Nature Environment and Pollution Technology
An International Quarterly Scientific Journal
p-ISSN: 0972-6268
(Print copies up to 2016)
e-ISSN: 2395-3454
Vol. 22 No. 2 pp. 1041-1045 2023
Original Research Paper
https://doi.org/10.46488/NEPT.2023.v22i02.052
Open Access Journal

Stabilisation of Spent Wash by Polyhydroxybutyrate (PHB) Producing Microorganisms Isolated from Karad Region, Maharashtra

G. V. Patil*† and G. R. Pathade*
*Krishna Institute of Allied Sciences, Krishna Institute of Medical Sciences (Deemed University), Karad, Maharashtra, India
†Corresponding author: G. V. Patil; gayatri25patil.gp@gmail.com

Nat. Env. & Poll. Tech.
Website: www.neptjournal.com

Received: 07-10-2022
Revised: 01-11-2022
Accepted: 14-11-2022

Key Words:
PHB producers
Spent wash
COD
Stabilization

ABSTRACT

In recent years, there has been a significant increase in the needs of the overgrowing population. Naturally, industrial belts increased worldwide to satisfy the variety and quantity of needs. While producing the products, a huge quantity of waste is generated and added continuously to the environment, causing hazardous effects on the flora and fauna. Spent wash is one of the most important types of industrial waste since it is the liquid waste that is left over after making alcohol and it pollutes the environment. Despite effluent quality standards, untreated or partially treated sewage frequently enters water courses or soils. It is always overloaded with organic and inorganic substances. The stabilization of the effluent by microorganisms is reflected in the form of COD reduction. The present study attempted to isolate the PHB-producing organisms and use them to stabilize the spent wash.

The locally isolated, characterized, and identified 11 PHB-producing microorganisms from the Karad region of Maharashtra were used to stabilize spent wash. They include *Candida orthopsilosis, Bacillus subtilis, Bacillus cereus, Bacillus megaterium, Klebsiella grimontii, Citrobacter fruendii*, and members of *Staphylococcus* and *Rhodococcus*. All potential organisms were reported to stabilize the spent wash. The degree of stabilization was measured in terms of the percent reduction of COD and BOD. The % reduction was reported at 95.31% and 81.39% of COD and BOD, respectively, by *Klebsiella grimontii,* followed by 92.18% and 80.46% reductions of COD and BOD, respectively, by *Staphylococcus.* These organisms are promising in the treatment of spent wash.

INTRODUCTION

Interest in low-carbon and more sustainable energy production has grown in several nations worldwide due to worries about climate change, energy security, and the depletion of fossil fuels (Chooyok et al. 2013). Bioenergy is a carbon-neutral, renewable energy source that may be used to create any fuel. The need for sustainable and renewable biofuels, particularly bioethanol, is increasing globally, and ethanol distillery businesses are expanding quickly to meet this demand (David et al. 2015).

Molasses waste wash is the term for the high volume of strong wastewater produced by the ethanol distillery industry. The world's water quality and soil quality are seriously threatened by the ever-increasing output of molasses waste wash, which produces undesired residual liquid waste (Mohana et al. 2009). Distillery waste wash is a caramelized and recalcitrant waste with extremely high COD, BOD, SS, inorganic solids, color, and low pH. Melanoidins, which make up 2% of the total wasted wash by mass, are primarily responsible for the spent wash's deep, dark brown color (Naik et al. 2010). For melanoidin, including distillery effluent, color removal is difficult. Typically, 10-15 L of wastewater is produced for every liter of alcohol (Biradar 2003).

