Biodegradation of Benzidibie by Alkaliphilic Strain Bacillus badius D1

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Abstract: Benzidine biodegradation was studied for 48 hr. using Bacillus badius D1. Various concentrations ranging from 100 to 500 mg/L of benzidine were used to judge the biodegradation percentage. The effect of different parameters like pH, temperature, salinity, carbon and nitrogen sources was also studied. The enzyme activities of certain biotransformation enzymes like CYP450, SOD, Acetanilide hydroxylase, Aminopyrine N-demethylase were carried by standard methods and were found induced during biodegradation. Complete degradation observed at 100 mg/L. 92% benzidine (500 mg/L) was degraded at 32 °C, pH 9.00 in shaking incubator with 110 rpm.

Key Words: Biodegradation, Benzidine, Bacillus badius D1

Abbreviations: SOD- Superoxide dismutase, CYP450- Cytochrome P450

I. Introduction:

Benzidine is a toxic aromatic amine. It causes the bladder cancer in mammals. Azo dyes account for the majority of all textile dyestuffs produced and are the most commonly used in synthetic dyes, textiles, and paper industries. These are also used in printing technology leather and cosmetic industries [1]. Many azo dyes on biotransformation turn into benzidine as one of the metabolite of catabolic pathway. It is threat to health due to benzidine induces tumors in the liver of hamsters. It also affected the Harderian gland, Zymbal's gland. Mammary gland, and intestine of rats [2, 3]. Other aryl amines structurally related to benzidine induced tumors through alteration in critical cellular constituents by reactive aryl amine metabolites formed within the target organs.

N-Hydroxylation is regarded as the initial step in metabolic activation of these aryl amines and aryl amides; subsequent esterification of the N-hydroxyl moiety yields electrophilic reactants capable of binding to nucleophilic centers in protein, RNA, and DNA. Several biotransformation occurs in benzidine during detoxification. Some of these changes in benzidine appear to cause many of the chemical's harmful effects [4]. The disposal of such dye stuff or aromatics can be carried by aerobic, anaerobic microbes or both in combination. [5, 6, 7].

II. Methodology:

2.1 Chemicals: Yeast extract, peptone was purchased from Hi-media Mumbai. Other chemicals were taken from SRL chemicals. Pure bezidine was obtained by the curtesy of Department of Chemistry, Pune University.

2.2 Composition of the media:

Yeast Extract – 5g/L, Peptone- 5g/L, NaCl- 3g/L, KH2PO4- 170 mg/L, Na2HPO4- 290mg/L, (NH4)2SO4- 100mg/L, MgSO4- 4.87mg/L, FeSO4- 0.05mg/L, CaCO3- 0.2mg/L, ZnSO4- 0.08mg/L, CuSO4-0.016mg/L, CaSO4- 0.015mg/L, Boric Acid-0.006mg/L, pH 9.00 sterilized by autoclaving at 121 °C for 20 minutes.

2.3 Biodegradation study: Nine 500 ml Conical flasks containing sterilized 250 ml alkaline broth of pH-9.00 were inoculated by 1 % Bacillus badius D1 culture possessing 1.6 OD at 600 nm aseptically. These culture flasks were incubated for 24 hrs at 37 °C with shaking on Orbital shaker at 110 rpm. The 24 hrs grown culture flasks were induced by adding appropriate concentration of benzidine. These flasks were removed sequentially from 0 to 48 hours by 6 hr. interval. The removed flasks were used for OD at 600 nm to check the growth and then spun to DuPont Sorvoll Cold centrifuge at 10000 x g. Similarly one another flask was kept as abiotic control by adding experimental concentration of benzidine. All experiments were carried in the dark. The study was extended to verify the effects of various parameters like concentration, pH, temperature, salinity, carbon and nitrogen sources on biodegradation. The bio-catalytic induction of biodegradation was studied by standard methods.

2.4 Spectrophotometric analysis:

An aliquot of cell free centrifuged supernatant was used for solvent extraction to know the spectral changes in benzidine at various time intervals and remaining was used to evaluate the concentration remaining after biodegradation using spectrophotometer Jasco Varien 630 at 285 nm.
2.5 Cytosolic preparation and enzyme activities: Cell mass was harvested after 24 hours induction with benzidine by Du-Pont Sorvall RC-5B centrifuge by spinning at 10000 x g for 15 min at 4°C. The cell mass was washed with phosphate buffer pH 8.0 twice and physiological saline. Cell disruption was carried by sonicator Ultra O Sonic (Mumbai) in Tris buffer pH 7.50. The resulting homogenate was centrifuged in cold condition at 15000 x g for 20 min. The protein in cytosole was estimated by Lawry [8]. Cytochrome P450 was determined by Omura and Satof [9,10]. Superoxide dismutase activity was carried by Mishra and Fredovic [11]. Aminopyrine N-demethylase and Acetanilide hydroxylase activities were performed by Shenkman, Weiseberg and Goodal [12,13].

