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Effect of bacteria treated and untreated post-methanated distillery effluent (PMDE) on seed germination, seedling growth and amylase activity in *Phaseolus mungo* L

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ABSTRACT

Present study deals the effect of bacteria treated and untreated post-methanated distillery effluent (PMDE) on germination, seedling growth and amylase activity in *Phaseolus mungo* L. seeds. Results revealed that untreated PMDE was highly toxic in nature carrying high BOD, COD values along with high metals content. But, after bacterial treatment, these values were reduced by 64.58 and 74.20%, respectively. It was observed that 40% untreated PMDE has no inhibitory effect on seed germination but 60, 80 and 100% PMDE has inhibited 20, 40 and 60% germination, respectively while 100% germination was recorded up to 60% treated PMDE. Moreover, 40 and 60% PMDE has shown deleterious effects on seedling growth parameter and seeds treated with 80 and 100% PMDE showed no root development. However, 20% bacteria treated PMDE was found most suitable for plant growth possibly due to presence of optimum level of nutrients. Further, *Phaseolus* seeds treated with 60 and 80% untreated PMDE showed reduced amylase activity and no amylase activity was observed in seeds treated with 100% untreated PMDE. But, seeds treated with bacterial degraded PMDE showed amylase activity and molecular weight of α-amylase enzyme determined by SDS-PAGE was approximately 47.5, 46 and 44.5 kDa, respectively.

1. Introduction

Post-methanated distillery effluent (PMDE) is a dark brown to black complex, cumbersome wastewater discharged from distillery and fermentation industries utilizing sugarcane molasses as raw material for alcohol production. The dark brown colour of PMDE is mainly due to the presence of a complex biopolymer called melanoidin, which is generated by the non-enzymatic Maillard reactions during the processing of sugarcane juice in sugar industries and distillation process of sugarcane molasses in distilleries [1,2].

In distillery and fermentation industries, during the course of alcohol production, around 12–15 l of effluent are generated for per liter of alcohol produced [2]. In India, there are more than 300 distilleries producing a total of 3.5×10^{15} l of effluent annually [3] and this large volume of effluent is the major source of soil and water pollution. It causes inhibition of seed germination and depletion of vegetation by reducing the soil alkalinity and manganese availability in agricultural land receiving this waste effluent [4]. In addition, it reduces the penetration power of sunlight due to increased colouration of water and ultimately reduced the photosynthetic activity and dissolved oxygen level affecting the normal life cycle of aquatic fauna and flora [2,5].

In many developing countries including India, farmers are irrigating their crop plants with industrial effluents carrying high concentration of various toxic metals due to the non-availability of alternative sources of irrigation water. Moreover, Kulkarni et al. [6] have characterized the PMDE as a dilute liquid organic fertilizer as it contains both organic and inorganic nutrients with high potassium content and different trace elements due to which the diluted PMDE have been reported to have a beneficial effect on crop yields [7–9]. But, the direct use of PMDE in agricultural field for irrigational purposes is generally not recommended safe for environmental health and plant growth due to high BOD, COD values, high concentration of potentially toxic trace elements (Cu, Cd, Zn, Fe, Ni, Mn and Pb) and ions (SO_4^{2-}, PO_4^{3-}, K^+ and Cl^-) present in PMDE [8].

Hence, it will be better that PMDE should be biologically treated (aerobic treatment) at tertiary stage to bring down its toxic parameters because the reported physical methods are energy intensive and require large volume of water for dilution resulting an inflated volume of effluent and ultimately require very large area for drying. Whereas chemical methods are very expensive requiring huge amount of chemicals for removal of organic matter resulting the generation of huge amount of sludge as well as secondary pol-

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lutants [10]. But, biological treatment is an environment friendly nature's own self-purification process carried out by microbes under controlled conditions. After biological treatment, a suitable toxicity/risk assessment test should be done with the approved aquatic and terrestrial test models prior to its discharge from industries to judge whether the treated PMDE is suitable to discharge from industries without any harmful effects on soil and aquatic environment. Further, it is also important that farmers should make adequate dilution of such effluents before using in agricultural practices. Therefore, this study deals with the toxicity evaluation of PMDE before and after bacterial treatment by using *Phaseolus mungo* L. seeds in terms of seed germination, seedling growth and amylase activity for environmental safety.

