IMPROVED BIOLOGICAL TREATMENT EFFICIENCY OF PULP MILL WASTE WITH CO-FEEDING STRATEGY ALONG WITH POLYHYDROXYBUTYRATE CO-PRODUCTION

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By

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CANDIDATE'S DECLARATION

I hereby declare that the work presented in dissertation entitled "**IMPROVED BIOLOGICAL TREATMENT EFFICIENCY OF PULP MILL WASTE WITH CO-FEEDING STRATEGY ALONG WITH POLYHYDROXYBUTYRATE CO-PRODUCTION**" submitted in partial fulfilment of the requirements for the award of degree of Master of Technology in Bioprocess Engineering, Indian Institute of Technology Roorkee, is an authentic record of my work carried out under the supervision of Dr. Bijan Choudhury, Associate Professor, Department of Biotechnology, IIT Roorkee. The matter embodied in this has not been submitted by me for the award of any other degree.

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CERTIFICATE

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

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ABSTRACT

This study is focused on the use of pulp process waste as the medium for growth of *Halomonas sp.* MCC 2171. It was found that halophile is efficiently able to grow in the press filtrate that was produced during the dewatering stage of pulp. Initial characterization of pulp process waste shows that, it can be used as the suitable growth medium, without any further treatment. However, to enhance the reduction of organic content of the waste, co-feeding strategy was employed. Glycerol, a major by-product of the biodiesel industries was used as the co-substrate. Addition of glycerol not only increased the reduction of Biological Oxygen Demand and Chemical Oxygen Demand but also resulted in production of polyhydroxybutyrate (PHB). Further study was performed by addition of black liquor and crude glycerol in the press filtrate medium. Also assimilations of carbohydrate and lignin degradation products during growth of *Halomonas sp.* was observed in press filtrate medium. Thus using this strategy, it is possible to detoxify the press filtrate and conversion of some organic carbon compounds of pulp process waste into PHB and biomass. Treated wastewater can be recycled to the pulp washing stage which can improve the washing efficiency of pulp.

Keywords: *Halomonas sp.*, Pulp Mill Effluent, Black Liquor, Crude Glycerol

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ABBREVIATIONS AND SYMBOL

BOD	Biological Oxygen Demand
BL	Black Liquor
COD	Chemical Oxygen Demand
FTIR	Fourier Transform Infrared Spectroscopy
GC-MS	Gas Chromatography Mass Spectroscopy
min	Minute
PF	Press Filtrate
PHB	Polyhydroxybutyrate
TGA	Thermogravimetric Analysis
TIC	Total Ion Chromatograph
TOC	Total Organic Carbon
TSS	Total Suspended Solid
%	Percentage
°C	Degree Celsius
mg	Milligram
w/v	Weight by Volume
v/v	Volume by Volume

1. INTRODUCTION

1.1. Background

Paper and pulp industry is considered as a large producer and user of biomass based materials and energy (Svensson & Berntsson, 2014). The crucial step towards the pulp production is removal of lignin form the cellulose. Alkaline extraction (use of sodium hydroxide and sodium sulfite) process also known as kraft pulping generally employed for extraction of lignin and produce considerable amount of wastewater from pulp and paper mills (Yang et al., 2008). In general, 60 m^3 of water utilized for production of a ton of paper (Nemerow & Dasgupta, 1991). The alkaline wastewater generally characterized with pH range form 11 – 13 and more than 100000 mg/L of chemical oxygen demand (COD) (Huang et al., 2007). It is known as black liquor because of presence of chromophores of lignin and its derivatives (Yang et al., 2010).

Black liquor produced from the mills are generally associated with huge number of problems (Sumathi & Hung, 2006). Such as, dark brown color of black liquor causes the reduction of light penetration, if entered in water bodies, hence affecting the aquatic habitat. The problems associated with black liquor are:

- Effluents of pulp mill causes the water bodies discoloration.
- Large content of the organic matter, cause the increase in Biological Oxygen Demand, hence results in depletion of dissolved oxygen in the ecosystems.
- Persistent, toxic pollutants and bio-accumulative.
- Organic halide can be absorbed in the receiving ecosystems.
- Transport of organic halides to long distance for example, chloroguaiacols resulting in contamination of remote seas and lakes.
- Cross-media pollutant transfers through volatilization of compounds and absorption of chlorinated organics to wastewater particulates and sludge.

