ENTEROBACTER CLOACAE SUBSP. DISSOLVENS STRAIN TJ1812 IN ACRYLAMIDE BIODEGRADATION

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Abstract - **Extensive use of acrylamide in industries has contributed to the environmental pollution and to the human health. Increasing demands to seek the sustainable and controllable process which do not burden the environment significantly is becoming an area of interest. There are several advanced treatment plants employed, biodegradation strategies using bacterial strains has its own credits in rapidly degrading hazardous organic pollutants to environmentally safe levels in soils. In this work, acrylamide degrading bacteria was isolated from industrial wastewater. The isolate was identified as *Enterobacter cloacae subsp. dissolvens* strain TJ1812 by 16SrRNA gene sequencing. The growth specific characterization, optimum growth conditions were analyzed and tested for its acrylamide degradation efficiency from 0.5% with significant degradation reported upto 4%. This was followed by the influential effect of heavy metals along the degradation process and other amides degradation. GCMS analysis revealed the compounds of acrylamide degradation. These results highlighted the potential aspects of the bacterial strain in treating such hazardous pollutants as a bioremediation approach.**

Keywords - **Acrylamide, *Enterobacter cloacae*, industrial wastewater, biodegradation**

I. INTRODUCTION

Environmental pollution caused by increased levels of industrial chemicals, manufacturing wastes and biocides in water and on land, poses a considerable problem for society. In spite of the present treatment technology, the organic pollutants are found persisting in the soil-water environment above their acceptable level. There was an alarming report of the use of acrylamide in oil industries (Khayat, M. E *et al.*, 2017). Many reports have suggested that acrylamide seems to be found in foods that have been processed by heat-treatment methods other than boiling (Riboldi, B.P *et al.*, 2014). Acrylamide is a neurotoxin could be absorbed through unbroken skin, mucous membranes, lungs, and the gastrointestinal tract (Kay-Shoemake, J.L. *et al.*, 2000; ALKarim, S *et al.*, 2015; Kusnin, N *et al.*, 2015) One of the carcinogenic agents in the polluted environment (K. Labahn *et al.*, 2010). Human exposure to acrylamide is primarily

occupational from skin contact with the solid monomer and inhalation of dust and vapour. Probable exposure to the general public is through drinking water treated with polyacrylamide flocculants (Xiong, B, *et al.*, 2018). Acrylamide may not be completely removed in many water treatment processes with some remaining after flocculation with polyacrylamides probably due to its water solubility and is not absorbed by sediment. It is also one of the chemical intermediates in *N*-methylol acrylamide and *N*-butoxy acrylamide production and in disposable diapers, medical as a super absorbent. Small amounts of acrylamide are also used in sugar beet juice clarification, adhesives, binders for seed coatings, printing ink emulsion stabilizers, thickening agents for agricultural sprays, latex dispersions, textile printing paste, and water retention aids Shanker, R *et al.*, 1990). *Enterobacter aerogenes* reported to degrade acrylamide at 5000 mg/L among bacteria *Bacillus sp.*, *Pseudomonas sp.*, *Geobacillus sp.*, and *Rhodococcus sp.*, (Oksińska, M.P *et al.*, 2016; Madmanang, R *et al.*, 2018). Biodegradation is one of the classic methods for removal of undesired organic compounds to concentrations that are undetectable or below limits established as acceptable by regulatory agencies. Major limitations are the bioavailability of the organic matter and the finding of efficient bio-degraders. This study aims in biodegradation of acrylamide monomer using isolates obtained from industrial waste water samples.

II. MATERIALS AND METHODS

A. Isolation and Identification of bacteria

Samples of Industrial wastewater collected from different locations in and around Chennai, processed by standard spread plate method. About 1 ml of the waste water sample was added to 100 ml of peptone water incubated at 37^0C for 1 hour. 1 ml of the sample was serially diluted in 9 ml of sterile saline till 10^{-7} dilution.0.1 ml of the sample from the last dilution was spread plated on nutrient agar plates incubated at 37^0C for 24 hours. A loopful of the culture was inoculated on to Nutrient agar, Mac conkey agar medium and incubated at 37^0C for 24 hours. After incubation, the colony morphology of different strains was observed. Gram staining, motility, biochemical tests, catalase and oxidase was performed for the obtained isolates.