2. Materials and methods

2.1. Collection of effluent sample

Post-methanated distillery effluent (PMDE) used in this study was collected from the anaerobic digestion plant of *M/s* Unnao distillery and breweries, Unnao (U.P.), India. The sample was taken in pre-sterilized plastic jerry cane (capacity 25 l) (Tarsons Production Pvt. Ltd., USA), brought to laboratory and used in experiments.

2.2. Decolourization and detoxification of PMDE by developed bacterial consortium at optimized conditions

The decolourization experiments were carried out in triplicate in 250 ml Erlenmeyer flasks containing 100 ml of sterile modified GPYM broth containing ($g\,l^{-1}$): D-glucose, 10; peptone, 1.0; K_2HPO_4, 1.0, and $MgSO_4{\cdot}7H_2O$, 0.5 and supplemented with PMDE to obtain optical density 3.5 at 475 nm and pH was adjusted at 7.0 ± 0.5. The flasks were inoculated with 1% (v/v) over night grown bacterial consortium [10] and incubated at 37 °C under shaking flask condition (125 rpm, Innova 4230 Refrigerated shaker, New Brunswick, USA) for 6 consecutive days. The decolourization of PMDE was monitored spectrophotometrically (Techcomp, UV-2300 spectrophotometer, Korea) in terms of bacterial growth, and decrease in colour density (absorbance) at 620 and 475 nm, respectively and was expressed as decrease in absorbance at 475 nm against the initial absorbance at the same wavelength [11,12].

2.3. Physico-chemical analysis of PMDE before and after bacterial treatment

The physico-chemical analysis of PMDE before and after bacterial treatment was done as per standard methods for the examination of water and wastewaters [13]. The pH of samples was measured by Orion ion meter (Model-960), BOD by 5 days method, COD by open reflux method, total solids (TS) by drying method and total nitrogen (TN) by TOC-V_{csn} analyzer (Shimadzu, Japan). Phosphate and sulfate was measured by vanadomolybdo phosphoric acid colorimetric and $BaCl_2$ precipitation methods, respectively [13].

2.4. Preparation of bacteria treated PMDE samples for seed germination test

Bacteria treated PMDE at 144 h incubation period was centrifuged at $5000 \times g$ for 20 min to remove bacterial biomass and left over night to settle down the remaining suspended particles. If growth occurs, the effluent were centrifuged again to remove it, stored in screw-caped glass bottle and used in seed germination experiment.

2.5. Seed germination experiment

For seed germination experiment, the concentration of bacteria treated and untreated PMDE used was 5, 10, 20, 40, 60, 80 and 100% (v/v). Subsequently, 10 seeds of *P. mungo* L. were placed in sterilized glass petri dishes of uniform size lined with two Whatman No. 1 filter paper discs. These filter discs were then moistened with 5 ml of tap water for control and with the same volume of different concentrations of bacteria treated and untreated PMDE followed by incubation at 28 °C in a BOD incubator for a period of 6 consecutive days. The experiment was performed in triplicate. The seeds were surface sterilized with 2.0% $HgCl_2$ solution for 2 min to avoid any fungal contamination [14] and then washed thrice with double distilled water.

The seeds that germinated were counted and removed from petri dishes at the time of first count on each day until there was no further germination. The criterion of germination which we have taken was the visible protrusion of radical from seed coat and it was expressed in percentage. The germination rate was determined for seeds kept at ambient temperature at every 24 h interval of incubation period up to 6 days. The germinated seeds were counted to the initial appearance of the radical by continuous visual observation for 6 days. The germination index (GI) was calculated according to the method of Ana et al. [15].