Black liquor is generally utilized for either lignin recovery or used as an energy source in recovery boiler due to high content of lignin. However**,** in the process, sugar and lignin degradation products like alpha hydroxyl organic acid, organic acids which constitutes around 30% are not utilized efficiently as these products have less calorific value in comparison to

lignin. Various techniques for treatment of black liquor have been anticipated such as chemical coagulation (Ganjidoust et al., 1997), alkali recycling (Dafinov et al., 2005), adsorption of organic pollutants (Zhang & Chuang, 2001) and acidification (Dafinov et al., 2005). Alkaline recovery process and acid precipitation are the best-known available methods for alkali recovery and lignin removal respectively (Yang et al., 2008). However, high operation cost, production of secondary pollutants such as chlorine and sulphur, and huge consumption of mineral acid make the process not economical (Xiong et al., 2007).

Utilization of microorganism is another approach for wastewater treatment. Various microorganism such as white rot fungi have been reported to remove lignin form wastewater (Font et al., 2003; Wu et al., 2005), *Acinetobacter calcoaceticus* and *Aeromonas formicans* has also been reported for treatment of black liquor (Gupta et al., 2001; Jain et al., 1997). However, these microorganisms were operated under pH less than 9.0 and COD less than 10000 mg/L. Hence, the insufficient tolerance to high COD and pH made these microorganism industrially impractical (Yang et al., 2008). Along with high COD and pH values, black liquor is also rich in alkalinity. In a word, during kraft pulping process use of alkali for lignin removal makes the wastewater high in alkalinity. Hence leaving the wastewater unsuitable for microbial growth. In an alternative strategy, black liquor after suitable dilution can be used as fermentation substrate with a drawback of significant increase in water consumption and simultaneous increase in waste volume. One possibility of recovery of sugar degradation products is to use press filtrate which is generated from dewatering stage of washed pulp. This press filtrate is the diluted form of black liquor containing all the components at much lower concentration. Press filtrate is generally recycled back to washing of pulp in a counter current washer which finally ends up as a black liquor. Thus this press filtrate can be an attractive fermentation substrate in spite of presence of lower concentrations of sugar degradations products as it doesn't need any pre-treatment. Further treated black liquor with lower BOD and COD can enhance the washing efficiency of pulp. In other words, it can reduce the consumption of fresh portable water.

Number of halophiles, alkaliphiles have been isolated form extreme hypersaline and alkaline environments (Horikoshi, 2004; Oren, 2002). It is well known fact that these microorganisms have high potential for removal of toxic components form the extreme

environment (Yang et al., 2010). Halophiles and alkaliphiles are reported in reduction of dye colors (Asad et al., 2007), organic compounds degradation (Alva & Peyton, 2003; Bhatt et al., 2005) and in treatment of wastewater (Woolard & Irvine, 1995). Yang et al, isolated two halophilic strains i.e. *Halomonas sp*. Y2 and *Halomonas sp.* 19-A form alkaline black liquor and reported to degrade organic compounds as well as production of alkali-tolerant hydrolytic enzyme (Yang et al., 2010). The alkaline consortium was constructed and reported to reduce (27.4%) COD, pH and (35.4%) color of wheat straw black liquor (Yang et al., 2008).

In this study, *Halomonas sp.* MCC 2171 was investigated as a potential candidate for reduction of organic load of pulp and paper mill wastewater.

Scope of The Study

This work is focused on using pulp and paper mill waste as a medium for growth of *Halomonas sp.* MCC 2171. Press filtrate, a diluted form of black liquor and mixture of press filtrate and black liquor was also used along with crude glycerol was used to determine potential for organic load reduction, secondary metabolite production by the *Halomonas sp.* The results show that *Halomonas sp.* MCC2171 is a potential candidate for organic load reduction in pulp and paper mill waste. Co-feeding with glycerol doesn't only improves the PHB production but also enhances the COD and BOD reductions of the waste and also found to involve in assimilation of degradation products of carbohydrate and lignin.