B. **Screening of *Enterobacter sp***

For the selective isolation of *Enterobacter* species from the obtained isolates the following tests were performed. A loopful of the culture of selected isolates was inoculated on to Eosin methylene blue agar, Tergitol-7 medium (HIMEDIA M850) and incubated at 37^0C for 24-48 hours. After incubation, the colony morphology was observed. Carbohydrate fermentation test for arabinose, raffinose, xylose was performed. Amino acid decarboxylation test with amino acids such as Phenylalanine, arginine, ornithine was tested.

C. **Screening of Acrylamide- degrading bacteria**

Acrylamide monomer in the medium serves as the sole carbon and nitrogen source for the bacterial growth. Acrylamide utilization by the obtained isolates in minimal media with Dipotassium hydrogen phosphate-0.7g, Potassium dihydrogen phosphate-0.2g, Tri-sodium citrate-0.05g, Magnesium sulphate-0.01g, Agar-1.8g supplemented with 1.0g acrylamide in 100ml distilled water and the pH was maintained at 7.0 respectively (Prabu, C.S *et al*., 2007). 1% acrylamide media was prepared and the bacterial isolates were inoculated and incubated at 37^0C for 24-48 hours. The plates were examined for visible growth of the isolates.

D. **Bacterial Identification by 16SrRNA Sequencing**

Bacterium identification was performed by InstaGene Matrix (Bio-Rad, USA, perform 35 amplification cycles at 94℃ for 45s, 55℃ for 60s, and 72℃ for 60s. A positive control (*E. coli* genomic DNA) and a negative control in the PCR are included. Purification of PCR products by using Montage PCR Clean up kit (Millipore). Sequencing were performed by using Big Dye terminator cycle sequencing kit. Sequencing products were resolved on an Applied Biosystems model automated DNA sequencing system (Altschul, S.F *et al*., 1990). The culture sequence obtained were subjected to BLAST analysis, the phylogenetically similar type strains sequence and other phylogenetic related sequence were selected from the GenBank and they were subjected to multiple sequence alignment and were subjected to phylogenetic tree (neighbour joining) construction using MEGA 6(Tamura, K *et al*., 2013; Kumar, S *et al*., 2018).

E. **Influence of growth parameters in biodegradation of acrylamide**

The efficiency of the selected isolate in degrading acrylamide monomer was tested in minimal media with varying concentrations of acrylamide 0.5%, 1%, 2%, 3%, 4% respectively with pH maintained at 7.0 inoculated with the selected bacterial strains and incubated at 37^0C and growth was determined by spectrophotometer at regular intervals. The degrading acrylamide with a minimum concentration of 0.5% was studied with varying growth parameters pH 5,6,7,8 and temperature 25^0C, 37^0C and 45^0C in triplicates.

F. **Influence of heavy metals in biodegradation of acrylamide**

Minimal medium broth with 0.5% acrylamide was introduced with varying concentrations of metals (0.5%, 1%, and 1.5%) as copper sulphate (Cu), cobalt chloride (Co), mercury chloride (Hg), zinc sulphate (Zn) and potassium chromate (Cr). The tubes were inoculated with selected bacterial strains and incubated at 37^0C and the growth was determined by spectrophotometer at regular intervals.

G. **Degradation of specific amides by select strains**

The potency of the strain in degrading other amides was also detected by substituting N, N-methylene bisacrylamide and sodium azide instead of acrylamide in the medium. The amides were added in varying concentrations such as 0.5%, 1%, 2% respectively in the minimal medium and observed for degradation.

H. **Gas Chromatography- Mass Spectrometry**

Biodegradation of acrylamide was monitored by gas chromatography. An Agilent 6890 gas chromatograph with a 15m Alltech EC-5column (250μ I.D., 0.25μ film thickness). The split ratio was set to 10:1 with temperature programmed at 35ºC, hold for 2 minutes, then ramp at 20ºC per minute to 300ºC and hold for 5 minutes. The helium carrier gas was set to 2 ml/minute flow rate (constant flow mode). A JEOL GC mate II benchtop double-focusing magnetic sector mass spectrometer operating in electron ionization (EI) mode with TSS-20001software was used for all analyses. Identification of the components was matched with their recorded spectra with the data bank mass spectra of NIST library V 11.

III.   RESULTS AND DISCUSSION

Acrylamide degrading bacteria were isolated industrial waste water by standard spread plate method and twenty isolates were obtained after morphological and biochemical identification.

