BIODEGradation OF DISTILLERY MELANOIDINS BY A NOVEL FUNGUS IN OPTIMIZED MEDIUM

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ABSTRACT: The present study aims to explore the feasibility of using a highly potent novel fungus coded **VT-NSK** with **GenBank Accession number JN570507** isolated from the effluent contaminated soil from Kerala, India for decolorization and degradation of the recalcitrant compound namely Melanoidin in distillery effluents from distillation and fermentation industries . An optimum medium both physical and nutritional was formulated for VT-NSK for maximum enzyme production (0.059U/ml) and colour reduction (92%) at 48 hrs. The colour removal efficiency of VT-NSK through the breaking down of substrates both distillery spent wash and synthetic melanoidin at various concentrations with respect to biomass, pH etc. were studied.

Key Words: Biodegradation, Distillery effluent, Synthetic melanoidin, Noval Fungus, Optimized medium, Molasses spent wash, Decolorization.

HIGHLIGHTS

- A highly potent novel fungus coded **VT-NSK** was isolated and deposited in **NCBI with GenBank Accession No. JN570507.**
- The isolate producing melanoidin degrading enzymes with remarkable properties was characterized.
- Preliminary work showed that it was also thermophilic compared to many other fungi and had high optima with respect to pH and temperature, which makes the wild isolate a good candidate for wide application in industries like textiles, paper and pulp, distillation and fermentation industries etc.

INTRODUCTION

Today industries are the major source of pollutants to the ecosystem. Distilling industries are those concerned with the production of ethanol and distilled spirits such as rum, whisky, brandy, gin, cordials and liquors. Now a day's alcohol distilleries have emerged as a prominent sector worldwide due to the large-scale industrial applications of alcohol in pharmaceuticals, food, perfumery etc. Moreover alcohol is used as an alternate fuel. There are more than 319 distilleries producing 3.25 x 10^9 l of alcohol and generating 40.4 x 10^{10} l of wastewater annually in India alone (Pant and Adholeya, 2007). Apart from other industries distillery is one of the industries which produce wastes characterized by high organic matter, disagreeable colour and odour. In fact alcohol distilleries are listed at the top in the "Red Category" industries as per the Ministry of Environment and Forests (MoEF) due to their high polluting potential (Tewari *et al* ., 2007).

The residue of the fermented mash which comes out as liquid waste is generally known by the term spent wash (Pathade 2003; Chandraraj and Gunasekharan 2004; Singh *et al* ., 2004). Spent wash is a very complex, caramelized and cumbersome recalcitrant agro- industrial waste with a temperature range of 70-80^{0}C at discharge point , deep black brown colour, low pH , high concentration of organic materials and solids. However the distillery effluent pollution load largely depends on the quality of molasses, unit operations for processing the molasses and process recovery of alcohols respectively (Pandey *et al.*, 2003). Distillery effluent is characterized by high biological oxygen demand (BOD), chemical oxygen demand (COD), phenol compounds, sulphate and heavy metals (Pant and Adholeya, 2007).

For the present study, digested molasses spent wash (DMSW) was collected from United Spirits Ltd. Aleppey, India after the biomethanation step in an anaerobic digester plant. Physical, chemical and biological characterization of the effluent was performed as per standard methods (APHA, 1995). Parameters analyzed were pH, electrical conductivity, TDS, D O, chlorides, nitrates, Biological Oxygen Demand (BOD 3 days), Chemical Oxygen Demand (COD) and reducing sugars.

MATERIALS AND METHODS

Preparation and characterization of synthetic Melanoidin

Synthetic melanoidin was prepared by dissolving equimolar amounts of analytical grade glucose and glycine (1mole each) and sodium carbonate (0.5 moles) in 1L deionized water. The sample was heated in an autoclave for 3 h at 121^o C (Migo et al. 1993). The resulting brown stock solution was lyophilized and stored in the refrigerator prior to use.