2.6. Preparation of enzyme extract and assay for α-amylase activity

For the preparation of enzyme extract, twenty seeds from each treatment were homogenized with 0.1 M sodium acetate buffer (pH 4.8), filtered through two layers of cheese cloth to remove large particles and the supernatant obtained was centrifuged at $15{,}000 \times g$ for 20 min. All the preparations were carried out at 4 °C. The supernatant obtained was used as crude enzyme extract for α-amylase assay. For enzyme assay, the reaction medium (3 ml) contained 1 ml of 0.1 M acetate buffer, pH 4.8, 0.5 ml of enzyme extract diluted to 1 ml using acetate buffer, and 1 ml of 0.1% soluble starch solution. The enzyme extract was diluted to obtain an absorbance change of less than one during the enzyme assay. The reaction medium was incubated for 10 min at room temperature and then the reaction was stopped by adding 1 ml of 0.1% iodine reagent and 3 ml of 0.05 N HCl. The absorbance was measured at 620 nm and decrease in absorbance was expressed in terms of amylase activity [16].

2.7. Concentration and purification of enzymes

The supernatant containing crude α-amylase enzyme was concentrated by adding the double volume of cold acetone (−20 °C) and the precipitated proteins were collected by centrifugation at $15{,}000 \times g$ for 20 min. After removing the acetone, the precipitated proteins were dissolved in the minimal volume of 0.1 M sodium acetate buffer (pH 4.8). To purify, the soluble proteins were passed through a column (80 cm × 2.0 cm) containing Sephadex G-100 [17] previously equilibrated with the same buffer and the protein fractions (2.0 ml) eluted at the flow rate of 0.5 ml min^{-1} were stored at −20 °C and used in further studies.

2.8. SDS-PAGE and molecular weight determination of enzyme

The molecular weight of concentrated and purified α-amylase enzyme was determined by denaturing SDS-PAGE performed on 10% polyacrylamide gel. The α-amylase enzyme (Sigma–Aldrich, USA) and protein ladder (Bangalore Genei, India) was used as marker standards to compare and estimate the molecular weight of enzyme. After gel electrophoresis, the protein bands were stained with Coomasie Brilliant Blue R-250 dye and destained with destain-

ing solution. After destaining, the gels were visualized and stored in a gel documentation system (Syngene, UK).

2.9. Statistical analysis

To confirm the variability and validity of results, the data obtained were subjected to statistical analysis using one-way analysis of variance (ANOVA) to make comparison between more than two means followed by Tukey's test [18] to compare the individual means using the Graph Pad software (Graph Pad Software, San Diego, CA).

3. Results and discussion

3.1. Physico-chemical characteristics of PMDE before and after bacterial treatment

The physico-chemical analysis of PMDE before bacterial treatment has revealed that it was an alkaline, dark black coloured wastewater carrying very high BOD, COD values, total solids, sulfates, phosphates and phenolic compounds with high concentration of different heavy metals (Cu, Cd, Zn, Fe, Ni, Mn and Pb) (Table 1). But, after bacterial treatment, it was biotransformed into a neutral, pale coloured wastewater. The developed bacterial consortium was found effective to decolourize the PMDE up to 70.66% with considerable reduction in BOD, COD values, total solids, sulfates, phosphates and phenolic compounds up to 64.58, 74.20, 66.61, 59.58, 55.54 and 71.01%, respectively (Table 1). The developed bacterial consortium was also found highly effective to reduce metals content (both by extra and intracellular process). The reduction of metals content might be due to either the bioaccumulation of metals inside the cells or binding with liposaccharides of extra cellular membrane [19]. The reduction of BOD, COD, total solids, sulfates, phosphates and phenolic by developed bacterial consortium might be attributed to the bacterial degradation of complex recalcitrant compounds (i.e. melanoidins) and other organic and inorganic substances present in PMDE to meet the nutritional requirements.

Table 1
Physico-chemical characteristics of post-methanated distillery effluent (PMDE) before and after bacterial treatment.