A. **Isolation and Identification of acrylamide degrading bacteria**

They were tested for the primary ability in acrylamide degradation with 1% acrylamide agar plates. Among them, eight isolates showed growth, five isolates of *Pseudomonas* species and three isolates of *Enterobacter* species. Since *Pseudomonas* sp is very much known for its degradative property on various toxic substances, further study was carried with *Enterobacter* sp. Morphological identification revealed Gram-negative rod-shaped bacteria, motile, catalase positive. Biochemical identification indicated positive for Voges Proskauer, Simmon's citrate, arginine dihydrolase, ornithine decarboxylase and nitrate and negative results for urease, cytochrome-oxidase, lysine decarboxylase, indole, methyl red and H_2S. Acid and gas

produced in carbohydrate fermentation test from glucose, maltose, sucrose, mannitol arabinose, xylose and raffinose.

B. Sequencing Analysis

16SrRNA gene sequencing of the isolate of *Enterobacter sp* with the accession number: KX621310.1 is identified as *Enterobacter cloacae subsp. dissolvens* strain TJ1812. 16SrRNA gene was consistently 99% similar to that for *Enterobacter cloacae* including *Enterobacter cloacae strain VITPASJ1*(accession no. KU598849.1), *Enterobacter cloacae* strain AJ1(accession no. KJ872526.1), *Enterobacter sp*. BAB-3164(accession no. KF984462.1), *Enterobacter sp*. BAB-3144(accession no. KF984443.1), *Enterobacter sp*. BAB-3135(accession no. KF984435.1) and *Enterobacter cloacae* strain R1(accession no. MK064178.1). Phylogenetic tree was constructed by neighbour-joining method showed close relation among above mentioned strains supporting the similarities in Fig. 1. Acrylamide biodegradation in wastewater by *Enterobacter aerogenes* was reported in Thailand (Buranasilp, K *et al*., 2011).
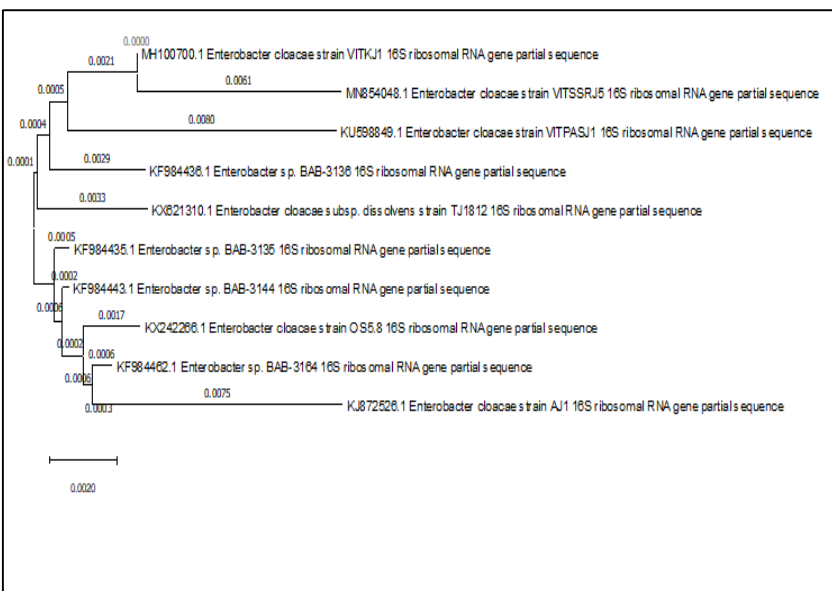

Fig. 1 A Phylogenetic tree using neighbour-joining method

C. Growth specific characterization of *Enterobacter cloacae subsp. dissolvens strain TJ1812*

Acrylamide biodegradation by *Enterobacter cloacae subsp. dissolvens* strain TJ1812 was evident in minimal medium with 0.5% and 1% acrylamide, however there is steady decline in the growth with an increase upto 4% as shown in Fig. 2. However, the efficient degradation was reported at concentrations 1-2% as reported (Prabu, C.S *et al*., 2007; Guezennec, A.G *et al*., 2015). The highest concentration of acrylamide degradation is at 4% in the current study. Polyacrylamide degradation was reported at 50-1000mg/L by two bacterial strains HWBI and HWBII isolated from activated sludge and soil in an oil field (Wen, Q *et al*., 2010; Bao, M *et al*., 2009; Muslim, S.N *et al*., 2018). Joshi, S.J., &

Abed, R. M. (2017) reported 16-91% degradation of polyacrylamide by different aerobic and anaerobic bacteria with an emphasis on yeast to degrade at 60-80%. It was also reported in sulphur-reducing bacteria and mixed bacterial population (Nyyssölä, A., & Ahlgren, J. (2019).