If distillery waste is disposed of improperly, it poses a major risk to the nearby land and water bodies because it blocks sunlight from penetrating the aquatic environment, depleting the dissolved oxygen concentration. This happened due to the melanoidin pigment, a dark brown pigment produced by the Maillard reaction between sugar and amino compounds (Pandey et al. 2003, Satyawali & Balkrishnan 2007, Khandegar & Saroha 2012). Investigations into how to decolorize it physically or chemically were unsuccessful. Utilizing microbial activity to decolorize the spent wash has received more attention recently. Environmentalists and academics face challenges in distillery waste wash's economical and environmentally responsible management. Distillery waste wash can be treated using various physical, chemical, and biological techniques, particularly for decolorization and COD removal. The method most frequently used to treat distillery waste wash is bio-methanation, followed by aerobic treatment (Yadav 2012). Chemical oxygen demand

(COD) and biological oxygen demand are reduced by aerobic treatment by up to 50-70%. (Shruti et al. 2013). Since biological treatment with bacterial cultures has a reduced operating cost, it might be a useful alternative to reduce COD in wastewater. In the current investigation, isolated PHB producers were utilized locally to reduce COD and BOD to stabilize the organic matter and make it easier to dispose of without endangering the local flora and fauna.

MATERIALS AND METHODS

Physiochemical Characterization of Distillery Spent Wash Sample

The effluent was analyzed in triplicate, before and after the addition of potential organisms, for its color, odor, pH, Chemical Oxygen Demand (COD) by Dichromate reflux method, and Biochemical Oxygen Demand (BOD) by Winkler's modified Alkali-Azide method (Greenberg et al. 1998).

Use of Potential Isolates for Stabilization of Effluent

Out of 75 locally isolated microorganisms with PHB production ability, 11 isolates with excellent PHB production potential (Table 1) were identified and used to test their abilities to stabilize the spent wash in terms of reduction in COD and BOD.

The COD Determination

The extent of organic matter degradation was determined mainly by the reduction in COD values. To determine the COD, 100 mL of mineral salt medium supplemented with 10% spent wash was inoculated with 1 % inoculum containing 1×10^8 CFU.mL^{-1} potential PHB producers separately and incubated for 96 h at 30°C. After incubation, it was diluted to 1:1500, and samples were used to determine COD values by the dichromate reflux method (Hu & Grasso 2005). These values were then used to calculate the % reduction of COD, which directly revealed the extent of stabilization of effluent. The controls were without inoculum added.

The BOD Determination

The reduction in BOD values determined the extent of organic matter degradation. To determine the BOD, 100 mL of mineral salt medium supplemented with 10% spent wash was inoculated with 1 % inoculum containing 1×10^8 CFU.mL^{-1} potential PHB producers separately and incubated for 96 h. After incubation, it was diluted, and samples were used to determine BOD values by modifying Winkler's alkali azide method (Sawyer & McCarty 1978, Greenberg et al. 1998) calculates the amount of oxygen absorbed by bacteria during organic matter breakdown. Five days at 20°C are used to measure the change in DO concentration. The BOD is determined by subtracting the starting DO from the final DO. These values were then used to calculate the % reduction of BOD, which directly revealed the extent of stabilization of effluent. The controls were without inoculums added.

Table 1: Potential PHB-producing microorganisms.

Sr. No.	Isolate No.	Isolate identified as	Accession Number
1.	1	*Candida orthopsilosis*	OP393929
2.	10	*Bacillus subtilis*	OP415431
3.	20	*Bacillus cereus*	OP430573
4.	24	Member of *Bacillus*	-
5.	33	Member of *Rhodococcus*	-
6.	37	*Klebsiella grimontii*	OP458827
7.	38	Member of *Bacillus*	-
8.	41	*Citrobacter freundii*	OP434597
9.	45	Member of *Staphylococcus*	-
10.	46	Member of *Staphylococcus*	-
11.	72	*Bacillus megaterium*	OP435363

RESULTS AND DISCUSSION

Physicochemical analysis of raw spent wash (Table 2) reflects its heavy organic load in terms of high values of COD and BOD. The color of the spent wash was observed as deep dark brown with an unpleasant odor and acidic pH.

The high values of COD and BOD in raw spent wash warn of the danger caused to the environment and make more attention to proper treatment to stabilize the organic load. This can be achieved effectively by the use of microbial treatment. In the present study, 11 potential PHB producers attempted to degrade the organic contents and reduce the COD and BOD.

Use of Potential Isolates for Stabilization of Spent Wash

The results of stabilization of spent wash in terms of COD value and % reduction are summarized in Table 3;

Table 2: Physicochemical analysis of spent wash sample.