Parameters	Value(s)		%Reduction after 168 h
	PMDE before treatment	PMDE after treatment	
Colour appearance	Dark black	Light brown	–
Colour intensity (Co.pt)	80000 ± 2127	5656 ± 173	70.7
pH	8.5 ± 0.17	7.2 ± 0.20	15.3
BOD ($mg\,l^{-1}$)	12000 ± 160	7315 ± 118	64.6
COD ($mg\,l^{-1}$)	21000 ± 321	6417 ± 136	74.2
TSS ($mg\,l^{-1}$)	29810 ± 274	9470 ± 156	68.2
TDS ($mg\,l^{-1}$)	17612 ± 284	8364 ± 123	52.5
TS ($mg\,l^{-1}$)	47422 ± 336	17834 ± 238	66.6
TOC ($mg\,l^{-1}$)	28696 ± 345	1246 ± 321	95.7
TN ($mg\,l^{-1}$)	4096 ± 218	1716 ± 246	58.1
SO_4^{2-} ($mg\,l^{-1}$)	2786 ± 112	1126 ± 218	59.6
PO_4^{3-} ($mg\,l^{-1}$)	1625 ± 108	724 ± 52	55.4
K^+ ($mg\,l^{-1}$)	537 ± 27	213 ± 17	60.3
Cl^- ($mg\,l^{-1}$)	7842 ± 134	2842 ± 128	63.8
Phenolics ($mg\,l^{-1}$)	6893 ± 147	1998 ± 116	71.0
Heavy metals ($mg\,l^{-1}$)			
Cu	0.75 ± 0.21	0.26 ± 0.04	65.3
Cd	1.30 ± 0.10	0.80 ± 0.02	38.5
Zn	1.24 ± 0.20	0.53 ± 0.05	57.3
Fe	57.50 ± 3.90	20.60 ± 2.52	64.2
Ni	0.31 ± 0.03	0.18 ± 0.02	41.9
Mn	43.63 ± 3.41	16.20 ± 2.13	62.9
Pb	0.23 ± 0.04	0.12 ± 0.02	47.8

All the values are means of three replicate ($n = 3$) ± SD. BOD—biological oxygen demand; COD—chemical oxygen demand; TSS—total suspended solids; TDS—total dissolved solids; TS—total solids; TOC—total organic carbon; TN—total nitrogen.

3.2. Effect of bacteria treated and untreated PMDE on seed germination and seedling growth

The physico-chemical analysis has revealed that untreated PMDE was highly toxic in nature and has inhibitory effect on seed germination and seedling growth. But, after bacterial treatment its toxicity was reduced significantly and has shown improved seed germination compared to untreated PMDE. In general, various crop plant species differed widely in response to different concentration of PMDE with respect to seeds germination, seedling growth and productivity. The seeds of *P. mungo* L. were treated with different concentration (5, 10, 20, 40, 60, 80 and 100%, v/v) of bacteria treated and untreated PMDE, kept under observation for a period of 6 consecutive days to record percent germination, seedling growth (i.e. shoot length and root length) and α-amylase activity, which is an indicator of gibberlic acid (GA) level in germinating seeds [16].

In present study, it was observed that up to 40% (v/v) concentration of untreated PMDE has no inhibitory effect on seed germination while at higher concentration (>40%), decrease in percent germination was recorded as 60, 80, and 100% (v/v) concentration of untreated PMDE has shown only 80, 60, and 40% seed germination (Table 2). But, after bacterial treatment, 100% seed germination was recorded up to 60% (v/v) concentration of treated PMDE and only 10 and 20% seed germination was inhibited at 80 and 100% (v/v) concentration of treated PMDE, respectively (Table 2). These results were also supported by the previous studies made by Kaushik et al. [20] who reported that 50% (v/v) concentration of PMDE had no adverse effects on seed germination and seedling growth. Further, regarding the seedling growth (i.e. shoot length and root length), the present study has revealed that up to 10% (v/v) concentration of untreated PMDE has supported the seedling growth, i.e. maximum root length and shoot length was recorded for the seeds treated with 5 and 10% (v/v) concentration of untreated PMDE. These findings are in accordance with the observations of Subramani et al. [14] who reported that the promotion of seedlings growth at 10% effluent concentration might be due to the presence of optimum level of plant nutrients (nitrogen and other minerals) in effluent. But, at higher concentrations (40, 60, 80 and 100%), PMDE has deleterious effects on seedling growth parameters (i.e. shoot length, root length) showing reduction in shoot length with highly reduced roots at 40 and 60% (v/v) concentration and no root development was observed in seeds treated with 80 and 100% (v/v) concentration of untreated PMDE. The deleterious effects of PMDE might be due to the higher concentration of heavy metals (Cu, Cd, Zn, Fe, Ni, Mn and Pb) and ions (SO_4^{2-}, PO_4^{3-}, K^+ and Cl^-), which retard the seedling growth by affecting the water absorption and other metabolic activities.