The growth of the bacterium started at pH 5 and gradually increased till 7 and then decreased to 8 (Lakshmikandan, M *et al*., 2014). Similar reports were recorded with *Enterobacter aerogenes* with maximum at pH 9 because of low stability of plasma membrane, inhibition of enzymes and transport proteins in the membrane as shown in the Fig.3.

Shukor, M.Y *et al.,* 2009 reported the optimum incubation temperature recorded at 37ºC and a steady drop observed at temperature 25ºC and 45ºC, depicting the mesophilic characteristics as shown in the Fig. 4. (Jebasingh, S.E.J *et al*., 2013).
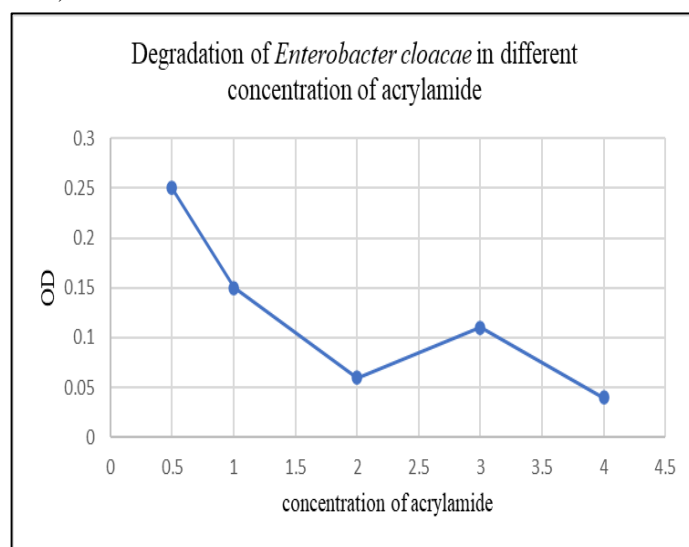

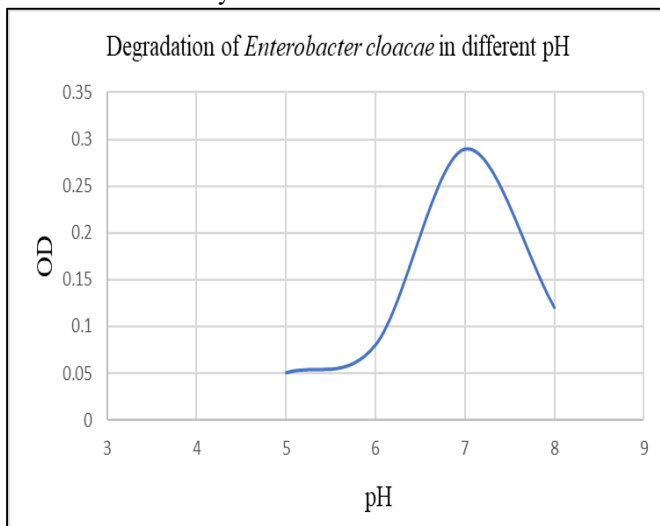Fig. 2. Degradation of *Enterobacter cloacae* in different concentration of acrylamide

Fig. 3. Degradation of *Enterobacter cloacae* in different pH

Acrylamide degradation was catalysed by enzyme amidase and there are several studies that supported the production of amidase and its influence in the degradation. However, the present study demonstrated its influence with a concentration