1.2. Objectives

- To use the pulp mill waste as a medium of growth for *Halomonas sp.* MCC 2171.
- To check the organic load reduction capability of microorganism during growth in pulp mill waste.
- To develop the strategy of using other carbon substrate for enhancement of waste treatment efficiency.
- To check the potential of production of secondary metabolite in the developed process.
- Identification of assimilated carbohydrate and lignin degradation compounds in press filtrate due to growth of *Halomonas sp.*

2. LITERATURE REVIEW

Industrial sector plays an impressive role in raising the living standard and shaping the economy of a nation. Paper and pulp mills are one of the largest global industries with high capital investments involved in production of paper on machines 10 m wide at speeds in excess of 2000 m per minute. Today, the making of tissues, paperboards and papers is highly cost-sensitive. The requirement of enhancement of quality and productivity, along with environmental regulatory pressures, has resulted in an enhanced demand of chemical additives in paper and pulp mills. Generally, 98% natural material founds in paper (Bajpai, 2015). Paper and pulp mills are the largest consumers of fresh water, chemicals and pulp biomass during the process of paper manufacturing. The history of paper and pulp industry in India dates back to the year 1812. Presently, the number of pulp mills operating in India are 726 (Sharma et al., 2016). Pulp & Paper mills is now not only limited to production of paper only but also extended its work form paper production to biofuels (Jönsson et al., 2011), carbon fiber (Maradur et al., 2012), bioethanol production & electricity generation (Phillips et al., 2013). However, along with their industrial expansion, the environment and human health are also deteriorated because of increased toxic waste production (Murillo-Luna et al., 2011).

The degree of toxicity and pollution depends upon the bleaching methods, pulping methods and raw material used by the paper and pulp mills. The pollution load from softwood is higher than hardwood. On the other hand, silica content is generally higher in the spent liquor generated from pulping. Wastewater volumes discharged varies significantly depending on the size of the mill, manufacturing process and the raw material utilized. Thus, the changeability of characteristics of effluents and volume from one mill to another highlights the requirement for a variety of treatment technologies and pollution prevention, tailored for a specific mill (Bajpai, 2013).

2.1. Pulp and Paper Making Process

For the lignin removal from the wood chips, the kraft pulping technique is the most commonly used process in the pulp mills (Figure 1) (Chakar & Ragauskas, 2004). The brief process description of the paper making processes is described below:

2.1.1. Raw material preparation

The woody material, taken as a raw material are initially debarked and chipped into smaller pieces and then fed into the digester for further pulping process. If the lignocellulose feedstock consists of recycled fiber and agro residues, then the chipping and debarking process are not required.

2.1.2. Pulping Process

The process of pulping is based on breaking of wood fibers and unwanted residues removal from them. In kraft pulping method, the wood chips were heated at 180 °C for 2 h in presence of sodium sulfide and sodium hydroxide under pH 10 – 14 (Zakzeski et al., 2010). Hydrosulfide and hydroxide break the aryl-alkyl bonds of lignin resulting in dissolution of lignin in liquor and turning it in black color (Chakar & Ragauskas, 2004; Chandra et al., 2008).

