C) were estimated using Standard Methods (APHA 2005).

### Effect of Different Initial Concentration

Degradation of MFR by the isolated strain was conducted with initial MFR concentrations of 100, 150, 200 and 250 mg/L in a screw capped Erlenmeyer flasks. For this, equal proportion of the cells of *Klebsiella pneumoniae strain CSMB6* and *Enterobacter cloacae strain CSMB2* were inoculated in MSM. A 5% head space was provided for controlled oxygen and incubated in an orbital shaker with a shaking speed of 50 rpm. The percentage degradation was measured at different time intervals.

### Effect on Intermediate Metabolites

Studies on the metabolic versatility was evaluated by inoculating equal proportions

of *Klebsiella pneumoniae* strain CSMB6 and *Enterobacter cloacae* strain CSMB2 to the intermediate metabolites of MFR such as formaldehyde, melamine, Ammeline, ammelide, cyanuric acid and biuret. Concentrations of intermediate metabolites were determined by comparing the calibration with those of authentic standards. The mineral medium was amended with the respective substrates at 50 mg/L. The percentage degradation was estimated by using the formula: Degradation (%) = [(Ao-A)/Ao]\*100, where Ao is the initial concentration of the substrate and A is the residual concentration after degradation.

### **Gas Chromatography – Mass Spectrum Analysis**

The ethyl acetate extracts of cell-free medium were analyzed for the degraded metabolites of the MFR by GCMS for mass analysis. The GCMS analysis was carried out using JEOL-GC-mate-II benchtop double-focusing GC mass spectrometer operating in electron impact ionization (EI) mode. Helium was used as carrier with a flow rate of 25 ml/min. The injector temperature was maintained at 220 °C (temperature range 70 - 250 °C) and the rate of increase in temperature was set to 15 °C/min. The ethyl acetate extract of the products were dissolved in methanol and subjected to GC/MS analysis. The compounds were identified on the basis of retention time and mass fractions.

## **Results and Discussion**

### **Isolation**

The bacterial strain CSMB6 was isolated from a microaerobic reactor, based on its ability to degrade formaldehyde. The strain CSMB6 was identified as *Klebsiella pneumoniae* strain SDM45. It is a Gram-negative, rod-shaped bacterium, non-motile

in nature, encapsulated, lactose-fermenting and facultative anaerobe. It occurs naturally in the soil, and about 30% of strains can fix nitrogen in anaerobic conditions (Postgate, J 1998). The strain CSMB2 was identified as *Enterobacter cloacae* strain SJ6. It is a rod-shaped Gram-negative bacterium, facultative anaerobe, bears peritrichous flagella for motility. It is oxidase-negative and catalase-positive. It is generally present in the normal gut flora of many humans.

### **Biodegradation of MFR**

To evaluate the degradability by the isolated bacterial strains, studies were conducted using MFR in a microaerobic reactor under optimized conditions (DO of 0.9 mg/L, pH of 7 and temperature of 30°C) Biodegradation was determined by estimating TOC and monitoring biomass growth in mineral medium containing MFR as the sole carbon and nitrogen source (Fig 2). The results indicated that a maximum of 83% MFR was degraded with the residual TOC concentration of 30 mg/L after 48h of incubation. Maximum biomass production of 165 mg/L as dry cells was observed at 36h and reduction in biomass noticed from 48h may be due to limitation of substrate. Simultaneous production of ammonia was observed from 12h of incubation and it continued till 48h. The release of ammonia is due to the deamination of melamine degradation followed by successive deamination of its secondary metabolites. It is reported that deaminations of melamine and other intermediates occur outside the cytoplasmic membrane (Shelton *et al.*, 1997). Though release of ammonia was continuous during the degradation of MFR, the pH of the culture was maintained may be due to the accumulation of formic acid by the degradation of formaldehyde. It is reported that formic acid is formed as the intermediate product of formaldehyde by the catalytic reaction of dismutase in bacteria