In case of the seeds treated with different concentration of bacteria treated PMDE, they all showed complete development of root and shoot. But, 20% (v/v) bacteria treated PMDE was found most suitable concentration for plant growth (shoot length: 5.72 cm and root length: 4.86 cm) possibly due to the presence of optimum level of primary and secondary nutrients required for plant growth. Although, the higher concentration (>20%) of bacteria treated PMDE also showed adverse effects on seedling growth, i.e. reduction in shoot length and root length but in compression to untreated PMDE, the seeds exposed to treated PMDE have shown better growth. The reduction in seed germination and seedling growth at higher concentrations of PMDE might be attributed to the high salt load in PMDE, which induces high osmotic pressure and anaerobic conditions [8]. This high osmotic pressure and anaerobic conditions render various biochemical processes such as movement of solute, respiration process of seeds and enzymatic steps of seed germi-

Table 2
Effect of bacteria treated and untreated (control) PMDE on seed germination and seedling growth in *Phaseolus mungo* L.

Concentration (%) of bacteria treated and untreated PMDE used in experiments	%Germination In		Speed of germination index		Shoot length (cm) In		Root length (cm) In		Root/shoot ratio In	
	Control PMDE	Treated PMDE	Control PMDE	Treated PMDE	Control PMDE	Treated PMDE	Control PMDE	Treated PMDE	Control PMDE	Treated PMDE
5%	100	100	230 ± 3.12	224 ± 2.21^{ns}	3.04 ± 0.07	3.56 ± 0.02*	3.74 ± 0.14	2.80 ± 0.04*	1.23 ± 0.04	0.78 ± 0.02*
10%	100	100	238 ± 3.32	240 ± 2.16^{ns}	3.82 ± 0.05	4.34 ± 0.04*	3.50 ± 0.18	3.46 ± 0.06^{ns}	0.92 ± 0.02	0.79 ± 0.06*
20%	100	100	232 ± 3.16	244 ± 2.32*	2.64 ± 0.04	5.72 ± 0.06*	3.24 ± 0.07	4.86 ± 0.05*	1.23 ± 0.05	0.85 ± 0.02*
40%	100	100	190 ± 2.10	226 ± 2.26*	2.21 ± 0.03	3.36 ± 0.03*	2.24 ± 0.02	3.40 ± 0.03*	0.56 ± 0.03	1.01 ± 0.03*
60%	80	100	162 ± 2.24	220 ± 2.32*	2.20 ± 0.06	2.54 ± 0.02*	0.43 ± 0.01	1.23 ± 0.04*	0.19 ± 0.10	0.48 ± 0.02*
80%	60	80	110 ± 2.16	222 ± 2.10*	0.52 ± 0.03	3.20 ± 0.07*	0.00 ± 0.00	1.74 ± 0.02*	0.00 ± 0.00	0.54 ± 0.01*
100%	40	60	92 ± 2.11	216 ± 2.18*	0.30 ± 0.04	2.42 ± 0.05*	0.00 ± 0.00	1.42 ± 0.02*	0.00 ± 0.00	0.58 ± 0.02*
Control (tap water)	100	100	228 ± 2.34	228 ± 2.34^{ns}	2.70 ± 0.08	2.70 ± 0.03^{ns}	2.93 ± 0.04	2.93 ± 0.02*	1.08 ± 0.03	1.08 ± 0.03^{ns}

All the values are means of triplicate ($n = 3$) ± SD. The statistical significance between the values of control to their respective treated samples was valuated by ANOVA. PMDE: post-methanated distillery effluent.
* Significance level: $p < 0.05$.
^{ns} Significance level: $p > 0.05$.

nation. Further, it has been reported that high PMDE content act as inhibitor for plant hormones (s) (amylases, auxins, gibberlines and cytokinins), which are mainly required for seed germination, seedling growth and development of plants, respectively [21]. The reduction in plant growth parameters at higher PMDE content might be also due to the entrance of potentially toxic trace elements into protoplasm resulting in the loss of intermediate metabolites, which are essential for further growth and development of plants as cadmium is specifically reported to have inhibitory effect on root growth more than on coleoptiles growth prior to germination of seeds [22].