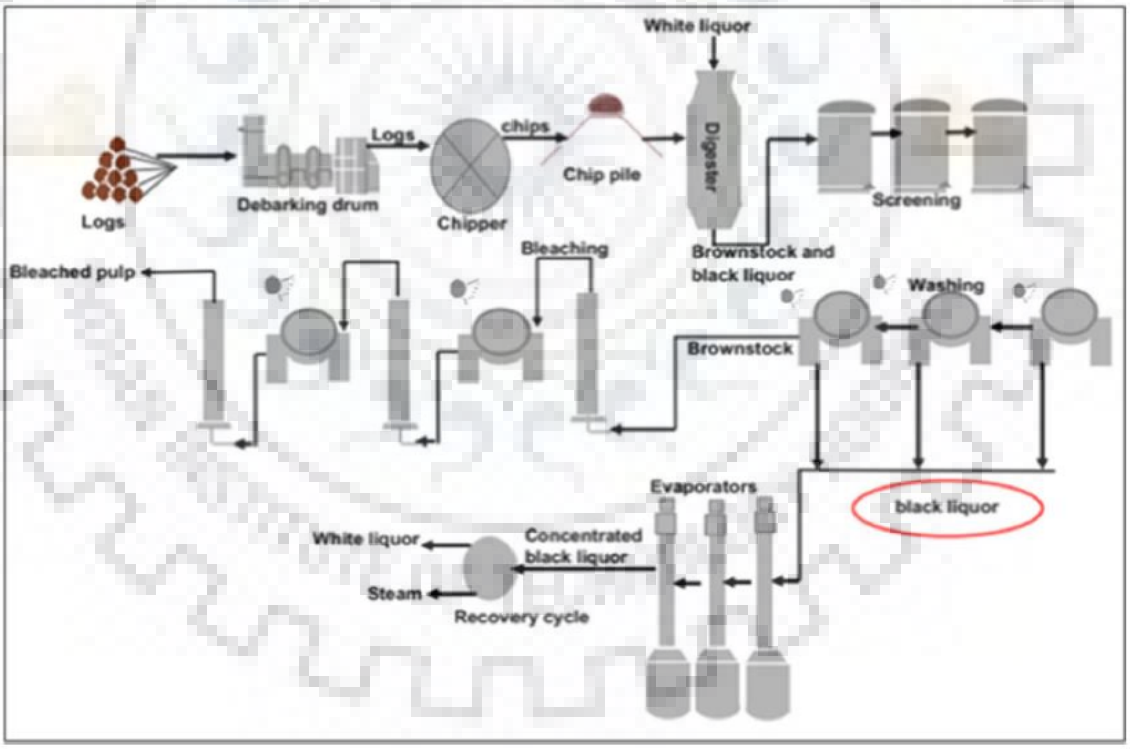

Figure 1 . An illustration of the kraft pulping process in the paper and pulp industries (Mathews et al., 2014).

Chemical recovery is an essential part of the pulp production process (Bajpai, 2008; Biermann, 1996). Chemical pulp fiber generally utilizes the half of the wood raw material. The rest half of wood raw material is utilized as fuel for heat and electricity generation. In fact, a pulp mill has two important lines. Wood is converted into pulp on the fiber line. Energy is generated in the chemical recovery line using the wood material heated in the liquor; the cooking chemicals are recovered for reuse. In the chemical recovery line, black liquor is evaporated and combusted in a recovery boiler, and the energy content of the dissolved wood material is recovered as steam and electricity. The process of chemical pulping process produces more energy than it uses. A pulp mill are able to produce energy for its own use and for selling purpose (Bajpai, 2012).

Production of pulp is followed by removal of impurities form the pulp. Various methods are available for the reduction of impurities such as defibering, screening and deknotting. Washing of pulp is done using brown stock washing using residual cooking liquor. Efficient washing is important to decrease the carryover of cooking liquor into the bleach plant and to increase the return of cooking liquor

2.1.3. Bleaching of Pulp

For the enhancement of pulp brightness, the bleaching process are employed to removes the residual lignin from the pulped fiber. For this, the alkali solutions and oxidizing agents for example ozone, hydrogen peroxide, chlorine are used.

2.1.4. Black Liquor

Black liquor is a dark-colored alkaline waste stream containing inorganic chemicals and wood residues (Chandra et al., 2011). During the brown stock washing stage, the pulp is thoroughly washed to remove the residual liquor. The wastewater collected is also known as press filtrate (PF) which finally ended up by mixing in black liquor for recovery process. The current method in practice is the black liquor incineration in the recovery boiler to produce steam for thermal energy and chemicals recovery (Mathews et al., 2014; Singhal & Thakur, 2009). However, burning of black liquor results in the generation of numerous toxic volatile organic compounds (Singhal & Thakur, 2009). Moreover, the amount of black liquor created by pulping can surpass the quantity of black liquor that the recovery boiler can efficiently process (Mathews et al., 2014). Disposing of the black liquor as effluent without any treatment,

will severely affects the aquatic life of flora and fauna and will also responsible for the persistent high concentration organic pollutants (POPs) (Kortekaas et al., 1998; Wang et al., 2013).