Sr. No.	Parameter	Results
1.	Color	Deep dark brown
2.	Odor	Unpleasant burn
3.	pH	5.5
4.	COD	128000 mg.L^{-1}
5.	BOD	43000 mg.L^{-1}

Table 3: COD value and % COD reduction of effluent after treatment with potential isolates. (Initial COD at 0 hr was 1,28,000 mg.L^{-1}).

Sr.No.	Isolate No.	COD value (after h)				% COD reduction (after h)				
		24	48	72	96	24	48	72	96	120
1.	1	1,20,000	90,000	48000	18,000	6.25	29.68	62.5	85.93	86.98
2.	10	36,000	36,000	18,000	12,000	71.87	71.87	85.93	90.62	92.12
3.	20	60,000	30,000	18,000	11,000	53.12	76.56	85.93	91.40	93.20
4.	24	1,20,000	18,000	18,000	18,000	6.25	85.93	85.93	85.93	87.43
5.	33	96,000	24,000	12,000	12,000	25	81.25	90.62	90.62	92.62
6.	37	24,000	18,000	12,000	6,000	81.25	85.93	90.62	95.31	97.31
7.	38	90,000	84,000	36,000	18,000	29.68	34.37	71.87	85.93	86.98
8.	41	66,000	36,000	24,000	12,000	48.43	71.87	81.25	90.62	92.50
9.	45	66,000	60,000	42,000	18,000	48.43	53.12	67.18	85.93	87.20
10.	46	1,20,000	60,000	24,000	10,000	6.250	53.12	81.25	92.18	93.20
11.	72	60,000	36,000	24,000	18,000	53.12	71.87	81.25	85.93	87.10

From Table 3, it can be noticed that COD values decreased as the time of reaction increased. It is evident from the table that the % COD reduction for the isolate was maximum after 96 hrs. of incubation, while after 120 hrs. of incubation, the % COD reduction was meagerly increased up to 1-2%, and hence 96 hrs. of incubation was taken as values as after the period of reduction. Hence COD values due to remaining isolates were determined and considered only up to 96 hrs. The range of COD values at the end of 96 hrs was recorded between 18000 to 6000. When these values were compared with values of COD from the original sample, i.e., 128000, a tremendous decrease in COD was noticed. The range of % reduction in COD for all these 12 isolates was found to be 84.37% to 95.31%, which indicates the excellent efficiency of isolates for stabilization of spent wash in terms of % COD reduction. A maximum % reduction of COD, i.e., 95.31, was reported using isolate 37, identified as *Klebsiella grimontii*. As Mohana et al. (2009) noted, the considerable potential for pollution makes the release of untreated wasted wash effluent into the environment hazardous to the ecosystem. Because of this, the stability of spent wash that shows a decrease in COD is considerably more important.

Ramachandra (1993) noticed COD reduction by *Pseudomonas stutzeri, P. acidovorans,* and *Enterbacter* sp. *Alcaligenes eutrophus* growing in the spent wash. Kumar et al. (1997) reported 41% and 39% COD reduction by LA-1 and D-2 isolates under optimized conditions in eight days. Sharma et al. (2000) measured 60 % COD reduction by several lactic acid bacteria from anaerobically digested molasses spent wash (ADMSW). Sudarshan et al. (2012) utilized *Azotobacter vinelandii* ATCC 12837 and ATCC 13705 to reduce COD levels (1100 mg.L^{-1}) of DSW to 200 mg.L^{-1} after fermentation was completed. Thus they successfully reduced the COD value to the permitted level.

In the present study, we have also achieved these two purposes of obtaining higher PHB quantity, and maximum COD reduction in distillery spent wash. Thus present results are on the same track of research giving suitable solutions for disposing of spent wash after proper treatment and sustaining the environment. The results of the stabilization of spent wash in terms of BOD value and % reduction are summarized in Table 4.

From Table 4, it is observed that values of BOD were recorded between 14200 to 8000. When these values were compared with values of BOD from the original sample, i.e., 43000, a large extent decrease in BOD was noticed. The range of % reduction in BOD for all these 11 isolates was found to be 66.98% to 81.39%, which indicated the excellent efficiency of isolates for stabilization of spent wash in terms of % BOD reduction. The maximum % reduction of BOD, i.e. 81.39 was reported using isolate number 37, identified as *Klebsiella grimontii* (Table 5).

