Bioremedial Potential of Bacillus humi AAY-1 for Eco-friendly Treatment of Environmental Pollutants

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ABSTRACT

*The discharge of dyes in natural marine water causes severe environmental problems because they are toxic to aquatic life and damage the aesthetic nature of the environment. Reductive cleavage of azo bond, leading to the formation of aromatic amines, is the initial reaction during the bacterial metabolism of azo dyes. An isolate capable of degrading the textile azo dye Acid Yellow 25 was isolated from the soil. Based on biochemical and phylogenetic analysis based on 16s rRNA gene sequence, the isolate AAY-1 was identified as Bacillus humi AAY-1 (**Accession No. LC596502**). Decolorization and Degradation of Acid Yellow 25 were carried out using the acclimatized Bacillus humi AAY-1 isolated from soil. The bacterium was able to effectively decolorize the azo dye in 24 hours. The decolorization of the azo dye Acid Yellow 25 in nutrient broth and half strength nutrient broth was up to 92.00% and 90.00% respectively in 24 hours. The percent decolorization of the dye was also studied by cell-free extract and was observed that the present isolate can decolorize the dye by 80.00 % in 24 hours. The percent decolorization of the dye was also studied in presence of different 1% co-substrates Viz. glucose, yeast extract, and starch was found up to 92.67%, 94.21% and 93.45.00% respectively. From the results, it can be concluded that the isolate could decolorize the dye very effectively. The percent decolorization of the dye Acid Yellow 25 was determined spectrophotometrically. The percent COD reduction of the dye by the isolate was 92.00%. The degradation products formed after degradation were analyzed by Gas Chromatography-Mass Spectroscopy (GC-MS) analysis and it was found that this promising bacterium degraded Acid Yellow 25 to the products having molecular weights 70, 97, 125, 140, 168, 205, 83, 111, 154. The microbial toxicity study revealed the degradation of Acid Yellow 25 into a non-toxic product by Bacillus humi AAY-1.*

Keywords: Azo dye, Soil bacteria, biodegradation, GC-MS, Microbial toxicit.

INTRODUCTION

According to one estimates globally more than 7 lack metric ton of dyes are produced and used in single year. Of them, 10 to 15 percent of wasted colours are released into the wastewater after dyeing and washing [1]. Colorful wastewater with poor biodegradable complex components, high pH, temperature, and COD concentration is discharged from the dye, food, textile, and leather, cosmetic, paper, and printing sectors [2]. Even in minute amounts, colour renders anything useless and worthless. Dye effluents need to be treated before they can be safely discharged into water streams because they are unsightly and environmentally problematic when left untreated or only partially treated. Azo dyes are the most widely used synthetic dyes and make up the majority of all textile dyestuffs produced. Moreover leather pharmaceutical cosmetics and paper industries use these dyes.

Azo dyes are artificial organic colorants with a wide range of structural characteristics. They share a common chromophore, N=N, and many auxochromes, including NH2, NR2, and OH groups [6]. Due to their toxicity and risk for cancer, azo dyes and their transitional amines represent a threat to the environment when present in large concentrations [7]. The most prevalent class of textile dyestuff is azo-aromatic. Many bacterial species are capable of reductively cleaving these dyes into colorless amines [8,9]. When microorganisms use reduced carbon compounds as the precursor molecule, the breaking of azo bonds can happen gratuitously [10]. When compared to traditional treatments, microbial decolorization techniques have the benefit of being simple in design and inexpensive. To remove contaminants from wastewater, a variety of physicochemical wastewater treatment techniques are now used.

In the bioremediation of dye wastewater textile colours, microorganisms are used as agents [11]. The process of bioremediation involves enhancing the natural ability of bacteria to break down waste and hazardous materials [12]. According to numerous reports, a wide range of microbes has been implicated in the biodegradation and bioremediation of dyes. These microbes include certain bacteria like Pseudomonas sp SUK 1 [13], Exiguobacterium sp RD 3 [14], Penicillin ochrocloron [15], and yeasts such Saccharomyces cerevisiae [16]. Because of their intricate structure, azo dyes have been shown to be challenging to degrade [17]. High levels of residual soils, dissolved solids, insoluble dyestuff (colour), and other auxiliary chemicals used in the numerous steps of dyeing and other processes are present in the wastewater produced by the textile processing industries.