of 0.5% to 1.5% in the process of degradation (Jangkorn, S *et al*., 2018). The influence of copper completely inhibited the acrylamide degradation, chromium, zinc and cobalt lowered the process, whereas mercury never showed any signs of inhibition of acrylamide degradation as shown in the Fig. 5. Nawaz, M.S *et al* 1993 investigated heavy metal influence such as copper, mercury, nickel and cobalt inhibited amidase activity whereas iron and chromium enhanced its activity significantly.
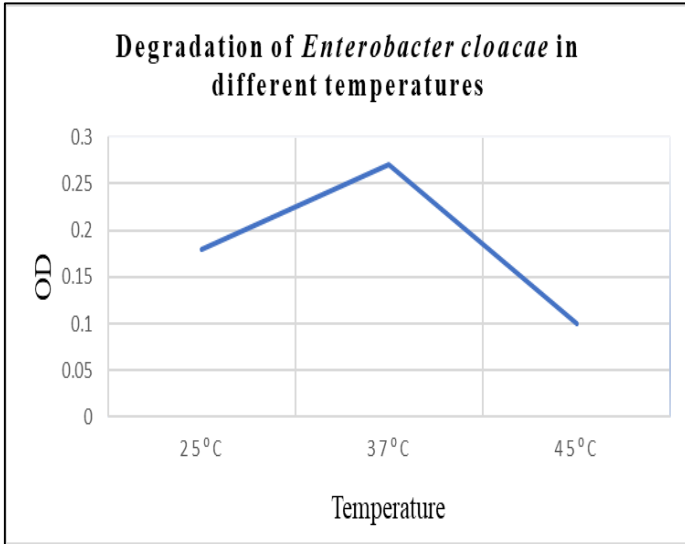

Fig.4. Degradation of *Enterobacter cloacae* in different temperatures
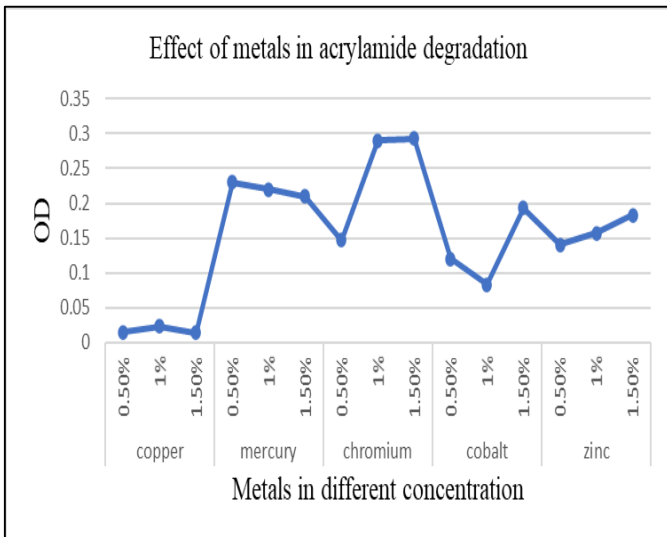

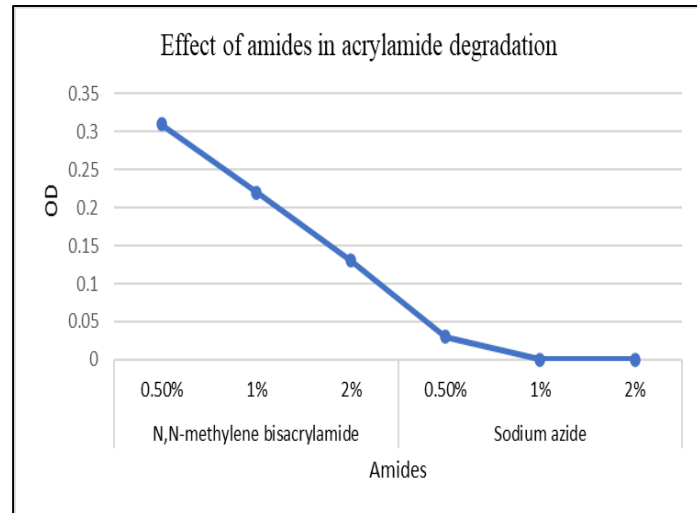Fig. 5. Effect of metals in acrylamide degradation

