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BIOREMEDIATION OF DISTILLERY SPENT WASH BY *Clonostachys rosea* AND *Penicillium citrinum* UNDER CARBON AND NITROGEN SUPPLEMENTATION

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ABSTRACT

Distillery spent wash (DSW) is a highly polluted industrial effluent characterized by dark colour, high organic load and elevated nutrient concentrations. An experiment was conducted during 2025-26 at the Soil and Water Management Research Unit, NAU, Navsari to evaluate the effect of carbon and nitrogen source on fungal bioremediation of DSW using *Clonostachys rosea* and *Penicillium citrinum*. Five treatments namely M_0 (without inoculation), M_1 (*Clonostachys rosea* @ 1%), M_2 (*Clonostachys rosea* @ 1% with carbon and nitrogen source), M_3 (*Penicillium citrinum* @ 1%) and M_4 (*Penicillium citrinum* @ 1% with carbon and nitrogen source) were evaluated over a 15-day incubation period. In this study sucrose and peptone @ 1% were used as external carbon and nitrogen source, respectively. Supplementation of carbon and nitrogen source significantly enhanced fungal activity and biodegradation efficiency. Among the all treatments, M_4 exhibiting the highest biodegradation efficiency followed by treatment M_2. Treatment M_4 reduced N (41.99%), P (42.13%), K (41.56%), Ca (41.97%), Mg (41.57%), Na (41.99%), SO_4^{-2} (42.21%), HCO_3^- (26.62%) and Cl^- (20.02%) compared to treatment M_0. Inoculation period and interaction with fungal treatments significantly influenced biodegradation. The results demonstrate that nutrient assisted fungal bioremediation is an efficient, eco-friendly and sustainable approach for safe management of distillery spent wash before agricultural reuse and other sustainable applications.

Key words: bioremediation; *Clonostachys rosea;* bioremediation; distillery spent wash; *Penicillium citrinum.*

Introduction

Distillery spent wash (DSW), also known as vinasse or molasses spent wash, is the principal liquid by-product generated during the production of the ethanol from molasses in distilleries. With the rapid expansion of the sugar and ethanol industries, particularly under India's E20 ethanol blending programme, the volume of distillery spent wash generated has increased substantially, creating serious environmental management challenges (Department of Food and Public Distribution, 2025; All India Distiller's Association, 2025). Approximately 15 L of spent wash is produced of every litre of ethanol manufactured, resulting in enormous quantities of wastewater requiring safe disposal and treatment (Dahiya and Vimal, 1984). It is one of the most problematic industrial effluents because of its high organic load, intense dark brown colour, low pH, high salinity and the presence of recalcitrant compounds such as melanoidins. It is characterized by extremely high chemical oxygen demand (COD) and biological oxygen demand (BOD), making its direct discharge into the environment hazardous (Sirianuntapiboon *et al*., 1988). Disposal of untreated spent wash contaminates soil and water resources, reduces dissolved oxygen in aquatic ecosystems, inhibits

seed germination and adversely affects soil health and crop productivity. Conventional treatments including physical, chemical and physico-chemical processes are often expensive, generate secondary pollutants and show limited efficiency in removing recalcitrant coloured compounds. Biological treatment using microorganisms has emerged as an eco-friendly, economical and sustainable approach for DSW remediation. Among various microorganisms, filamentous fungi are considered highly effective because they produce extracellular enzymes capable of degrading recalcitrant compounds. Fungi also more tolerant than bacteria to various in pH, temperature and nutrient availability, making them suitable for industrial wastewater treatment (Knapp, 2001). However, efficient fungal degradation often requires supplementation with readily available carbon and nitrogen sources, as spent wash contains limited easily metabolizable carbon. External carbon promotes fungal growth and enzyme production, while nitrogen sources, particularly nitrogen source enhance decolourization and biodegradation efficiency when applied at optimum concentrations (Raghukumar and Rivonkar, 2001; Kumar *et al*., 1998; Dahiya *et al*., 2001 Viswanath *et al*., 2008). Therefore, the present study was undertaken to evaluate the effect of selected fungal isolates with and without carbon and nitrogen supplementation on the biodegradation of distillery spent wash as an eco-friendly and cost-effective bioremediation strategy.

Material and Methods

Collection of samples

The distillery spent wash and soil sample were collected from the Distillery Unit of Gandevi Sugar Factory located at Ambheta, Gandevi, Navsari, Gujarat. It is situated between 20.78405° N Latitude and 72.98555° E Longitude. The spent wash was collected in the month of August 2025 and used for the further study.