Table 4: BOD value and % BOD reduction of effluent after treatment with potential isolates BOD value at 0 hrs was 43000 mg.L^{-1}.

Sr.No.	Isolate Number	BOD Value mg.L^{-1} after 5 days	% Reduction of BOD
1.	1	11400	73.48
2.	10	9200	78.60
3.	20	14200	66.98
4.	24	10200	76.28
5.	33	9200	78.60
6.	37	8000	81.39
7.	38	11400	73.48
8.	41	8800	79.53
9.	45	13800	67.90
10.	46	8400	80.46
11.	72	12800	70.23

Table 5: Physicochemical analysis of spent wash treated with *Klebsiella grimontii*.

Sr. No.	Parameter	Results
1.	Color	Faint brown
2	Odor	Unpleasant burn
3.	pH	5.7
4.	COD reduction	95.31%
5.	BOD reduction	81.39%

The pioneering research on bacterial wasted wash decolorization was carried out by (Kumar et al. 1997). They found that under ideal conditions, two aerobic bacterial isolates, LA-1 and D-2, achieved the highest levels of decolorization (36.5% and 32.5%) and COD reduction (41% and 39%) in just eight days. Ramachandra (1993) named four bacterial species as *Pseudomonas stutzeri*, *P. acidovorans*, *Enterbacter* sp., and *Alcaligenes eutrophus*, which were primarily found thriving in the wasted wash. They saw a significant reduction in BOD and COD and a decolorization of up to 60%. Sharma et al. (2000) isolated numerous lactic acid bacteria (ADMSW) from anaerobically digested molasses wasted wash.

Some isolates lowered COD by 60% and decolored ADMSW (25% v/v) by 70%. The viability of utilizing *Pseudomonas putida* for decolorizing used wash was discussed by Ghosh et al. (2003). The organism exhibited the capacity to extracellularly transform glucose into gluconic acid and hydrogen peroxide using glucose oxidase. The hydrogen peroxide created by this enzymatic oxidation removed the color from the used wash by oxidizing melanoidins. According to Sirianuntapiboon et al. (1988), Mycelia sterile D90 was the most effective strain, decolorizing molasses pigment by nearly 93% after eight days. When nutrients such as glucose (2.5%), $NaNO_3$ (0.2%), KH_2PO_4 (0.1%), and $MgSO_4.7H_2O$ (0.05%) were added to molasses wastewater, this strain also induced a BOD drop of nearly 80%. However, when these nutrients were absent, the decolorization yield was only 17.5%.

Our present results of COD reduction are on the same line of research as that of Kumar et al. (1997), Ramachandra (1993), Sharma et al. (2000) and Ghosh et al. (2003). On the other hand, the results of BOD reduction are similar to the results of Sirianuntapiboon et al. (1988).

A high % reduction in COD and BOD reflects these isolates' abilities to degrade the effluent's organic content. Thus converting the original hazardous effluent, such as a form of effluent that is safe to discharge into public water bodies by a suitable division nearby Karad city and safeguarding flora and fauna of the ecosystem.

CONCLUSIONS

The isolated 11 PHB producers efficiently degrade the organic content and stabilize the spent wash. The *Klebsiella grimontii* has the potential to stabilize the spent wash to a maximum degree by achieving a 95.31% reduction in COD and an 81.39% reduction in BOD. Thus biological treatment using *Klebsiella grimontii* culture may be a good alternative to stabilize the spent wash to a good extent.

ACKNOWLEDGMENT

We are very much thankful to the management of the Krishna Institute for inspiration and for providing all necessary laboratory facilities for the present research work

REFERENCES

Biradar, A. 2003. Physico-chemical and Biological Methods for the Treatment of Post Anaerobic Distillery Spent Wash. Ph.D. thesis. Centre for Environmental Science and Engineering Indian Institute of Technology, Mumbai.