2.1.5. Paper Making Process

Dry pulp is mixed with water to form a slurry using pulpers. Paper refining is one the crucial step in paper making process (Baker, 2000). Paper is then sent for the beating purpose in the Hollander beater. After the beating of pulp to certain extent, it is then sent to the refining process. Refining can be done in series or in parallel. Refining is done on the bases of requirement of type of paper fiber. The main aim of the refining is to improve the bonding of fibers, reducing the drainage resistance and minimizing the damaging of fibers hence, does mot reduces the strength of individual fibers.

At the end of paper making process, paper sheets are sent for their winding on the specific roll diameter. The paper making process is complete for the production of corrugated paperboard. Final finishing and converting of paper done, generally off line form the paper machine. The operations such as coating, winding, and calendaring occurs in last step of paper making process.

2.2. Sources of Pollution

The process of pulping generally associated with utilization of huge amount of water, which ends up with production of large amount of effluent. Different stages in pulp process such as preparation of wood, pulping, washing of pulp, screening, bleaching of pulp, and paper drying are associated with generation of polluted wastewater. Among all the stages, pulping process generates highly polluted wastewater because of alkali chemicals, dissolution of lignin resulting in high chemical oxygen demand, high pH and dark color wastewater. This wastewater consists of soluble wood materials and wood debris. Bleaching process using for whitening the pulp with the help of chlorine, hence generates toxic substances in form of chlorine compounds. Variety of plants are generally employed for pulp fires production such as bamboos, wood, reeds and canes, grasses and straw. Wood is best source of pulp fiber. It contains carbohydrate, extractives and lignin, and generally washed away in the effluent during screening, washing and dewatering processes. Different pulping process associated with generation of toxic compounds such as juvaniones, unsaturated fatty acids, resin acids and

chlorinated resin acids. The pollutant production during different stages are shown in Figure 2.

2.3. Impact on Environment

It was estimated that the demand of paper has crosses the 400 million tons of biomass feedstock yearly in India (Singh et al., 2011). Pulp and paper are now not only producing paper but also involved in production of other components such as biofuels (Jönsson et al., 2011), carbon fiber (Maradur et al., 2012), and ethanol (Fornell et al., 2012). Hence, this industry has emerged as profitable and necessary sector in the world. However, this industry is also associated with production of highly polluted wastewater. If discharges in water bodies, it may cause death to the aquatic animals due to its dark color and toxic components (Saraswathi & Saseetharan, 2010). Colored effluent results in the following detrimental effect upon the receiving water bodies:

- Inhibitory compounds in form of color that are presence because of various lignin derivatives.
- Color recreational value and decreases the visual appeal of water.
- Color substances can form complex compounds such as tar residues when reacted with metal ions for example copper, iron etc.
- BOD of wastewater increases because of chromophores and that cannot be measured using 5-day BOD method.
- Color also affects the downstream municipal and the industrial water uses, and also increases the cost and difficulty of wastewater pre-treatment.
- Color reduces the transmission of sunlight hence, reducing the aquatic life productivity by interfering with photosynthesis process.

Figure 2. Illustration of Stages of the pollution in paper and pulp mill (USEPA, 1995).

It was studied that kraft lignin and the derivatives of phenolic groups are the major environmental pollutants that constitutes the black liquor (Hooda et al., 2015; Raj et al., 2014). Apart from the naturally occurring polymers (lignin, tannins, etc.) paper and pulp mill effluent also contains xenobiotic components (chlorinated resin acids, chlorolignins, chlorinated, polyaromatic and phenols hydrocarbons) that get employed or formed while carrying out the pulping processes and paper making process (Chandra & Singh, 2012).