and by dehydrogenase in yeast (Glancer, 2001). From the results obtained it is confirmed that the bacterial strains *Klebsiella pneumoniae* strain CSMB6 and *Enterobacter cloacae* strain CSMB2, as consortium, utilise MFR as energy for its growth and degrade both formaldehyde and melamine simultaneously. Our results are similar to those observed on degradation of lindane by yeast (Abdul Salam *et al.*, 2013), metabolism of triazines by *Arthrobacter aurescens* TCI (Lisa *et al.*, 2002) and *Streptomyces* strain PSI (Mirdamadi *et al.*, 2005), where the increase in biomass correlated with the degradation of the respective substrate. When strain CSMB6 was used as the sole inoculant, the degradation of MFR was delayed with a lag period and only 60% reduction was observed at 48h.

But when it was co-cultured with the strain CSMB2, the degradation potential was enhanced with absence of lag period. As a mixed culture, they were able to metabolize both melamine and formaldehyde simultaneously, as observed from the reduction in TOC within 48h. It may be due to the induction of relevant enzymes for the catabolism of MFR and adoption to metabolize new chemical compounds by evolving the needed enzymes (Seffernick, 2001). It has been reported that *Klebsiella pneumoniae* 99 grows only with ammelide and its lower intermediates utilizing them as sole nitrogen source (Cook, 1987) and the *Enterobacter cloacae* strain 99 was able to degrade only cyanuric acid to biuret acid (Cheng, 2005). It is reported that stepwise adaptation is needed for *pseudo alcaligenes* strain (Mirdamadi, 2005) *Methylobacterium* strains BIP and ROS1 and Marine Microalga *Nanno chlorop sisocolata* ST-3 Strain, (Chongcharoen, 2005, Yoshida, 2009) for the effective degradation of formaldehyde. The reason may be due to the activity of

specific enzymes to catalyze different reactions. The results observed are in agreement with the degradation of melamine formaldehyde resin by *Micrococcus* sp. Strain MF-1. They suggested that the Strain MF-1 was able to split melamine moieties and remove formaldehyde prior to melamine degradation (El-Sayed, 2006).

### **Effect of Initial Substrate Concentration**

The ability of the bacterial strains *Klebsiella pneumoniae* strain CSMB6 and *Enterobacter cloacae* strain CSMB2 as consortium to tolerate high MFR concentrations was investigated in the microaerobic reactor at an optimized condition as mentioned earlier. The degradation experiment was carried out with the different initial MFR concentrations as TOC concentration of 400, 800 and 1200 mg/L and the TOC removal efficiency are depicted in Fig. 3. Maximum TOC reduction of 75% was obtained with in 48h when TOC of 400 mg/L concentration of MFR was used. Though the bacterial strains tolerated TOC of 800mg/L concentration of MFR, only 16% degradation was achieved. It may be due to substrate inhibition. Further increasing the concentration to TOC of 1200 mg/L, inhibition is observed.

### **Effect on Intermediate Metabolites**

Batch reactor studies were performed to evaluate the degradation of formaldehyde, melamine and its intermediate metabolites by the mixed culture of *Klebsiella pneumoniae* strain CSMB6 and *Enterobacter cloacae* strain CSMB2. 10% of exponential culture was inoculated in minimal medium amended with (50 mg/L) formaldehyde, melamine and other secondary metabolites independently. The initial dissolved oxygen (DO) concentration observed was 0.9 mg/L. At the end of 48h,