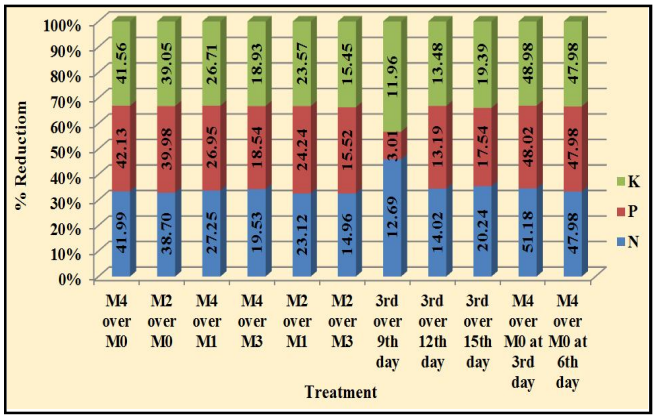


Fig. 1: Effect of microbial inoculation and incubation period on reduction of N, P and K content in distillery spent wash.

Table 1: Effect of microbes and incubation period on pH of distillery spent wash.

Treatment	pH					Mean (M)
	Incubation period (Day)					
	3rd	6th	9th	12th	15th	
M_0	4.62	5.15	5.30	5.06	4.83	**4.99**
M_1	4.40	4.48	5.39	5.15	4.86	**4.85**
M_2	3.76	3.60	3.47	3.44	3.36	**3.52**
M_3	4.45	4.49	4.83	5.08	4.98	**4.76**
M_4	3.69	3.51	3.45	3.45	3.39	**3.50**
Mean (D)	**4.18**	**4.25**	**4.49**	**4.43**	**4.28**	
Source	**S.Em. (±)**			**CD at 5%**		
M	0.02			0.05		
D	0.02			0.05		
M × D	0.04			0.11		
CV (%)	**1.79**					

Isolation of fungi

Fungi were isolated using Carboxy Methyl Cellulose (CMC) agar medium containing K_2HPO_4 (0.5 g/L), $MgSO_4$ (0.25 g/L), Gelatin (2 g/L), CMC (2 g/L) and congo-red dye (0.2 g/L). One gram of the collected sample was inoculated into a flask containing 50 mL of CMC broth and subjected to three successive enrichment cycles at three-day intervals by transferring 1 mL of broth into fresh sterile CMC broth. After enrichment, 1 mL of broth culture was inoculated onto PDA plates using streaking and spreading techniques and inoculated under static conditions for fungal growth. Morphologically distinct fungal colonies were purified by subculturing onto fresh PDA plates. The purified isolates were screened on CMC agar plates and those producing prominent clear halo zones around the colonies, indicating cellulolytic activity, were selected for further study.

Identification of fungi

Two potent fungal isolates were identified by ITS sequencing methodology at the laboratory of SLS Research Pvt. Ltd., Surat, Gujarat. Genomic DNA was

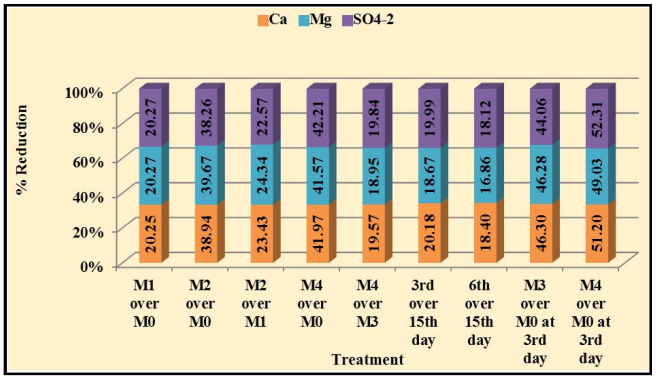


Fig. 2: Effect of microbial inoculation and incubation period on reduction of Ca, Mg and SO_4^{-2} content in distillery spent.

Table 2: Effect of microbes and incubation period on EC (dS/m) of distillery spent wash.

Treatment	EC (dS/m) Incubation period (Day)					Mean (M)
	3rd	6th	9th	12th	15th	
M_0	9.31	9.48	9.46	9.50	9.55	**9.46**
M_1	8.70	8.65	8.70	8.90	9.10	**8.81**
M_2	8.80	9.13	9.30	9.40	9.70	**9.27**
M_3	8.50	8.09	8.52	8.70	8.75	**8.51**
M_4	8.65	9.15	9.02	9.50	9.70	**9.20**
Mean (D)	**8.79**	**8.90**	**9.00**	**9.20**	**9.36**	
Source	**S.Em. (±)**			**CD at 5%**		
M	0.06			0.17		
D	0.06			0.17		
M × D	0.13			0.38		
CV (%)	**2.98**					

Table 3: Effect of microbes and incubation period on N content (mg/L) of distillery spent wash.