III. Results:

3.1 Spectral changes observed in Benzidine during biodegradation.

![Fig. 1 Spectral changes in Benzidine during biodegradation by Bacillus badius D1](image)

Spectrophotometric determination of benzidine was carried at 285 nm. The initial spectrum 285 nm shifted to right at 320 nm which undergone several changes indicated biotransformation in benzidine molecule (Fig.1). Further quantitative analysis was carried by monitoring reduction in absorption at 285 nm.

3.2 Biodegradation of Benzidine at various concentration:

![Fig.2 Biodegradation of Benzidine by Bacillus badius D1 at various concentrations](image)

It was observed that after inoculation of benzidine in Bacillus badius D1 in alkaline medium pH 9.00 at 37°C with experimental concentration ranging from 100 mg/L to 500 mg/L; that the higher concentration negatively affects the percentage degradation (Fig.2). The concentration of benzidine (100 mg/L) was completely degraded within 48 hr. It also degraded the higher concentration (500 mg/L); but the percentage degradation was found 78% in the same experimental condition.
3.3 Biodegradation of Benzidine at various pH:

Fig. 3 Biodegradation of Benzidine at various pH by Bacillus badius D1

The effect of pH on percentage degradation of benzidine was studied at 500 mg/L, 37°C in shaking incubator at 110 rpm, PH ranging from 7.00 to 11.00. The (fig. 3) shows the optimum pH 9.00 for degradation. Marginal change at pH 8.00, 9.00, 10.00 and was noted; while the lowest degradation rate was found at pH 7.00.

3.4 Biodegradation of Benzidine at various Temperatures:

Fig. 4 Biodegradation of Benzidine at various Temperatures by Bacillus badius D1

The effect of temperature (22°C - 42°C) on biodegradation of benzidine was studied using Bacillus badius D1. Benzidine was inoculated in fully grown culture of alkaline medium of pH 9.00 and at the experimental condition. The Fig (4) showed that the temperature 32°C was positively affected the percentage degradation. Least degradation percentage was noted at 42°C.
3.5 Biodegradation of Benzidine at different Salinity:

The (Fig. 5) indicate the biodegradation of benzidine by Bacillus badius D1 at salinity range 0.5 % to 2.5%. The experimental conditions were same as that of other experimental parameters except salinity difference. It indicated that at 0.5 % salinity, the percentage degradation was high and at higher salinity the percentage degradation was less.

3.6 Biodegradation of Benzidine with carbon Sources:

The (Fig. 6) showed the effect of additional carbon source in the culture medium at similar experimental condition; marginal changes in % degradation of benzidine by Bacillus badius D1 were noted.
3.7 Biodegradation of benzidine at various nitrogen sources:

![Graph showing biodegradation of benzidine](image)

**Fig. 7** Biodegradation of benzidine by Bacillus badius D1 using various nitrogen sources

The (Fig. 7) indicates the percentage degradation of benzidine (500 mg/L) at various additional mineral nitrogen sources. The addition of 0.02g/L inorganic nitrogen source marginally affected the percentage degradation of benzidine at experimental condition except additional nitrogen sources. Addition of NaNO₃ had not stimulated the percentage degradation of benzidine as compared to other sources but all nitrogen sources positively affected on degradation.

3.8 Induction of Biotransformation enzymes by benzidine:

![Graph showing enzyme activities](image)

**Fig. 8** Induction of Biotransformation enzymes in Bacillus badius D1 by benzidine at 24 hr.

Cytocrome P450 concentration was found increased 0.06 mg/ml in experimental flask compared to control flask (without benzidine) on induction for 24hr. The enzyme activities of SOD, Acetanilide hyddroxyase and Aminopyrine N-demethalase were carried by standard methods. The (Fig. 8) illustrate the enzymes induced during biodegradation. Acetanilide hydroxylase activity was observed more among all the enzymes studied.
IV. Discussion