In the current investigation, a bacterial strain was shown to be capable of removing the textile dye Acid Yellow 25 from wastewater that the textile sector discharges into the environment's water supply. This strain was tested for its ability to decolorize the dye Acid Yellow 25 under a variety of circumstances, including cell-free extract, complete nutrition medium, and half-strength nutrient media. Spectrophotometric monitoring was used to track the dye's decolorization. COD analysis was used to determine the dye's percent COD reduction. GC-MS analysis proved that the dye Acid Yellow 25 had degraded.

1. MATERIALS

1.1 Samples – In order to capture microorganisms, soil samples were taken from native soil.

1.2 Dye – Acid Yellow 25 (λ max 392nm)

Figure 1 : Structure and properties of the dye Acid Yellow 25

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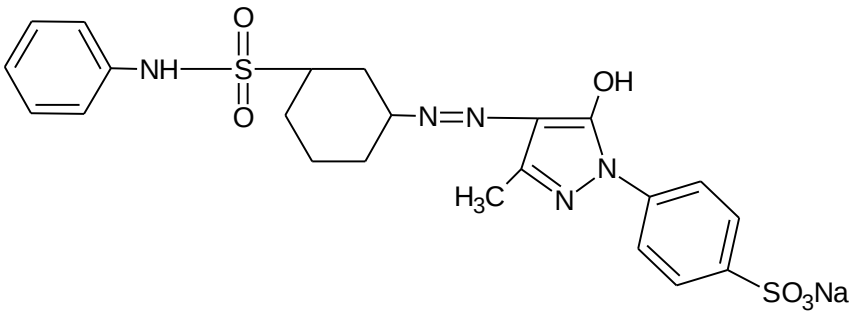
Structure



Properties

Molecular Formula = $C_{22}H_{24}N_5NaO_6S_2$

Formula Weight = 541.575629

Composition = C(48.79%) H(4.47%) N(12.93%) Na(4.24%) O(17.73%) S(11.84%)

λ max = 392nm

2. METHODS

2.1 Preparation of the dye solutions

Dye solutions of 1% (10,000µg/ml) were prepared in distilled water, which are stored as stock solutions and used for further study.

2.2 Acclimatization

In order to acclimate the microflora from the pulverized native soil samples, the dye Acid Yellow 25 was added for one month at progressively higher concentrations. After incubation, isolation was performed on nutrient agar that had been modified with Acid Yellow 25 at a final concentration of 1000 g/ml using 1 gram of acclimatized soil that had been inoculated in the nutrient broth (**Figure 1**).

2.3 Isolation of microorganisms

After the dye had acclimatized for a month in soil samples, isolation was performed. The soil was combined with nutrient broth in a flask and incubated for 24 hours at 37^0 C temperature to promote the growth of microorganisms in order to isolate them. The nutritional agar plates carrying the dye were then streaked with this nutrient broth. The strain that accomplished the best decolorization was given the name isolate AAY-1 and was collected for more research.

2.4 Decolorization of Dye in Nutrient Broth, Half strength nutrient broth and in presence of different co-substrates

20 ml of nutritious broth (peptone, NaCl, beef extract, distilled water, and 1000 g/ml dye concentration) were inoculated using an AAY-1 isolate. The dye in these tubes was examined for decolorization after a 24-hour incubation period at 37°C. Additionally, the ability of isolate AAY-1 to remove the color of the dye Acid Yellow 25 was examined using nutritious broth that was half as strong and had the same amount of dye (peptone 0.5g, NaCl 0.25g, Beef Extract 0.15g, Distilled Water 100.0ml). The dye decolorization was also examined with the same dye concentration when a number of co-substrates were present, including 1% glucose, 1% yeast extract, and 1% starch. All of the tubes were incubated for 24 hours at 37^0C before the decolorization % was determined.

The percent decolorization of the dye was determined by using the following formula.

$$\text{Decolourization (\%)} = \frac{A_0 - At}{A_0} \times 100$$

A_0 = Absorbance of the blank (dye solution)

At = Absorbance of the treated dye solution at a specific time.