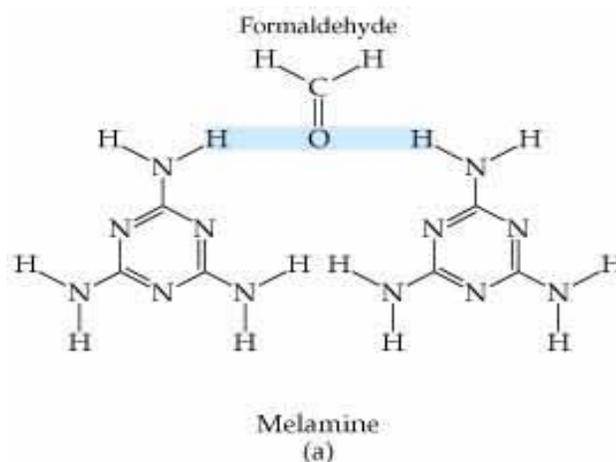
DO concentration of 0.02 mg/L was observed. About 80 to 90% reduction was observed as TOC measurement after 48h of incubation with all the tested intermediate metabolites as shown in Figure 6.4 confirming the degradation of the intermediate metabolites. Metabolism of melamine is based on enzyme catalyzed hydrolytic reactions (Cook, 1987). The amido hydrolase catalyze deamination of nitrogen containing heterocyclic ring

substrates (Seibert, 2005, Wackett, 2002). It is suggested that formaldehyde is metabolized either by formaldehyde dehydrogenase or by using a cyclic pathway with ribulose monophosphate cycle (Azachi *et al.*, 1995). Similar to this study, degradation of formaldehyde by different microorganisms (Yamazaki, 2001) and melamine deamination by microbes were reported (Wackett, 2002; Fruchey, 2003; Li, 2009; Seffernick, 2010; Cameron, 2011).

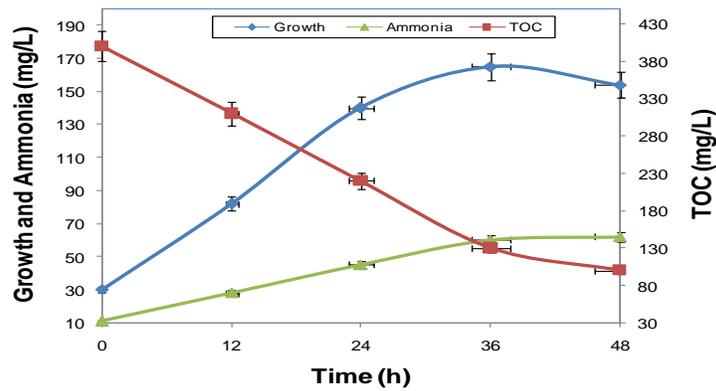
**Table.1** Chemical Structure, and Fragmentation Masses of the Degraded Metabolites of Melamine

Time	Retention time	Degraded metabolite	Molecular Weight	Fragment ion masses and Relative intensity (%)
24h	13.19	Ammeline	127	127 (75%)
		Methyl Butyl alcohol	88	83 (100%)
		Formic acid	46	51 (100%)
36h	15.08	cyanuric acid	129	129 (65%)
		2-Methoxymethyl formate	90	96 (100%)
48h	17.11	Biuret acid	104	102 (80%)
		3-Mercapto-3-methyl butyl formate	148	145 (100%)
		Methyl formate	60	52 (100%)

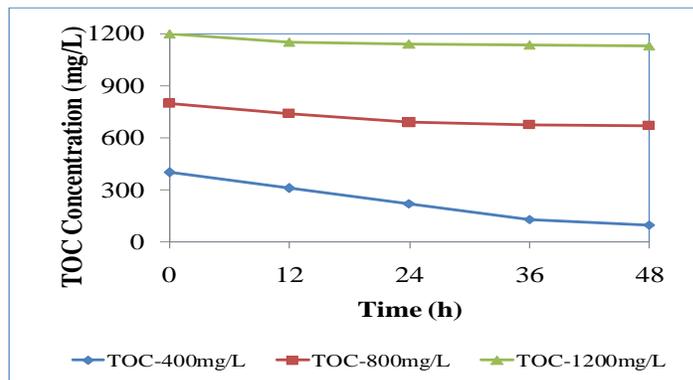
**Fig.1** Structure of Melamine Formaldehyde