2.4. Conventional Methods for Pulp Mill Waste Treatment

Many physical, electrochemical, and chemical measures including ozonation (Balcıoğlu et al., 2007), ultrafiltration (Puro et al., 2010), electrocoagulation (Uğurlu et al., 2008) and pretreatment technologies are present like ion exchange (Ciputra et al., 2010), chemical treatment (Babuponnusami & Muthukumar, 2012), and membrane processes (Chanworrawoot & Hunsom, 2012) for the contaminants mitigation from pulp and paper mill effluent. Sometimes these methods are also used in combination with each other for better treatment of pulp mill effluent (Rohella et al., 1996; Shawwa et al., 2001). However, when implemented practically, these techniques are suffered from various drawbacks such as energy intensive, high sludge generation, expensive capital investment, and secondary byproducts formations which further requires the treatment (Yang et al., 2008). Various techniques for treatment of black liquor have been anticipated such as chemical coagulation (Ganjidoust et al., 1997), alkali recycling (Dafinov et al., 2005), adsorption of organic pollutants (Zhang & Chuang, 2001) and acidification (Dafinov et al., 2005). Alkaline recovery process and acid precipitation are the best-known available methods for alkali recovery and lignin removal respectively (Yang et al., 2008). The released black liquor form pulp and paper mills are generally treated by ozonation, electrocoagulation, coagulants and adsorbents (Figure 3). However, these methods are having various loopholes, generation of inhibitors, and operational conditions severity sometimes degrade the cellulose moiety, which are essential for biomass saccharification (Potumarthi et al., 2013). All the anomalies convert these techniques into environmentally hazardous and economically unfeasible. Efficient strategies for delignification are still need to improve further, to avoid cellulose loose and to increase environmental friendly treatment methods.