Isolation of melanoidin degrading fungi

Soil samples were taken from various sites of United Spirits Ltd., Aleppey, India such as the dumping areas of distillery effluent. Isolation of soil fungi was carried out in Czapek Dox broth

(sucrose, 30g ; $NaNO_3$, 3g ; K_2HPO_4, 1g and $MgSO_4$ _$7H_2O$, 0.5 g ; KCl , 0.5 g ; $FeSO_4$, 0.01 g ; in 1L at pH 7.0) amended with 10% distillery effluent. The isolated organism which showed highest melanoidin degradation in the screening test was chosen for further studies. The isolate was systematically identified by microscopy and phylogeny by molecular techniques like 18 S rRNA gene sequencing followed by Blast analysis. The isolate was named as **VT-NSK** and was identified as "**unknown"** and the sequence has been deposited in Gen Bank with **Accession number JN570507.**

Decolorization/Degradation Assay

Melanoidin decolorization/degradation was measured as a decrease in optical density measurements at 475nm of culture media supernatant against un inoculated spent wash and expressed as the percentage decrease in absorbance (Fitz Gibbon *et al.*, 1995).

Growth in optimized medium

The organism VT-NSK was inoculated into 100ml of optimized medium viz. Czapek Dox broth (2g% glucose, 0.5g% urea , K_2HPO_4, 0.1 g, and $MgSO_4$.$7H_2O$, 0.05 g , KCl_2 , 0.05 g ; $FeSO_4$, 0.001 g ; and Tween 80 1%) containing 0.1% synthetic melanoidin, at pH 8.0 and incubated at 30^0C under shaking condition at 120 rpm in 500ml Erlenmeyer flasks. Samples were drawn at 24 to 240 hours at every 24hours interval, centrifuged at 4^{o}C at 6,000 rpm for 15 minutes and analyzed for biomass, total proteins, pH, colour reduction, enzyme activity etc.

Influence of concentration of synthetic melanoidin and distillery melanoidin on biomass and colour removal

Influence of time, concentration of effluent, pH and addition of glucose into the medium on biomass and decolorization were studied by inoculating the isolate into the above said optimized medium with different concentrations of synthetic melanoidin (0.1%, 0.2%, 0.5% and 1%) and distillery effluent(10%, 15% and 20%) respectively. Growth was monitored over 0-144 hrs with respect to pH, biomass (g L^1) and percentage of colour reduction in comparison with control at 475nm respectively.

RESULTS AND DISCUSSION

The effluent was characterized as per the standard methods APHA, 1995. Effluent had a dark black brown colour, highly acidic with a very low pH (3.5 -4.0) with electrical conductivity 24,500 , total solids 13,600 mg/l, dissolved oxygen nil , Chloride 4,165mg/l, Nitrate 10mg/l, BOD 65,000 and COD 1, 45,000 mg/l.

The potent isolate grew well in optimized medium at pH 8.0 and incubated at 30^0C under shaking condition at 120 rpm in 500ml Erlenmeyer flasks. Colour reduction was found to be proportional to increase in biomass. Decolorization obtained for synthetic melanoidin at different concentrations were (92%), (85%), (74%) and (66%) for 0.1%, 0.2%, 0.5% and 1% synthetic melanoidin (SM) respectively (Plates 1-4). Similarly decolorization obtained for various concentrations of distillery effluent were (90%), (77%) and (54%) for 10%,15% and 20% distillery effluent respectively (Plates 5-7).Whereas the biomass obtained for synthetic melanoidin concentrations were (34g L^1) , (35g L^1), (43g L^1) and (40g L^1) for 0.1, 0.2, 0.5 and 1% SM (Fig.1). Similarly, the biomass obtained for different distillery effluent concentrations were (35g $L^{1)}$, (30g $L^{1)}$ and (28 g L^1) for 10, 15 and 20% DE respectively at pH 8.0 and temperature 30^0C under shaking at 120rpm for 48 hrs of incubation (Fig .2). In all, the pH remained unchanged at pH 8.0 upto 72 hrs and there was a steady fall of pH from pH 8.0
after 72 hrs to pH 5.5 till 144 hrs. Figure.1 shows the effect of varying concentrations of synthetic melanoidin on growth. Irrespective of the concentrations from 0.1-1% there was 10-15g L^1 within the first 8 hrs. Beyond 8-12 hrs there was a decrease in the rate of growth up to 24 hrs. From 24 hrs in all concentrations except 0.1% there was a logarithmic increase up to 50 hrs, the biomass varied from 35-45g L^1. It was interesting to note that the growth became constant from 50 hrs -144 hrs.