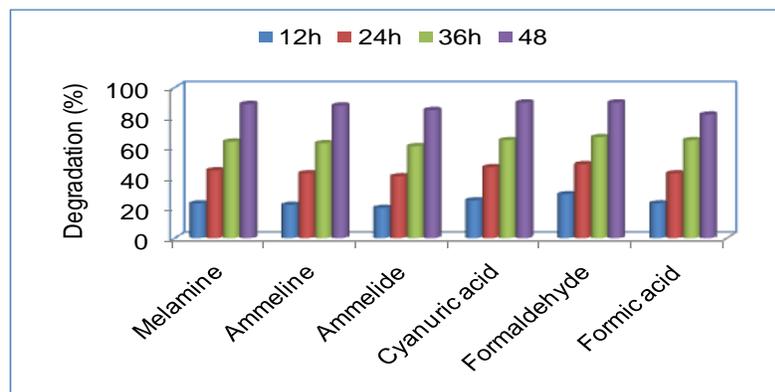
**Fig.2** Degradation Profile of Melamine Formaldehyde Resin (MFR)



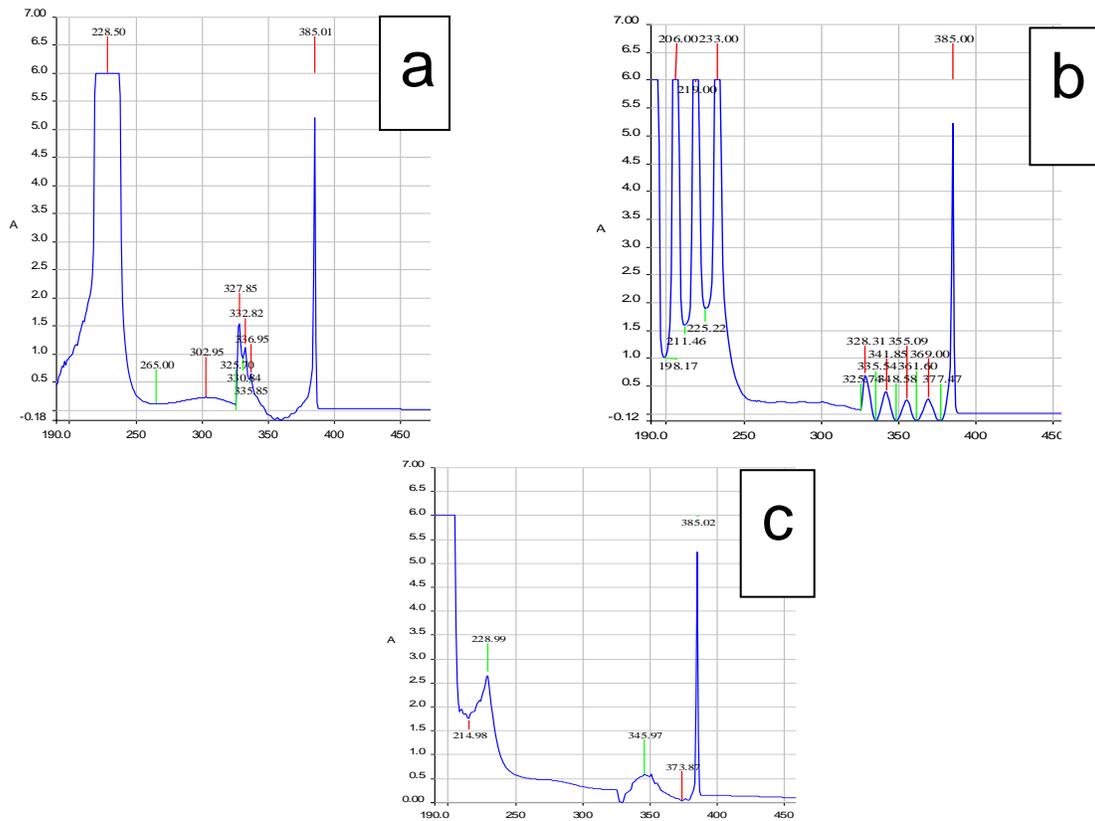
**Fig.3** Effect of Initial Concentration of MFR Observed as TOC Removal Efficiency