Treatment	N (mg/L) Incubation period (Day)					Mean (M)
	3rd	6th	9th	12th	15th	
M_0	393.25	399.20	414.00	403.31	401.25	**402.20**
M_1	289.17	279.23	324.13	358.25	352.65	**320.68**
M_2	211.25	222.60	234.88	236.30	327.75	**246.56**
M_3	254.15	266.80	294.25	306.25	328.25	**289.94**
M_4	192.00	207.65	242.65	254.25	270.00	**233.31**
Mean (D)	**267.96**	**275.10**	**301.98**	**311.67**	**335.98**	
Source	**S.Em. (±)**			**CD at 5%**		
M	4.35			12.25		
D	4.35			12.25		
M × D	9.72			27.39		
CV (%)	**6.51**					

extracted and the ITS (Internal Transcribed Spacer) region was amplified by universal primers followed by sequencing with the ITS4 reverse sequencing primer. The obtained gene sequences were analyzed using BLAST analysis with the NCBI GenBank database and deposited in GenBank and assigned unique accession numbers PX735669 and PX735670. Based on the highest sequence similarity, fungal isolate 1 was identified as *Clonostachys rosea* and fungal isolate 2 as *Penicillium citrinum*. Molecular identification was further confirmed by multiple sequence alignment with top ten homologous GenBank sequences and a phylogenetic tree. The identified fungal isolates were selected for subsequent biodegradation experiments.

Preparation of inoculation medium

Two separate 500 mL flasks containing 250 mL potato dextrose broth were aseptically inoculated with two fungal isolates and incubated for 7-10 days to obtain actively growing fungal broth cultures. The developed broth cultures were subsequently used for inoculation into distillery spent wash.

Preparation of 5% spent wash

After collection of spent wash from distillery, 5% spent wash solution was prepared by adding 125 mL of raw spent wash to 2.5 L amber-coloured glass bottles and making up the final volume to 2.5 L with distilled water.

Physico-chemical properties of distillery spent wash sample

The reduction in physico-chemical properties of the distillery spent wash such as pH, EC, N, P, K, Ca, Mg, Na, SO_4^{-2}, HCO_3^- and Cl^- was assessed before and after fungal treatments. All analyses were carried out following the standard procedures outlined by Gilcreas, (1966).

Methodology

Twenty-five amber-coloured bottles (2.5 L capacity) were filled with 5% diluted spent wash. Treatments M_1, M_2, M_3 and M_4 were inoculated with pure cultures of *Clonostachys rosea* @ 1%, *Clonostachys rosea* @ 1% with carbon and nitrogen source, *Penicillium citrinum* @ 1% and *Penicillium citrinum* @ 1% with carbon and nitrogen source, respectively while M_0 was kept as uninoculated as per treatment. Sucrose and peptone @ 1% were added as an external supplement as carbon and nitrogen source, respectively in M_2 and M_4 treatments. Each treatment was inoculated at room temperature for 15 days with daily shaking to ensure uniform mixing. For periodic sample analysis, five sets of each treatment with four replications were arranged and each one set was disturbed for periodic sample at 3rd, 6th, 9th, 12th &15th day and analysis for parameters such as pH, EC, N, P, K, Ca, Mg, Na, SO_4^{-2}, HCO_3^- and Cl^-.

Results and Discussion

Effect of microbes and incubation period on pH and EC of distillery spent wash in presence and absence of carbon and nitrogen source

Among the microbial treatments, uninoculated control (M_0) recorded the highest mean pH (4.99), whereas the lowest pH (3.50) was observed in treatment M_4 inoculated with *Penicillium citrinum* with carbon and nitrogen source, which was statistically at par with treatment M_2 (3.52). Treatments supplemented with carbon and nitrogen reduced the pH by 27.39 and 26.62% in M_2 and M_4 over M_1 and M_3, respectively, indicating enhanced fungal activity and biodegradation. During incubation period, the lowest pH (4.18) was observed on the 3rd day, followed by an increase to 4.49 on the 9th day and a subsequent decline to 4.28 on the 15th day. Among

Table 4: Effect of microbes and incubation period on P content (mg/L) of distillery spent wash.