Fig. 6. Effect of amides in acrylamide degradation

Complete degradation was reported in butyramide and urea, where as partial degradation was observed in the presence of acetamide, benzamide, formamide and no degradation in iodoacetamide, N, N-methylene bisacrylamide, sodium azide and thioacetamide (Nawaz, M. S *et al*., 1994; Yu, F *et al*., 2015; Singh, R. (2014); Bocian, A *et al*., 2017). There was partial degradation in the presence of N, N-methylene bisacrylamide and much lesser degradation in sodium azide in this study as shown in the Fig. 6. Thanyacharoen, U *et al*., 2012 reported moderate level degradation of formamide, butyramide, lactamide and also urea, 34% degradation of sodium azide by different bacterial strains.
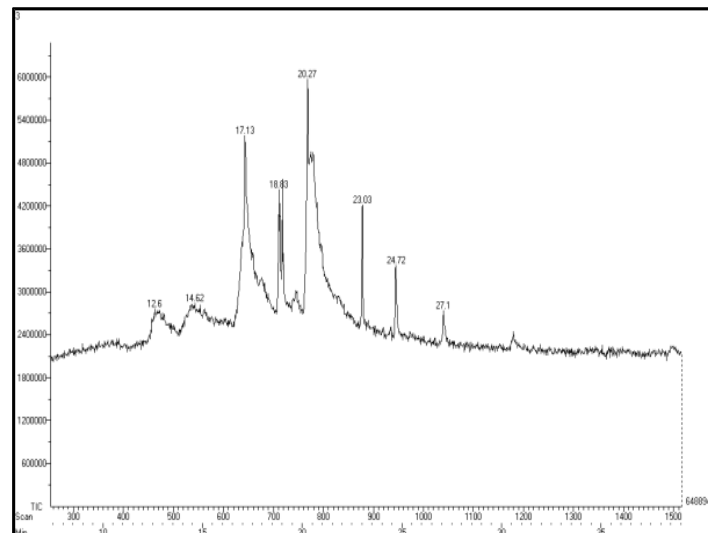

Fig. 7. Results of Gas chromatography- Mass spectrometry

The degradation of acrylamide was confirmed by HPLC (Chong, Y.M *et al*., 2017), but in this case it is concluded by GCMS. The oleic acid present in the table is an indicative of acrylic acid production, it is denoted as oleic acid due to the reaction of ethyl acetate which is used as a solvent for the extraction of cell culture supernatant prior analyzing in Gas chromatography- Mass spectrometry (Bedade, D.K.. *et al*., 2018; Nyyssölä, A., & Ahlgren, J. (2019).

Table -1 GCMS – Peak Report

Peak Report TIC

Peak No.	RT (Min.)	Compound Name	Peak Area	Peak Area (%)
1	12.6	Benzene, 1,4-bis[1-methylethyl]-	2765120	8.85
2	14.62	2[1H]-Naphthalenone,octahydro-4,4a-dimethyl-,[4a,4aa,8aa]-	2843824	9.10
3	17.13	Cyclopentaneundecanoic acid, methyl ester	5184592	16.59
4	18.83	Oleic Acid	4431952	14.18
5	20.27	Isopropyl stearate	5711136	18.27
6	23.03	Dibenzofurane-1,3-dicarbonitrile,2-amino-7-methoxy-4-[4-pyridyl]-	4215936	13.49
7	24.72	Estra-1,3,4[10],6-tetraene-3,17-diol,diacetate, [17a]-	3366224	10.77
8	27.1	6,9,12-Octadecatrienoic acid, phenylmethyl ester, [Z,Z,Z]-	2740608	8.77
		Total	31259392	100.00

The ethyl acetate which is ester of ethanol and acetic acid on combination with acrylic acid in the medium leads to esterification reaction resulting in the formation of oleic acid with the retention time of 18.83 minutes (Shukor, M. Y. (2018) as shown in the Fig. 7. The fourth peak with a retention time of 18.83 minutes as reported in the Table. 1 denotes oleic acid as a result of esterification reaction between ethanol of ethyl acetate and acrylic acid residues. Shanker R. *et al*., 1990 reported acrylamide degradation to acrylic acid and ammonia by *Pseudomonas sp* isolated from tropical garden soil. *Pseudomonas aeruginosa* BAC – 6 showed highest degradation of acrylamide at 500mg/L when supplemented with mannitol as carbon source (Chandrashekar, V *et al*., 2014). However, the *Pseudomonas sp* isolated in this study was not proceeded further with degradation studies. The acrylic acid produced by the degradation of acrylamide could be further degraded by microorganism using it as a carbon and energy source and proving that it is biodegradable organic compound (Madmanang, R *et al*., 2018)

IV.   CONCLUSION

Enterobacter cloacae subsp. dissolvens strain TJ1812 is an acrylamide degrading bacterium isolated from industrial waste water. Biodegradation property was analysed in this experimental study in different physical and influential parameters; hence this potential strain could be used as an important strategy to clean up toxic acrylamide contaminated sites. Thus, it is an efficient approach and could be implemented in much cost-effective way in restoration of ecological niche.

V.   ACKNOWLEDGEMENT

VI.   REFERENCE

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