Fig. 1, 2 & 3 shows the influence of distillery effluent on biomass. The picture was similar to synthetic melanoidin with 20-25g L^1 at 10, 15& 20% concentrations. After approximately 8-10hrs there was decrease in rate of multiplication up to 50 hrs in all concentrations followed by a stationary phase up to 144 hrs. Figure 4. shows the percentage colour reduction over time in media containing 0.1% synthetic melanoidin and 10% distillery melanoidin. The highest reduction was achieved within 10-12 hrs in both (80-85%). Further incubation had shown very little increase. They reached 92% for synthetic melanoidin and 90% for distillery effluent. Figure. 5 depicts the change in pH of media containing synthetic melanoidin and distillery effluent inoculated with VT-NSK. The initial pH of the medium remained constant at pH 8.0 up to 72 hrs followed by a sudden drop up to 96 hrs to about 5.5. It continued to be low till 144 hrs. This observation could be of great significance for continuous operations for large scale spent wash treatment in industry.

Another remarkable observation was that pH of the medium was at initial pH 8.0 even after three days of incubation which proves mineralization of the medium through biodegradation of the components (Dehorter *et al.*, 1992). Although spent wash contains huge amounts of sugar, its easily metabolisable carbon content is almost negligible (Kumar *et al.*, 1997). So addition of readily available external carbon source was found to be necessary for metabolism of microbes in the spent wash medium. Also addition of the secondary carbon sources enhanced the degradation of xenobiotic compounds (Veeranagouda *et al.*, 2004).

Growth pattern of the isolate with respect to color removal indicated that gradual increase in growth with decolorization was observed up to 72hrs. This is because the organism during its initial phase of growth, utilizes first the easily available carbon source added to the medium and later on starts to degrade the spent wash components for the carbon source (Kumar *et al.*, 1997).

It has been assumed that the decolorization of distillery spent wash occurs due to the growth of the culture on the refractile carbon source component of the spent wash, which suggests the role of the secondary metabolic reaction in degradation and mineralization process (Kumar *et al.*, 1997).

Decolorization was more in the presence of all carbon sources used with respect to control but was found to be highest in the presence of glucose. 2g% of glucose concentration as carbon source was optimized for decolorizing activity and with glucose above 4g% there was decrease in decolorization, which may be due to the acidic conditions produced in the medium after incubation, inhibiting the microbial growth.

Addition of organic nitrogen sources were found to enhance decolorization when compared to the inorganic nitrogen sources. Even though nitrogen was already present in distillery spent wash, the organism required additional organic nitrogen supplements for its growth and decolorization.

Growth of VT-NSK in liquid basal medium containing synthetic melanoidin- Plate 1
(0.1%), Plate 2 (0.2%), Plate 3 (0.5%), plate 4 (1%) with media controls, 24-48 hrs at 30 ^{0}C, pH 8.0 in shaking at 120 rpm.

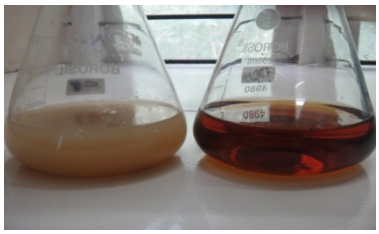

Plate 1 – Growth of VT-NSK in Liquid Medium containing synthetic melanoidin (0.1%) with control

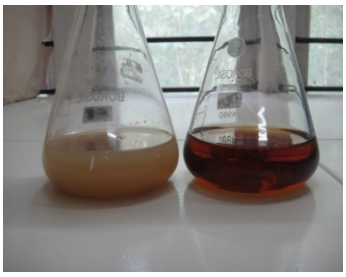

Plate 2 - – Growth of VT-NSK in Liquid Medium containing synthetic melanoidin (0.2%) with control