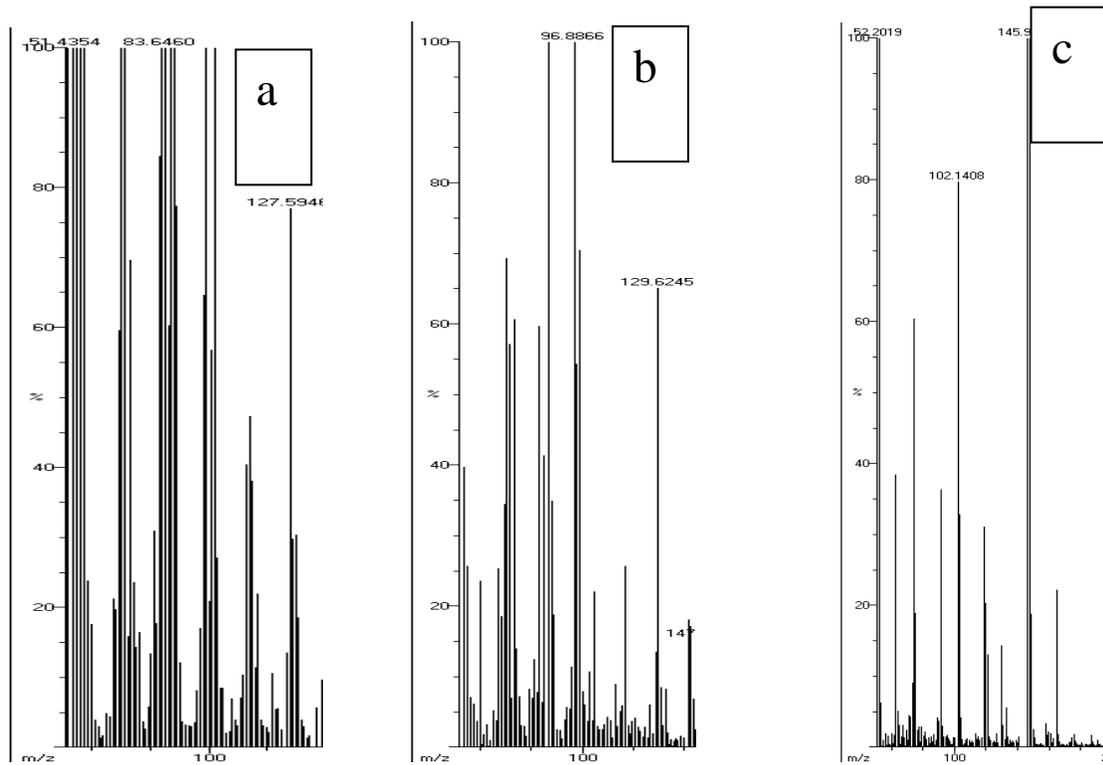
**Fig.4** Effect on Intermediary Metabolites of MFR