2.5 Decolorization of the dye by using Cell-Free Extract

The promising isolate's cells were cultured in the nutrient broth. Utilizing lysozyme, these cells were lysed. The cell-free extract was obtained by centrifuging the lysed cells at 8000 rpm for 15 minutes in a cooling centrifuge (Remi 412- LAG). After adding 10 ml of the cell-free extract's supernatant with 1000 µg/ml, the mixture was incubated at 37 ^{0} C for 24 hours to see if the dye had been decolored.

The percent decolorization studies were done by using the spectrophotometer (Systronics – 106 model).

2.6 Percent COD reduction Studies

The percent COD reduction value of the dye decolorized in nutrient broth by isolate AAY-1 was calculated by COD analysis using $K_2Cr_2O_7$ as a strong oxidizing agent under reflux.

2.7 GCMS Analysis

GC-MS analysis proved that the isolate was responsible for the dye's degradation. Di-Chloro Methane was used to extract the products and prepare the sample (DCM). For 20 minutes, the decolorized broth was centrifuged at 10,000 RPM. A separating funnel was used to decant and gather the supernatant. The separating funnel with the supernatant received an equal volume of DCM. For 20 minutes, the funnel was forcefully agitated to extract the products into DCM. For the separation of the solvent phase and the liquid phase, the separating funnel was left alone. The funnel was then opened, releasing the separated solvent.

By evaporating the solvent at room temperature, the products that were extracted in the solvent were consolidated in the vial. This was then analyzed by Gas chromatography and Mass spectroscopy (GCMS) using a Shimadzu 2010 MS Engine, equipped with an integrated gas chromatograph with HP1 column (60 m long, 0.25 mm id, non-polar). Helium was used as carrier gas at a flow rate of 1 ml min−1. The injector temperature was maintained at 280^0C with oven conditions as: 80^0C kept constant for 2 min and increased up to 200^0C with 10^0C min-1 raised up to 280^0C with 20^0C min−1 rate.

2.8 Isolation and Identification of microorganism

At the University of Pune Campus' National Center for Cell Sciences in Pune, the 16S rRNA was identified. According to the manufacturer's instructions, a Quaigen DNA isolation kit was used to isolate the isolate's genomic DNA. By utilizing BLAST to compare the contiguous 16S rRNA sequences with the reference and type strain data from publicly accessible databases GenBank, identification was made. The neighbor-joining (NJ) approach was used for the phylogenetic reconstruction together with bootstrap values [19].

2.9 Phylogenetic analysis and sequence alignment

The 16S rRNA gene sequence was first examined using the BLAST (blastn) program on the NCBI server (http://www.ncbi.nlm.nih.gov), and related sequences of similar species were then obtained and used for phylogenetic analysis. The neighbor-joining approach was used to infer the evolutionary history [20]. The branches were displayed next to the percentage of duplicate trees where the relevant taxa clustered during the bootstrap test (1,000 repetitions) [21]. The phylogenetic tree was modeled under the assumption that all lineages evolved at the same pace. It took 0.01 (time/node height) of clock calibration to convert the distance to time. The phylogenetic tree was inferred using evolutionary distances, and the tree was scaled using branch lengths in accordance with those measurements. The Maximum Composite method was used to calculate the evolutionary distances.[22] Phylogenetic analyses were conducted in MEGA4.

RESULTS AND DISCUSSIONS

Isolation and Identification

The organism was isolated from the soil on nutrient agar and was identified by using biochemical observations and 16s r-RNA analysis technique. From the analysis, the isolate was identified as *Bacillus humi AAY-1*. Biochemical results showed that the isolate was able to hydrolyze Starch and Gelatin but unable to hydrolyze casein, reduce nitrate, and fermentation of D-glucose showed acid and gas production whereas unable to ferment mannitol, D-fructose, and mannose. Biochemical results are given in **Table 1.** The phylogenetic tree was developed by using the Neighbor-joining method by Kimura-2-parameter with 1000 replicates in MEGA 4.0. [20] (**Figure 2**).