Treatment	P (mg/L)					Mean (M)
	Incubation period (Day)					
	3rd	6th	9th	12th	15th	
M_0	20.71	21.01	20.59	21.22	21.12	**20.93**
M_1	15.22	14.70	15.58	18.86	18.56	**16.58**
M_2	11.12	11.72	12.36	12.44	15.18	**12.56**
M_3	13.38	14.04	13.54	16.12	17.28	**14.87**
M_4	10.77	10.93	11.28	13.38	14.21	**12.11**
Mean (D)	**14.24**	**14.48**	**14.67**	**16.40**	**17.27**	
Source	**S.Em. (±)**			**CD at 5%**		
M	0.21			0.60		
D	0.21			0.60		
M × D	0.47			1.34		
CV (%)	**6.15**					

different interaction combinations, the lowest pH (3.36) was recorded in M_2 on the 15th day followed by M_4 (3.39), whereas the highest pH (5.39) was observed in M_1 on 9th day. The consistently lower pH in M_2 and M_4 suggests that carbon and nitrogen supplementation stimulated fungal growth, extracellular enzyme production and degradation of organic constituents leading to the formation of acidic metabolites such as citric, oxalic, gluconic and acetic acids. Similar findings were reported by Angayarkanni *et al.,* (2002), who observed reduction in pH of spent wash from 9 to 8.1 during treatment of *Aspergillus niveus* due to production of organic acids during the biodegradation process. Choudhary *et al.*, (2020) also observed slightly declined in pH during bacterial treatment of distillery wastewater from 7.71 to 6.99.

EC of distillery spent wash varied among the microbial treatments, the uninoculated treatment (M_0) recorded the highest EC (9.46 dS/m), whereas the lowest EC (8.51 dS/m) was recorded with M_3. Treatments M_2 and M_4 recorded higher EC values of 9.27 and 9.20 dS/m, respectively, which were 5.22% and 4.43% higher over M_1, and 8.93% & 8.11% higher than M_3, respectively. Across incubation periods, the lower EC (8.79 dS/m) was recorded at 3rd day, which gradually increased to the highest EC (9.36 dS/m) on 15th day. Regarding interaction effect, the lowest EC (8.09 dS/m) was recorded with M_3 at 6th day, whereas the highest EC (9.70 dS/m) was observed in treatments M_2 and M_4 on 15th day. The increase in EC under carbon and nitrogen supplemented treatments may be attributed to enhanced fungal metabolism, resulting in mineralization of organic matter and release of soluble ions such as Ca, Mg, K, Na and SO_4^{-2} into the medium. Similar findings were reported by Rane *et al.,* (2018) observed decrease in EC from 22.4 to 0.98 dS/m during treatment of molasses spent wash by *Fusarium solani* due to reduction in soluble salts and mineral constituents during fungal biodegradation process.

Table 5: Effect of microbes and incubation period on K content (mg/L) of distillery spent wash.

Treatment	K (mg/L)					Mean (M)
	Incubation period (Day)					
	3rd	6th	9th	12th	15th	
M_0	1,386.88	1,407.71	1,459.28	1,422.71	1,415.30	**1,418.38**
M_1	1,019.69	984.64	1143.13	1,263.56	1,243.86	**1,130.98**
M_2	744.93	784.96	828.24	833.27	1,130.75	**864.43**
M_3	896.21	940.82	1037.62	1,079.93	1,157.51	**1,022.42**
M_4	707.63	732.24	855.66	896.57	952.11	**828.84**
Mean (D)	**951.07**	**970.07**	**1,064.79**	**1,099.21**	**1,179.91**	
Source	**S.Em. (±)**			**CD at 5%**		
M	15.28			43.04		
D	15.28			43.04		
M × D	34.16			96.23		
CV (%)	**6.49**					

Effect of microbes and incubation period on N, P and K content of distillery spent wash in presence and absence of carbon and nitrogen source

The highest nitrogen (N) content (402.20 mg/L) was recorded in the uninoculated treatment (M_0), whereas the lowest N content (233.31 mg/L) was observed in treatment M_4. Treatments M_4 and M_2 was 41.99% and 38.70% over control. Treatment M_4 exhibited 27.25% and 19.53% reduction in N content over M_1 and M_3, respectively. Similarly, M_2 registered 23.12% and 14.96% reduction in N content over M_1 and M_3, respectively. Across incubation periods, the lowest N content (267.96 mg/L) was observed at 3rd day which was statistically at par with 6thday. Regarding the interaction effect, the highest N content (414.00 mg/L) was observed in treatment M_0 at 9th day, whereas lowest N content (192.00 mg/L) was recorded in M_4 at 3rd day, with represented reductions of 51.18 and 47.98% compared with M_0 at 3rd and 6th day, respectively. These findings indicated that fungal inoculation, particularly with carbon and nitrogen supplementation effectively reduced nitrogen content in spent wash through microbial assimilation and immobilization of available nitrogen into fungal biomass. The gradual increase in later stage may be due to mineralization of organic nitrogenous compounds and enzymatic decomposition of organic matter. Similarly,

Table 6: Effect of microbes and incubation period on Ca content (mg/L) of distillery spent wash.