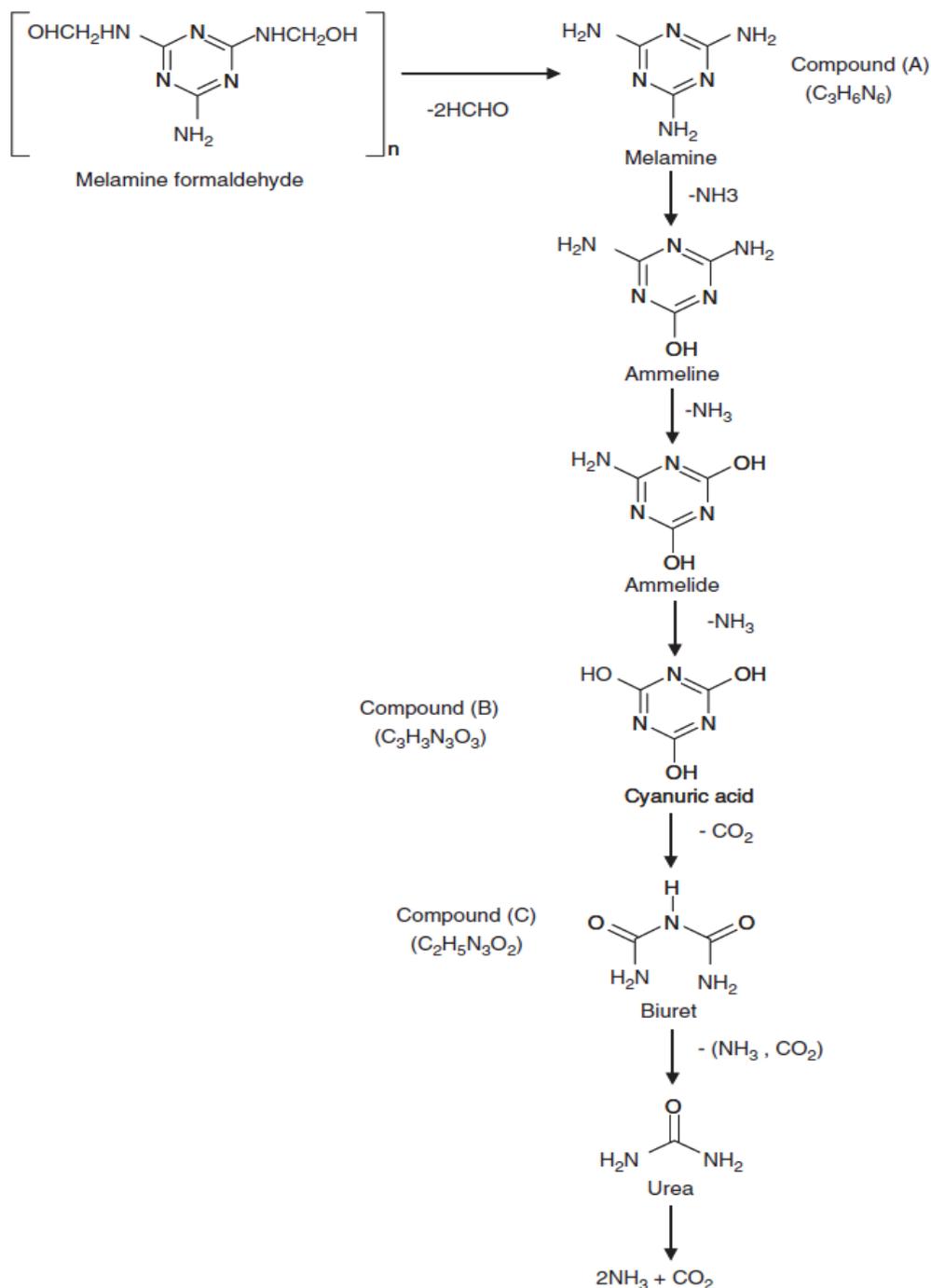
**Fig.5 a, b, c** Spectrum Showing Degradation of MFR



**Fig.6 a, b, c** GCMS - Degradation Profile of MFR



**Fig.7** Proposed Microaerobic Degradative Pathway for MFR



### UV-Visible Spectroscopy

MFR degradation was monitored using UV-Vis Spectroscopy. The culture supernatant obtained at 0h revealed absorption peaks ( $\lambda$

max) for MFR and are 385 and 228 nm (Fig. 5). The culture supernatant drawn after 24h incubation exhibited a number of new peaks representing oligomeric esters. The disappearance of the peaks at 48h might be

due to the successive deamination of melamine and degradation of formaldehyde to acids and esters. The observation on 48h culture sample confirmed the degradation of oligomeric esters representing 70-80% degradation of MFR. It is reported that since the carbon to nitrogen ratio of melamine is 0.43, it requires an external carbon source for the catabolism of melamine. Studies by Takagi (2011) revealed that a consortium of three strains *ATD6*, *CDB21*, and *CSBI* needed ethanol as external carbon source to degrade melamine completely. In the present study, the formaldehyde present in MFR was perhaps used as carbon source by the bacterial strains for the deamination of melamine.