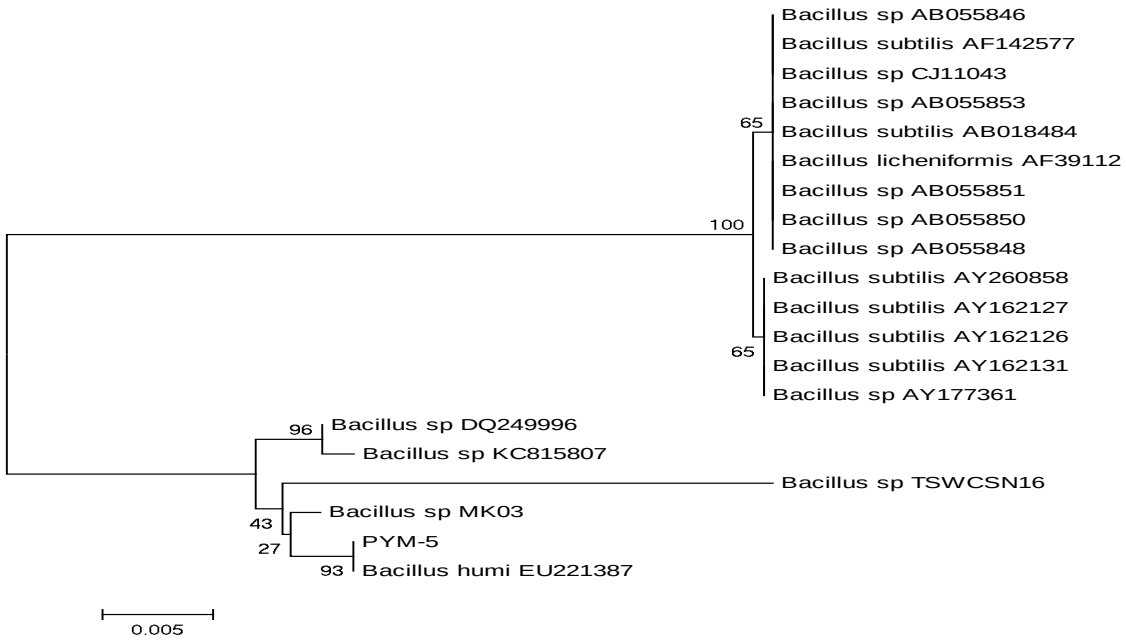


Figure 2: Phylogenic tree of ***Bacillus humi AAY-1***. Phylogenetic analysis of 16s rRNA gene sequence of *Bacillus humi AAY-1*. The percent numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 1,000 replicates. The scale bar (0.005) indicates the genetic distance.

Table 1 Biochemicals of the isolate

Characteristics of isolate AAY-1	***Bacillus humi AAY-1***
Gram nature and Motility	Gram-positive motile rods
Optimal growth temperature (^{0}C)	37^{0}C
Utilization of	
D-Glucose	+
Mannitol	-
D-Fructose	-
Mannose	-
Hydrolysis of	
Starch	+
Gelatin	+
Casein	-
Enzyme activity	
Amylase	+
Oxidase	+
Catalase	+
Urease	-
Nitrate reduction	+

Percent Decolorization of the Isolates

The ability of Bacillus humi AAY-1 to decolorize 1000 g/ml of dye in nutritional broth was investigated. Table 2 displays the outcomes of the Acid Yellow 25 dye's percent decolorization in nutritional broth. Malachite green deterioration has been previously documented by Li, et al (2010). Using *Shewanella strain* J18143 bacterial cells, Li, T., and Guthrie, J.T. (2010) investigated the dye removal efficiency from the aqueous medium of metal complex azo dyes.

Percent Decolorization in Half (½) Strength Nutrient Broth

The capacity of *Bacillus humi* AAY-1 to decolorize the dye in nutritional broth of half (12) strength with the dye at the same concentration of 1000 µg/ml as in complete nutrient broth was investigated. **Table 2** displays the findings of the % decolorization of the dye Acid Yellow 25 in half-strength nutritional broth.