Treatment	Ca (mg/L)					Mean (M)
	Incubation period (Day)					
	3rd	6th	9th	12th	15th	
M_0	269.17	273.28	283.34	275.95	274.22	**275.19**
M_1	197.85	191.05	222.02	245.33	241.10	**219.47**
M_2	144.54	148.50	160.82	162.09	224.25	**168.04**
M_3	174.20	182.76	201.44	209.82	224.59	**198.56**
M_4	131.37	142.08	166.16	173.99	184.90	**159.70**
Mean (D)	**183.43**	**187.53**	**206.76**	**213.43**	**229.81**	
Source	**S.Em. (±)**			**CD at 5%**		
M	2.97			8.38		
D	2.97			8.38		
M × D	6.65			18.73		
CV (%)	**6.51**					

Table 7: Effect of microbes and incubation period on Mg content (mg/L) of distillery spent wash.

Treatment	Mg (mg/L)					Mean (M)
	Incubation period (Day)					
	3rd	6th	9th	12th	15th	
M_0	351.86	357.18	370.42	360.85	359.01	**359.86**
M_1	258.73	249.83	290.01	320.54	315.53	**286.93**
M_2	189.01	201.55	210.15	211.43	273.34	**217.10**
M_3	227.40	238.72	263.28	274.01	293.70	**259.42**
M_4	179.33	185.79	217.11	227.49	241.58	**210.26**
Mean (D)	**241.26**	**246.61**	**270.19**	**278.86**	**296.63**	
Source	**S.Em. (±)**			**CD at 5%**		
M	3.99			11.23		
D	3.99			11.23		
M × D	8.91			25.11		
CV (%)	**6.68**					

Pawar *et al.,* (2011) who observed nitrogen reduced from 2,64,000 to 88,000 mg/L (33% reduction) by *Aureobasidium pullulans* treatment of distillery effluent due to active microbial assimilation, biomass formation and enzymatic degradation.

Among the microbial inoculations, the highest phosphorus (P) content (20.93 mg/L) was recorded in the uninoculated treatment (M_0), whereas the lowest P content (12.11 mg/L) was observed in inoculated with *Penicillium citrinum* with carbon and nitrogen source (M_4), which was statistically at par with M_2 (12.56 mg/L). Treatments M_4 and M_2 reduced P content by 42.13% and 39.98%, respectively over M_0. The P content of spent wash was reduced by 26.95% and 18.24% in M_4 and by 24.24% and 15.52% in M_2 over M_1 and M_3, respectively. During incubation periods, the lowest P content (14.24 mg/L) was recorded at 3rd day indicating a gradual increase in P content during incubation. Regarding the interaction effect, the lowest P content (10.77 mg/L) was recorded in treatment M_4 at 3rd day, representing reductions of 48.02 and 47.98% over M_0 on the 3rd and 6th day, respectively. These findings indicated that treatments supplemented with carbon and nitrogen source (M_2 and M_4) decrease in phosphorus content may be due to microbial sequestration of phosphate for ATP production and energy transfer reactions. Availability of readily utilizable carbon and nitrogen source might have stimulated fungal growth and accelerated assimilation of soluble phosphorus into microbial biomass. Similar results were reported by Rane *et al.,* (2018), who observed reduction in phosphorus from 26,600 to 12,788 mg/L during treatment of molasses spent wash by *Fusarium solani* supplemented with glucose and peptone due to microbial utilization and mineralization of phosphorus containing compounds during biodegradation process.

The uninoculated treatment (M_0) recorded the highest potassium (K) content (1,418.38 mg/L), whereas the lowest K content (828.84 mg/L) was observed in treatment M_4 inoculated with *Penicillium citrinum* along with carbon and nitrogen source, which was statistically at par with treatment M_2 (864.43 mg/L). The results further showed that 41.56%, 26.71% and 18.93% reduction in K content was observed with M_4 over M_0, M_1 and M_3, respectively. In case of M_2, 39.05%, 23.57% and 15.45% decrement in K content was registered with M_2 over M_0, M_1 and M_3, respectively. Across the incubation period the lowest K content (951.07 mg/L) was found at 3rd day. The results further revealed that K content at 3rd day registered 11.96%, 13.48% and 19.39% reduction over 9th, 12th and 15th day. Regarding the interaction effect, the lowest K content (707.63 mg/L) was recorded in treatment M_4 at 3rd day, representing reductions of 48.98% and 47.98% over M_0 at 3rd and 6th day, respectively. These findings indicated that fungal inoculation, particularly with carbon and nitrogen source, effectively reduced K content in spent wash through adsorption and assimilation of K into fungal biomass during active metabolism. The gradual increase in K content during prolonged incubation due to mineralization of K bearing organic compounds and their subsequent release into the spent wash. Bharagava and Chandra (2010) observed reduction in potassium content from 537 to 213 mg/L (60.3%) during bacterial treatment of post-methanated distillery effluent due to microbial utilization and biosorption of soluble K ions during biodegradation process.