### **GC-MS Analyses**

GC-MS analyses were carried out to find the metabolites formed during the biodegradation of MFR by the strain *CSMB6* and *CSMB2*. Metabolites were detected by GC at different retention times of 13 to 17 min. and were identified by comparing the mass with those of authentic standards from the NIST library. The molecular weight (MW) of the intermediates and the fragment ion-mass observed from MFR degradation is given in Table 1 and spectrum is shown in Fig. 6. The intermediates observed after biodegradation of MFR for 24h were ammeline, formic acid and methyl butyl alcohol. At 36h, appearance of cyanuric acid and 2-methoxymethyl formate were observed. The supernatant of 48h culture revealed the formation of biuret acid, methyl formate and 3-mercapto-3-methyl butyl formate. The results indicated that deamination of melamine may lead to the formation of ammeline. Further deamination of ammeline resulted in cyanuric followed by biuret acid. It is reported that the deamination of melamine proceeds stepwise with the loss of one, two or three amino

groups (Bozzi, 2004). Simultaneous degradation of formaldehyde resulted in accumulation of formic acid. Presence of formic acid, methyl butyl alcohol, methoxy methyl formate and 3-mercapto-3-methyl butyl formate confirmed the degradation of MFR which ultimately ends with the release of ammonia and CO<sub>2</sub>.

### **MFR Biodegradation Pathway**

Based on the evaluation studies under microaerobic condition on MFR by the consortium of the two isolated bacterial strains *CSMB6* and *CSMB2*, a biodegradation pathway is proposed as shown in Fig.7. The UV-Visible spectrum and GCMS results indicated the simultaneous biodegradation of formaldehyde and melamine, the main components in MFR. It also suggested that successive deamination of melamine resulted in the formation of cyanuric acid. Decarboxylation of cyanuric may lead to the accumulation of biuret acid with the release of ammonia. It is presumed that the methylol group of MFR (formed by an addition reaction of formaldehyde with amino group of melamine) on degradation was transformed to carboxylic acids and alcohols resulting in oligomeric esters with a release of CO<sub>2</sub>.

In conclusion, Melamine formaldehyde resin (MFR) possessing polymeric aromatic molecular structures are highly stable and hence highly resistant to biodegradation. MFR is one of the retanning agents used in leather industry. The ability of the bacterial strains *Klebsiella pneumoniae* strain *CSMB6* and *Enterobacter cloacae* strain *CSMB2* as a consortium for degradation of MFR concentration of 100 mg/L in a microaerobic reactor under optimized conditions was studied. The results obtained confirm the degradation of MFR for the TOC

concentration of 400 mg/L, with the residual TOC concentration of 100 mg/L and biomass (dry cells) production of 165 mg/L after 48h of incubation. Degradation of MFR with corresponding growth proves the utilization of MFR as sole carbon and nitrogen by the isolated strains. When tested for the inhibitory concentration of MFR, above 300 mg/L was found out to be inhibitory corresponding to TOC content of 800 mg/L. UV-Vis spectrum of the culture filtrate after 48h confirmed the degradation of oligomeric esters representing 70-80% degradation of MFR. Metabolites detected in the culture supernatant by GC-MS indicated the presence of formic acid, methyl butyl alcohol, methoxy methyl formate and 3-mercapto-3-methyl butyl formate, which confirmed the degradation of MFR. The significance of the present study is that the consortium consisting of *Klebsiella pneumoniae* strain CSMB6 and *Enterobacter cloacae* strain CSMB2 was able to degrade both melamine and formaldehyde simultaneously within 48h, utilizing them as sole carbon and nitrogen, which may be due to their adoption to the pollutants present in leather industrial wastewater. So the consortium of CSMB6 and CSMB2 may be used in the bioremediation of MFR contaminated site.

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