Percent Decolorization in Cell-Free Extract

Bacillus humi AAY-1 was studied for its ability to decolorize the dye in Cell-Free extract. The results of percent decolorization of dye Acid Yellow 25 in the cell-free extract is given in **Table 2**.

Percent COD reduction

The Percent COD reduction of the dye after decolorization of the dye by the *Bacillus humi AAY-1* is given in **Table 2.**

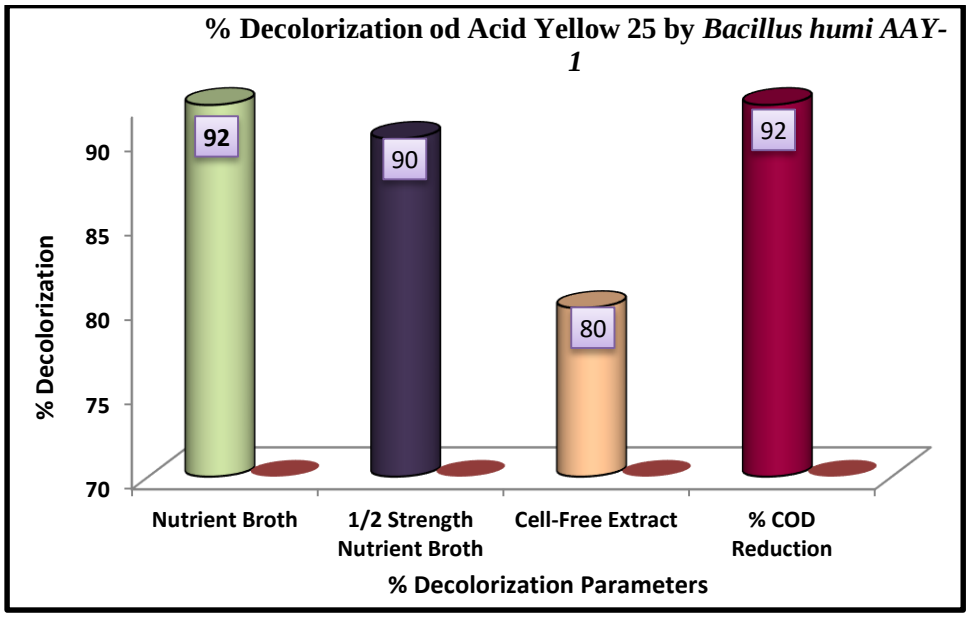


Figure 3: Percent Decolorization in Nutrient Broth and ½ Strength Nutrient Broth Cell-Free Extract in 24 hrs at λ max- 392nm and percent COD reduction value

The promising strains proved to be useful for the major removal of COD from that of the dyes. Telke, *et al*., [24] showed results similar with *Pseudomonas sp. SU-EBT, Citrobacter sp. KCTC* 18061P. Balamurugan, *et al*., [25] designed a Monod kinetic model which showed better COD removal for *Halomonas glacei* than any other strain.

Mabrouk, et al., [26] formulated a medium that proved to be the best mineral salt medium for the growth of *B. subtilis* HM for decolorizing the dye Fast Red. A novel bacterial consortium was found to decolorize the textile azo dyes Navy Blue HER, Blue 19, Porcion Golden Yellow HR, Red HE8B, Reactive Black B and Direct Black 22 in Bushnell and hass medium supplemented with 0.1% glucose and 0.6% yeast extract (w/v) [27]. The present study revealed that all the promising strains were able to decolorize the specific dyes with initial dye concentrations of 1000µg/ml to 10,000 µg/ml.

Percent Decolorization of Dye in presence of different Co-substrates

Bacillus humi AAY-1 was further studied for its ability to decolorize the dye Acid Yellow 25 in a nutrient medium and 1% Glucose, 1% Starch and 1% Yeast extract as Co-substrates and 1000µg/ml concentration of dye. The results of percent decolorization of dye Acid Yellow 25 in presence of different Co-substrates are given in **Table 3.**

Culture Code	**Identified As**	**% Decolorization in 1% Glucose**	**% Decolorization in 1% Yeast Extract**	**% Decolorization in 1% Starch**
AAY-1	*Bacillus humi AAY-1*	92.67%	94.21%	93.45.00%