Effect of microbes and incubation period on Ca, Mg and Na content of distillery spent wash in presence and absence of carbon and nitrogen source

Among the microbial treatments, M_0 recorded the

Table 8: Effect of microbes and incubation period on Na content (mg/L) of distillery spent wash

Treatment	Na (mg/L) Incubation period (Day)					Mean (M)
	3rd	6th	9th	12th	15th	
M_0	310.46	315.16	326.84	318.40	316.78	**317.53**
M_1	228.29	220.44	255.89	282.83	278.41	**253.17**
M_2	166.78	175.74	185.43	186.55	233.75	**189.65**
M_3	200.64	210.63	232.30	241.78	259.14	**228.90**
M_4	151.58	163.93	191.57	200.72	213.16	**184.19**
Mean (D)	**211.55**	**217.18**	**238.41**	**246.06**	**260.25**	
Source	**S.Em. (±)**			**CD at 5%**		
M	3.43			9.65		
D	3.43			9.65		
M × D	7.66			21.59		
CV (%)	**6.53**					

highest calcium (Ca) content (275.19 mg/L). Whereas, treatment M_4 and M_2 recorded significantly lower Ca content 159.70 and 168.04 mg/L, respectively. Treatment M_2 and M_4 reduced Ca content by 38.94% and 41.97%, respectively, over the control (M_0). Compared with their respective fungal treatments without supplementation, M_2 reduced Ca by 23.43% over M_1, while M_4 recorded a 19.57% reduction over M_3. Across incubation periods, the lowest Ca content (183.43 mg/L) was recorded at 3rd day indicating a gradual increase in Ca concentration with incubation. Regarding the interaction effect, the lowest calcium content (131.37 mg/L) was recorded in M_4 at 3rd day, representing reduction of 46.30% and 51.20% by M_2 and M_4, respectively, over M_0 on 3rd day. These findings indicated that fungal inoculation, particularly with carbon and nitrogen source, effectively reduced Ca content in distillery spent wash through microbial uptake, complexation and precipitation of calcium as insoluble salts during biodegradation. The gradual increase may be attributed to mineralization and decomposition of Ca containing organic constituents. Similar reductions also observed by Kalalarasl and Tamilselvi (2018), who observed reduction in calcium content from 2,799 to 2,419 mg/L by fungi *Aspergillus flavus* and *Penicillium* sp. due to utilization of calcium for microbial growth.

The uninoculated treatment (M_0) recorded the highest magnesium (Mg) content (359.86 mg/L), whereas the lowest magnesium content (210.26 mg/L) was observed in treatment M_4 inoculated with *Penicillium citrinum* along with carbon and nitrogen source, which was statistically at par with treatment M_2 (217.10 mg/L). Treatments M_2 and M_4 reduced Mg content by 39.67% and 41.57%, respectively, over control. Treatment M_2 reduced Mg content by 24.34% over M_1, while M_4

Table 9: Effect of microbes and incubation period on SO_4^{-2} content (mg/L) of distillery spent wash.

Treatment	SO_4^{-2} (mg/L) Incubation period (Day)					Mean (M)
	3rd	6th	9th	12th	15th	
M_0	1,055.57	1,071.54	1,111.26	1,082.56	1,077.04	**1,079.59**
M_1	776.18	749.50	870.02	961.62	946.59	**860.78**
M_2	590.49	597.51	630.45	634.28	879.75	**666.50**
M_3	682.19	716.15	789.83	822.04	881.09	**778.26**
M_4	503.39	557.38	651.32	682.46	724.74	**623.86**
Mean (D)	**721.56**	**738.41**	**810.58**	**836.59**	**901.84**	
Source	**S.Em. (±)**			**CD at 5%**		
M	11.42			32.71		
D	11.42			32.71		
M × D	25.53			71.93		
CV (%)	**6.37**					

recorded 18.95% reduction over M_3. Regarding the incubation periods, the lowest Mg content (241.26 mg/L) was recorded at 3rd day. Across the interaction effect, the lowest Mg content (179.33 mg/L) was recorded in treatment M_4 at 3rd day, representing reductions of 46.28% and 49.03% by M_2 and M_4 over M_0 at 3rd day of incubation, respectively. The data indicated that treatments supplemented with carbon and nitrogen source (M_2 and M_4) effectively reduced the Mg content through microbial uptake and immobilization into fungal biomass. Similar findings were reported by Chavan *et al.* (2006), who observed reduction of Mg from 2,160 to 200 mg/L during biodegradation of spent wash by using *Pseudomonas* sp.