Plate 1 - Growth of VT-NSK in Liquid Medium containing synthetic melanoidin (0.5%) with control

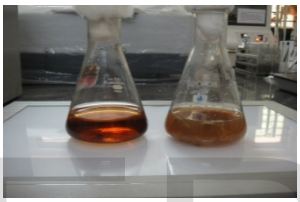

Plate 4 – Growth of VT-NSK in Liquid Medium containing synthetic melanoidin (1.0%) with control

Growth of VT-NSK in liquid basal medium containing distillery effluent- Plate 5 (10%), Plate 6 (15%), Plate 7 (20%) with media controls- 24-48 hrs at 30^{0}C, pH 8.0, in shaking at 120 rpm.

Plate 5 – VT-NSK in distillery effluent (10%) with control

Plate 6 – VT-NSK in distillery effluent (15%) with control

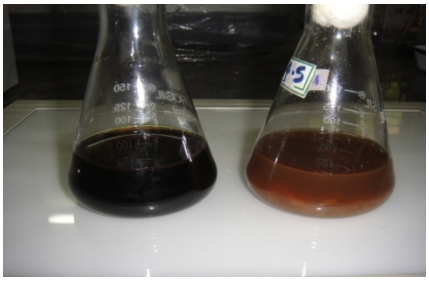

Plate 7 – VT-NSK in distillery effluent (20%) with control

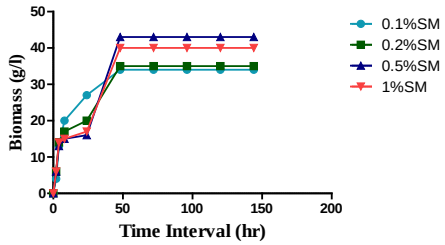

Fig .1 – Effect of concentration of synthetic Melanoidin (SM) and on growth of VT-NSK.

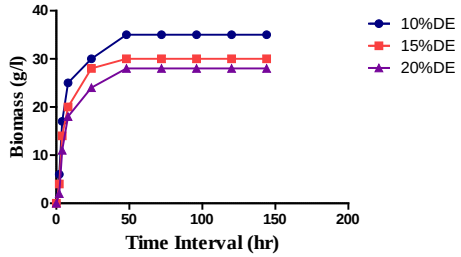

Fig .2 – Effect of concentration of distillery effluent (DE) on growth of VT-NSK.

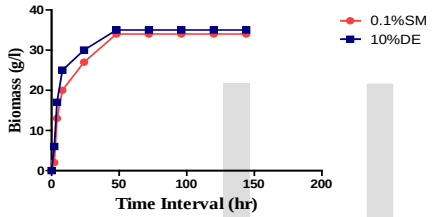

Fig.3 – Effect of 0.1% synthetic Melanoidin (SM) and 10% distillery effluent (DE) on growth of VT-NSK.

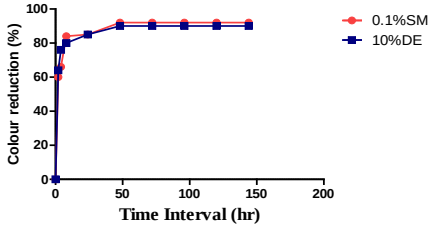

Fig .4 – Effect of 0.1% synthetic Melanoidin (SM) and 10% distillery effluent (DE) on colour removal of VT-NSK.

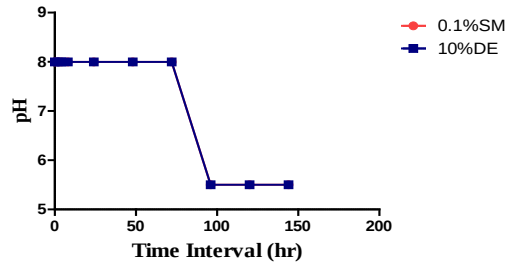

Fig .5 – Effect of 0.1% synthetic Melanoidin (SM) and 10% distillery effluent (DE) on pH of VT-NSK.

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