Table 3 Percent Decolorization in presence of Different Co-Substrates *Viz.* 1% Glucose, 1% Yeast Extract, and 1% Starch in 24 hrs at λ max- 392nm

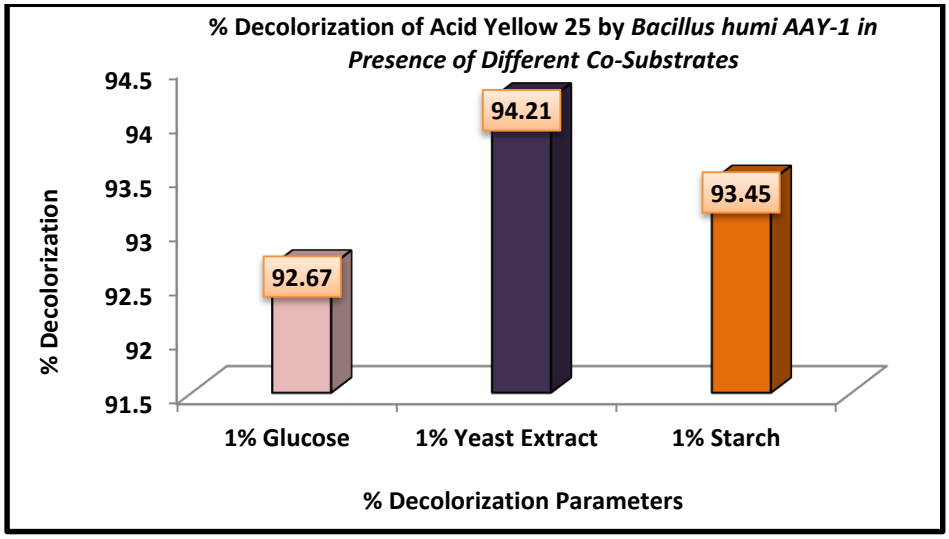


Figure 4: Percent Decolorization in presence of Different Co-Substrates *Viz.* 1% Glucose, 1% Yeast Extract, and 1% Starch in 24 hrs at λ max- 392nm

The presence of the co-substrate enhanced the process of decolorization of the dyes. It increased the rate of reaction and also the percentage of dye decolorization was also increased.

Jonstrup *et al*., [28] used glucose as a carbon source in the minimal medium for the treatment of the three azo dyes Remazol Red RR, Remazol Yellow RR, and Remazol Blue RR.

The strain AAY-1 showed maximum decolorization in the presence of the co-substrate rather than only in a nutrient medium. This proves that, in absence of co-substrate, the bacterial culture was unable to decolorize the dye and the carbon source seems necessary for the growth and decolorization of the dyes.

GC-MS analysis

The GC-MS analysis report of the dye is shown in **Figure 5**. The reports showed that the dye was degraded by the isolate having different molecular weights (**Table 4**). The results showed that the isolate from the acclimatized soil has good decolorization and degradation of the dye Acid Yellow 25. Confirmation of the biodegradation of the dye Acid Yellow 25 was done by analyzing the samples with GC-MS. The degradation products of the dye were of much lower mass than the original compounds.

Molecular weights of the degraded products of dye

The GC-MS analysis report showed that the dye Acid Yellow 25 was degraded and not only decolorized. The molecular weights of the degraded products are given in **Table 4.** Reports have been observed for the degradation of the dyes and their analysis by GCMS [29, 24, 30].

Culture Code	**Identified As**	**Molecular weights of the degraded products**
AAY-1	***Bacillus humi AAY-1***	**70, 97, 125, 140, 168, 205, 83, 111, 154 etc.**