3.3. Effect of bacteria treated and untreated PMDE on amylase activity

Seed germination is a complex physiological and biochemical process in plants that can be affected severely by several environmental factors. Starch is the major component of most of the world's crop yield and degradation of starch is essential for seed germination. In germinating seeds, starch degradation is initiated by α-amylase [23] producing soluble oligosaccharides from starch. These are then hydrolyzed by β-amylase to liberate maltose and finally, α-glucosidase breaks down maltose into glucose providing energy to germinating seeds.

In present study, the optimum amylase activity (0.6 U) was recorded in seeds treated with 10% (v/v) concentration of untreated PMDE and thereafter a continuous decline in α-amylase activity was observed at higher concentration (>10%) of PMDE (Fig. 1). However, the seeds treated with tap water (control) have shown low amylase activity (0.3 U) than the seeds treated with 10 and 20% (v/v) concentration of untreated and treated PMDE, respectively. Moreover, the seeds treated with bacterial degraded PMDE at 20% (v/v) concentration have shown the maximum amylase activity (0.9 U) (Fig. 1) indicating that the toxicity of PMDE is reduced significantly after bacterial treatment. The reduction in amylase activity at higher (>10 and 20%) concentration of untreated and treated PMDE might be due to the high salt load and metals content affecting various physiological and biochemical process of seed germination. Further, the denaturing SDS-PAGE of α-amylase enzyme extracted from germinating seeds treated with different concentration of untreated and treated PMDE has yielded three bands of different molecular weight and intensity/concentration. Band intensity has indicated the concentration of α-amylase enzyme produced in germinating seeds treated with different concentration of untreated and treated PMDE.

Fig. 1. Amylase activity shown by *Phaseolus mungo* L. seeds treated with different concentrations of bacteria treated and untreated PMDE. PMDE: post-methanated distillery effluent; UT: untreated; T: treated.

Results indicated that the concentration of α-amylase enzyme (i.e. band intensity) decreases gradually as the concentration of untreated PMDE increases. *Phaseolus* seeds treated with 60 and 80% (v/v) concentration of untreated PMDE have shown reduced α-amylase activity clearly and no α-amylase activity or enzyme production was observed in seeds treated with 100% (v/v) concentration of untreated PMDE. But, in case of the seeds treated with different concentrations of bacteria treated PMDE, they all showed α-amylase activity and enzyme production but the optimum amylase activity (0.9 U) and enzyme production was recorded in seeds treated with 20% (v/v) concentration of bacteria treated PMDE. Further, the molecular weight of α-amylase enzyme determined by comparing with protein marker and α-amylase standard has indicated that the molecular weight of three fragments of α-amylase separated by SDS-PAGE was approximately 47.5, 46 and 44.5 kDa, respectively. Moreover, the measurement of α-amylase activity in germinating seeds is a classical bioassay of gibberellic acid content determination, which mediates seed germination, cell elongation, flowering and various other physiological responses in plants.

4. Conclusions

In present study, it was observed that untreated PMDE has deleterious effects on seed germination and seedling growth parameters and at higher concentration (>40%), it proved to be lethal for plant growth parameters. But, after bacterial treatment, 60% (v/v) concentration of PMDE has shown 100% seed germination indicating that bacterial treatment has significantly reduced the toxicity of PMDE. The results obtained at 10% (v/v) effluent concentration were invariably better as compared to control. It appears that this concentration of effluent acts as a liquid fertilizer. Moreover, bacteria treated PMDE could be used for irrigation purposes at 20% (v/v) concentration only after making the proper dilution and satisfying the other conditions. The pronounced effects of untreated PMDE on seed germination and growth parameters at lower concentration might be due to the presence of optimum level of primary and secondary nutrients essential for plant growth. While the inhibitory effects at higher concentrations could be because of high salt load and high metals content. Over all, the nutrients present in PMDE played a significant role in seed germination and seedling growth parameters. Hence, this study concluded that the indiscriminate application of PMDE is health hazards during the irrigation in Indian agricultural practices. The study also revealed that the plants irrigated without proper dilution of PMDE will be not safe for human consumption.

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