Chooyok, P., Pumijumnog, N. and Ussawarujikulchai, A. 2013. The water footprint assessment of ethanol production from molasses in Kanchanaburi and Suphanburi province of Thailand. APCBEE Procedia, 5: 283-287.

David, C., Arivazhagan, M. and Tuvakara, F. 2015. Decolorization of distillery spent wash effluent by electrooxidation (EC and EF) and Fenton processes: a comparative study. Ecotoxicol. Environ. Safety, 121: 142-148.

Ghosh, M., Ganguli, A. and Tripathi, A.K. 2003. Treatment of anaerobically digested distillery spent wash in a two-stage bioreactor using *Pseudomonas putida* and *Aeromonas* sp. Process Biochem., 7: 857-862.

Greenberg, A.E., Eaton, A.D., Clesceri, L.S. and Franson, M.A.H. (eds). 1998. Standard Methods for the Examination of Water and Wastewater. Twentieth Edition. American Public Health Association (APHA), American water works association (AWWA), Water Environment Federation (WEF), Washington, DC.

Hu, Z. and Grasso, D. 2005. Water analysis: Chemical oxygen demand. Encycl. Anal. Sci., 14: 325-330.

Khandegar, V. and Saroha, A.K. 2012. Electrochemical treatment of distillery spent wash using aluminum and iron electrodes. Chin. J. Chem. Eng., 20(3): 439-443.

Kumar, V., Wati, L., Nigam, P., Banat, I.M., Mcmullan, G., Singh, D. and Marchant, R. 1997. Microbial decolorization and bioremediation of anaerobically digested molasses spent wash effluent by aerobic bacterial cultures. Microbios, 89: 81-90.

Mohana, S., Acharya, B.K. and Madamwar, D. 2009. Distillery spent wash: Treatment technologies and potential applications. J. Hazard. Mater., 163: 12-22.

Naik, N., Jagadeesh, K.S. and Noolvi, M.N. 2010. Enhanced degradation of melanoidin and caramel in bio methanated distillery spent wash by microorganisms isolated from mangroves. Iran. J. Energ. Environ., 14: 47-35.

Pandey, R.A, Malhotra, S., Tankhiwale, R. and Pande, S. 2003. Treatment of biologically treated distillery effluent: A case study. J. Clean Energy Technol., 14: 133.

Ramachandra, M. 1993. Development of Indigenous Technology (Microbial Strains) for Removing Sulfur Compounds and Color from Distillery Effluent. Annual Progress Report, Industrial Toxicology Research Centre, Lucknow.

Satyawali, Y. and Balakrishnan, M. 2007. Removal of color from bio methanated distillery spent wash by treatment with activated carbons. Bioresour. Technol., 98: 2629-2635.

Sawyer, C.N. and McCarty, P.L. 1978. Chemistry for Environmental Engineering. Third Edition. McGraw-Hill, NY.

Sharma, N., Wati, L. and Singh, D. 2000. Bioremediation of Anaerobically Digested Molasses Spent Wash By Lactic Acid Bacteria. Abstract Presented in AMI Conference, Birla Institute of Scientific Research, Jaipur, November, 25-27, pp. 175.

Shruti, M., Krishna, B., Usha, M. and Murthy, N. 2013. Colour removal from bio-digester effluent by electrochemical treatment with and without fenton reagent. Int. J. Curr, Eng. Technol., 6: 115-117.

Sirianuntapiboon, S., Somachai, P., Ohmomo, S and Atthasampunna, P. 1988. Screening of filamentous fungi having the ability to decolorize molasses pigments. Agric. Bio. Chem., 52: 387-392.

Sudarshan, S., Lakhawat, S., Amrendra, N., Pathak, M.V., Kulkarni, K. and Garikipati,V. 2012. Production of poly β-hydroxy butyrate from distillery spent wash using mutant *Azotobacter vinelandii*. Int. J. Adv. Biotechnol. Res., 3(2): 596-604.

Yadav, S. 2012. Degradation and decolorization of post methanated distillery effluent in biphasic treatment system of bacteria and wetland plant for environmental safety. Ph.D. thesis. School of Life Science, Pandit Ravi Shankar Shukla University.