Table 4 Molecular weights of the degraded products

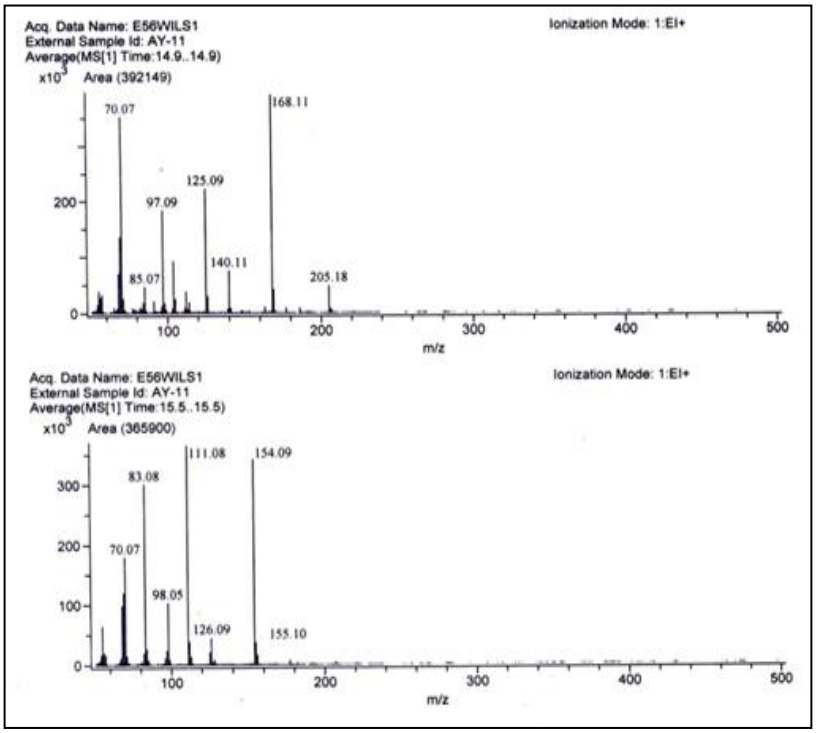


Figure 5: **GCMS analysis report of degraded products of Acid Yellow 25 dye by *Bacillus humi AAY-1***

Since the wastewater discharged from the dye-containing fabrics is largely multicolored, it can be seen [31]. The intricate delicate structure of the colorings is sensitive to ozone, light, and other environmentally damaging factors. As a result, traditional wastewater treatment continues to be unsuccessful. Additionally, due to the reactive fragmentation of azo groups, both anionic and non-ionic azo dyes result in toxic amines [32]. The treatment of dyes in fabric wastewater has been the focus of scientists' efforts up to this point, but it is still a difficult task [33]. (Dos Santos and coworkers, 2007) The declination of tropeolin O, orange II, azo color, and azure B by *Phanerochaete chrysosporium* was shown by Colleen et al. in their study [34]. Young and Yu [35] have reported that Acid Black 24 has undergone biological deterioration. According to the research done by Young and Yu [35], *P. chrysosporium* and *T. versicolor* prompted 98 percent of Acid Black 24 (9 days) color to be diminished. It has been demonstrated that facultatively anaerobic bacteria like *Proteus vulgaris* and *Streptococcus faecalis* as well as anaerobic bacteria like *Bacteroides sp. and Eubacterium sp.* can decolorize the various azo colorings under anaerobic circumstances by reducing the azo bond [36]. Decolorization only occurred when a carbon source and a source of energy were present in the growth medium. Glucose and an incentive extract were used as co-substrates since color decolorizing bacteria appear to require metabolizable carbon sources to function. [37]. According to Cripps et al. [38], P. chrysosporium could eliminate 87 to 93 percent of orange 11, tropeolin O, and azo hue in just five days. According to Spadaro et al. [39], *P. chrysosporium* was capable of mineralizing a variety of poisonous azo dyes, and as a result, the character of ring substituents played a role in the mineralization of sweet azo dye rings.

In the current investigation, *Bacillus humi AAY-1,* which was isolated from soil, demonstrated azo dye Acid Yellow 25's percent decolorization to an extent of 92.00 in a nutrient medium and 90.00 in a half-strength nutrient medium.

CONCLUSION

Bacillus humi AAY-1 was used to decolorize Acid Yellow 25 by looking at the colorful features of environmental and aesthetic conditions. The outcomes showed that the novel species had a significant capacity for azo colorings to be rapidly decolored. *Bacillus humi* AAY-1, a newly identified bacterium, decolorizes color extremely efficiently under a variety of aesthetic and environmental conditions. The treatment of textile wastewater can therefore be carried out on a broad scale using this culture.

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