Environmental pollutants are the substances entering into the environment at higher concentration and exerting toxicity to the living creatures in a specific chemical form at exceeding the threshold limit value [14]. These pollutants or xenobiotics substances are being a serious problem of nature. The xenobiotics like organochlorines, aromatic amine, phenolics , pesticides ,dyes ,drugs, paper and pulp industrial waste or wastes from refineries, distilleries are exerting toxic effects on environment. However, it is believed that microorganisms are capable of degrading almost all the different complex and resistant xenobiotics found on the earth [15, 16]. Thus environmental wastes have to be treated before they are introduced into the environment. Azo dyes are dominant synthetic dyes which on degradation converts into toxic aromatic amines [17]. Benzidine is one among the aromatic amines produced during biodegradation of such dyes. Swallowing contaminated dust of benzidine can also enter the body by passing through the skin. Some dyes may still contain small amounts of benzidine as a contaminant, or chemicals that may transform to benzidine [18]. Use of such dyes to dyeing cloth, leather, or other materials; one can expose to benzidine by breathing or swallowing dust, through skin contact. Benzidine is recalcitrant compound having carcinogenic, mutagenic and genotoxic effects. [19, 20, 21].

Biotransformation of these recalcitrants can dominate toxicokinetics and the metabolites may reach higher concentrations in organisms than their parent compounds [22, 23, 24, 25]. These recalcitrants can affect normal microflora of soil positively or negatively. Benzidine shown negative effect on the nitrogenase activity of bacteria [26]. The discharge of benzidine dyes into water bodies can causes toxic effects like DNA damage in human beings [27]. Benzidine based dyes affects on mixed function oxidase system [28].

Recent advancements have proved successful via the addition of matched microbial strains to the medium to enhance the resident microbe population's ability to break down contaminants. [29 - 34]. Still the reports of contaminant degradation are less in alkaline condition [35, -39].

In current studies Bacillus badius D1 was used for degradation of aromatic amine benzidine. The usual absorption spectrum of benzidine found altered in experimental flask and no change in abiotic control flask containing alkaline broth media of pH 9.00 at 37 °C at 110 rpm in shaking incubator. This indicates that benzidine had gone through several biotransformation steps. Fig. (1). During the breakdown or detoxification the biotransformation enzymes were increased comparative to control [40]. The Fig (8) showed that Bacillus badius D1 was actively involved in biodegradation process inducing biotransformation enzymes[41]. It is well reputed that effect of particular concentration, temperature, pH, carbon and nitrogen affect significantly on biodegradation [42- 45]. The biotransformation enzyme induction was reported in case of Y Proteobacterium and others [46-49].

It was tried to know the maximum percentage degradation of benzidine on inoculation of Bacillus badius D1 culture in alkaline broth possessing different concentration of benzidine ranging from 100 to 500 mg/L. at 37 0C, 110 rpm, pH 9.00. It was observed that at lower (100 mg/L) concentration was easily degraded by the bacteria. As the concentration increases the rate of percentage degradation was lowered [50]. Although, there was higher concentration 500mg/L benzidine 78% degradation of benzidine was noticed (Fig.2). To optimize the process of degradation various parameters like pH, temperature, salinity, additional carbon and nitrogen sources also studied.

Benzidine (500 mg/L) was inoculated with Bacillus badius D1 culture in alkaline broth at various pH, 37°C , 110 rpm to study the percentage degradation. The optimum pH for benzidine degradation was recorded pH 9.00; however Bacillus badius D1 shown broad pH range from pH 7.00 to pH 11.00 for degradation as in Fig. (fig.3). Some of the bacterial enzymes showed pH versatility and salt tolerance in azo dye degradation [51]. Thus pH parameter can affect the biodegradation [52].

Similar to pH parameter the degradation study for temperature parameter also carried to know the effect on percentage degradation of benzidine by Bacillus badius D1. Marked percentage degradation at 32°C was noted. Rest of other temperatures shown little less percentage degradation of benzidine as in (Fig.4). This might be due to negative effect of temperature on biodegradation enzymes.

The (Fig.5) indicate the biodegradation of benzidine by Bacillus badius D1 using different salinity. At 0.5% salinity 98% benzidine degradation was noted. Usually 0.3% salinity was used for other experimental parameters. Excess salinity or less salinity might have bothered the osmoregulation of Bacillus badius D1[53]. Some of the bacteria have high salinity tolerance [54].

Both carbon and nitrogen sources positively affected the biodegradation of benzidine by Bacillus badius D1.Fig (6) and Fig(7).Utilization of additional carbon and nitrogen sources was favored the biodegradation of pollutant in case of Stenotrophomonas maltophilia [55]

4.1 Conclusion: Bacillus badius D1 is potent nonpathogenic bacteria isolated from Lonar Crater Lake MS, India is able to degrade carcinogenic and genotoxic contaminant benzidine at higher concentration. Exploiting it to various other aromatic amines can be effective in bioremediation process at contaminated sites.
Acknowledgement:
Special thanks to UGC (WRO), New Delhi, India. Prof. Kr.Gawai, University of Pune, Department of Chemistry Pune.

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