The highest sodium (Na) content (317.53 mg/L) was recorded in the uninoculated treatment (M_0), while the lowest Na content (184.19 mg/L) was observed in M_4 inoculated with *Penicillium citrinum* along with carbon and nitrogen source, which was statistically at par with treatment M_2 (189.65 mg/L). Treatment M_4 reduced Na content by 41.99% and 19.53% over M_0 and M_3, respectively. Similarly, M_2 reduced Na content 40.27% and 25.09% over M_0 and M_1, respectively. Across incubation period, the lowest sodium content (211.55 mg/L) was recorded at 3rd day. Regarding interaction effect, the lowest Na content (151.58 mg/L) was recorded in M_4 at 3rd day. The reduced Na content in M_2 and M_4 may be attributed to availability of readily utilizable carbon and nitrogen source that stimulated fungal growth and accelerated assimilation of soluble sodium into microbial biomass. Furthermore, microbial metabolism and biochemical transformation during biodegradation process may have contributed to variations in Na content throughout the incubation period. Similar findings were

Table 10: Effect of microbes and incubation period on HCO_3^- content (mg/L) of distillery spent wash.

Treatment	HCO_3^- (mg/L) Incubation period (Day)					Mean (M)
	3rd	6th	9th	12th	15th	
M_0	2,132.31	2,064.63	2,035.81	2,070.31	1,968.00	**2,054.21**
M_1	1,856.75	1,794.50	1,759.50	1,717.00	1,659.50	**1,757.45**
M_2	1,621.00	1,469.63	1,342.19	1,442.19	1,593.06	**1,493.61**
M_3	1,801.75	1,766.44	1,700.44	1,718.00	1,652.38	**1,727.80**
M_4	1,654.25	1,311.38	1,480.31	1,593.81	1,496.75	**1,507.30**
Mean (D)	**1,813.21**	**1,681.31**	**1,663.65**	**1,708.26**	**1,673.94**	
Source	**S.Em. (±)**			**CD at 5%**		
M	18.59			52.38		
D	18.59			52.38		
M × D	41.57			117.11		
CV (%)	**4.87**					

Table 11: Effect of microbes and incubation period on Cl^- content (mg/L) of distillery spent wash.

Treatment	Cl^- (mg/L) Incubation period (Day)					Mean (M)
	3rd	6th	9th	12th	15th	
M_0	2,084.08	1,965.10	1,868.29	1,830.97	1,798.77	**1,909.44**
M_1	1,897.**02**	1,719.**97**	1,531.**14**	1,652.**50**	1,637.**38**	**1,687.60**
M_2	1,776.**02**	1,627.**60**	1,518.**29**	1,438.**29**	1,405.**24**	**1,553.09**
M_3	1,833.**92**	1,748.**49**	1,537.**82**	1,645.**39**	1,690.**37**	**1,691.20**
M_4	1,736.**52**	1,603.**10**	1,470.**79**	1,408.**29**	1,417.**74**	**1,527.29**
Mean (D)	**1,865.51**	**1,732.85**	**1,585.26**	**1,595.09**	**1,589.90**	
Source	**S.Em. (±)**			**CD at 5%**		
M	17.18			48.40		
D	17.18			48.40		
M × D	38.42			NS		
CV (%)	**4.59**					

reported by Tripathi *et al.*, (2021a), who observed reduction in sodium content at 6th day from 45.23 to 12.45 ppm during biodegradation of distillery wastewater by bacterial consortium due to microbial uptake of dissolved salts during biodegradation process on 6th day of incubation.

Effect of microbes and incubation period on SO_4^{-2}, HCO_3^- and Cl^- content of distillery spent wash in presence and absence of carbon and nitrogen source

Among the microbial treatments, the uninoculated treatment (M_0) exhibited the highest sulphate (SO_4^{-2}) content (1,079.59 mg/L), however the lowest sulphate content (623.86 mg/L) was observed in treatment M_4 inoculated with *Penicillium citrinum* along with carbon and nitrogen source, which was found statistically at par with treatment M_2 (666.50 mg/L). Treatment M_2 reduced sulphate content by 38.26% and 22.57% over M_0 and M_1, respectively. Similarly, M_4 reduced 42.21% and 19.84% over M_0 and M_3, respectively. Across incubation periods, the lowest sulphate content (721.56 mg/L) was recorded at 3rd day. Regarding interaction effect, the lowest sulphate content (503.39 mg/L) was recorded in M_4 at 3rd day. Availability of readily utilizable carbon and nitrogen source might have stimulated fungal growth and accelerated assimilation of soluble sulphate into microbial biomass. A portion of sulphate may be converted into organic sulphur compounds. Similar reduction in sulphate content was also observed by Chavan *et al.*, (2006), who recorded reduction in sulphate content from 1,100 to 400 mg/L during biodegradation of distillery spent wash by using *Pseudomonas* sp.

The highest bicarbonate (HCO_3^-) content (2,054.21 mg/L) was recorded in the treatment (M_0), whereas the lowest HCO_3^- content (1,493.61 mg/L) was observed in treatment M_2, which was statistically at par with treatment M_4 (1,507.30 mg/L). Treatment M_4 reduced HCO_3^- content by 26.62%, 14.23% and 12.76% over M_0, M_1 and M_3, respectively. With respect to incubation period, the lowest bicarbonate content (1,663.65 mg/L) was recorded at 9th day. Regarding the interaction effect, the lowest HCO_3^- content (1,311.38 mg/L) was recorded in treatment M_4 at 6th day. Treatments supplemented with carbon and nitrogen source maintained lower bicarbonate content throughout the incubation period, with M_2 and M_4 showing 37.05% and 38.50% reductions, respectively, compared with M_0 on the 3rd day. The active growth and metabolic activities of the fungus resulted in the utilization of HCO_3^- and carbonate ions present in the spent wash. The conversion of organic substrates into simpler metabolites and the release of acidic compounds during the biodegradation process further contributed to the decline in alkalinity. Similar reductions in alkalinity and bicarbonate during fungal treatment of distillery spent wash by *Aspergillus* species have been reported by Kalalarasl and Tamilselvi (2018).

Among the microbial inoculations, the highest chloride (Cl^-) content (1,909.44 mg/L) was recorded in the uninoculated treatment (M_0), whereas the lowest chloride

content (1,527.29 mg/L) was observed in treatment M_4, which was statistically at par with treatment M_2 (1,553.09 mg/L). The result further indicated that 20.01%, 9.50% and 9.69% reduction in chloride content was recorded in M_4 over M_0, M_1 and M_3, respectively. Across incubation period, the lowest chloride content (1,585.26 mg/L) was recorded at 9th day which was statistically at par with 12th and 15th day. 15.02% and 8.52% reduction in chloride content was observed at 9th day over 3rd and 6th day incubation period. The interaction effect between microbial inoculation and incubation period was non-significant. The reduction in chloride concentration may be attributed to enhanced fungal growth and microbial uptake of soluble salts during the biodegradation process, particularly in treatments supplemented with carbon and nitrogen source. Similarly, Tripathi *et al.*, (2021a) observed reduction in chloride concentration from 9,788.9 to 5,543 ppm during biodegradation of distillery wastewater by a bacterial consortium containing *Stenotrophomonas maltophilia*, *Bacillus cereus* and *Bacillus anthracis*. Similar reduction were also observed by Tripathi *et al.*, (2021b) that chloride reduced from 1,829 to 688.674 mg/L (84%).

Conclusion

The present study demonstrated that both *Clonostachys rosea* and *Penicillium citrinum* possess considerable potential for the biodegradation of distillery spent wash and their efficiency was markedly enhanced by supplementation of sucrose and peptone as external carbon and nitrogen source, respectively. Among the treatments, M_4 consistently exhibited the highest degradation efficiency, followed by treatment M_2. Significant reductions in nutrient and ionic constituents of distillery spent wash were achieved under treatment M_4, including nitrogen (41.99%), phosphorus (42.13%), potassium (41.56%), sodium (41.99%), calcium (41.97%), magnesium (41.57%), sulphate (42.21%), bicarbonate (26.62%) and chloride (20.01%) compared with uninoculated control (M_0). Incubation period significantly influenced the degradation process. Most physico-chemical parameters showed greatest reductions during the early incubation period, whereas gradual increases in nutrient concentrations during later stages were associated with mineralization and release of soluble ions from decomposing organic matter. Interaction effects further confirmed that carbon and nitrogen supplemented treatments maintained significantly lower nutrients and salts concentrations throughout the incubation period compared with uninoculated control treatment. Supplementation with carbon and nitrogen sources stimulated the fungal growth, extracellular enzyme production, microbial assimilation, biosorption and biotransformation of organic and inorganic constituents, thereby improving the biodegradation efficiency of distillery spent wash. Among the two fungal isolates, *Penicillium citrinum* with carbon and nitrogen source proved to be the most effective treatment. Therefore, nutrient-assisted fungal bioremediation is an effective, eco-friendly and economically viable strategy for the treatment of distillery spent wash before its environmental disposal or agricultural reuse and other sustainable applications.

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