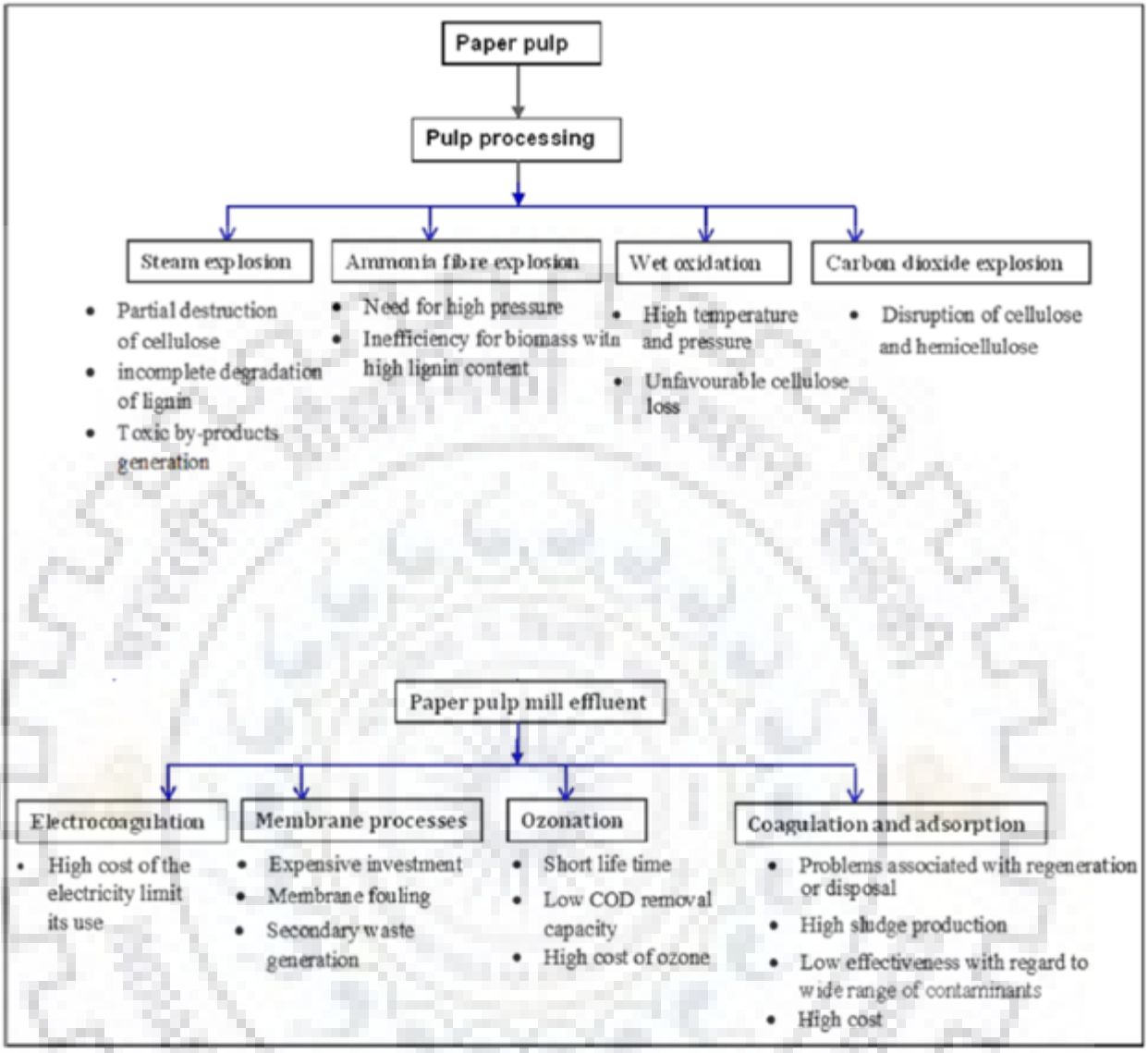

Figure 3. Diagram showing the anomalies in conventional methods for pulp mill waste processing and treatment.

2.5. Biotechnological Approach

Biotechnological approaches shown the new route for the abatement of pulp mill contaminants in an environmental friendly way. Use of this approach is also proven to be cost effective. It produces less toxic compounds, requires less amount of water, economical feasible, uses less energy for wastewater treatment then other physical or chemical methods (Banat et al., 1997; Rai et al., 2005). The biodegradability of components depends upon the

type of microbes, its adaptability in extreme conditions, metabolic activity. Naturally, bacteria and fungi degrades the toxic compounds into smaller products and reducing the stress on environment. Use of microbe for degradation of toxic components are known as bioremediation. It provided the various route for degradation, detoxification and decolorization of recalcitrant contaminants form pulp mill wastewater.

Most of the bio-remedial studies were done on brown rot and white rot fungi. They have shown the promising results in the field of bioremediation (D'Souza et al., 2006; Minussi et al., 2007). *Rhizopus oryzae* and *Pleurotus sajor caju* were reported to reduce the color about 72−74 % at 465 nm and the 25 – 46% relative absorbance at 250 nm, and ~81 % COD of bleached effluent after incubation for 10 days (Freitas et al., 2009). It was studied that *Emericella nidulans* var. *nidulans* was able to reduce 66.66% of color and 37 % lignin of the pulp mill effluents after employing the optimized process parameters of pH (5), rpm (125), inoculum size (7.5 %), temperature (30−35 °C), duration (24 h) including co-feeding of carbon sources for example tryptone (0.1 %) and dextrose (0.25 %) (Singhal & Thakur, 2009). However, intolerability of pH, substrate penetration by mycelium, necessity of carbon sources addition, necessity of long retention time hinders the commercial applicability of fungi (Banat et al., 1997; Swamy & Ramsay, 1999). In fact, because of high alkalinity and dark color, the pulp mill waste is deficient in oxygen and high in pH making fungal system unsuitable to grow under industrial conditions. Also, the plant lines may get choke because of fungal mycelia causing the bio-sludge formation, which will further increase the treatment expenses. Hence, scale-up of fungal system is difficult. Therefore, there should be other system, which can survive in extreme environment conditions and able to tolerate a wide range of pH.

Many bacterial species have been studied having a potential for pulp process wastewater treatment. *Paenibacillus sp*. strain LD1 was isolated (laccase-producing bacterium) and able to effectively reduce the parameters (such as 78 % of COD, 86 % of phenol, 54 % of lignin, color by 68 % and BOD 83 %) from the paper and pulp mill effluent when incubated for 144 h (Raj et al., 2014). In a similar study, Haq et al. (2016) reported that *Serratia liquefaciens* (LiP producer) significantly able to reduce the COD by 85 %, lignin by 58 %, color by 72 %, and total phenol by 95 % from the pulp process wastewater after the treatment for 144 h (Haq

et al., 2016). In these aforementioned work, the shift in pH towards acidic range was observed during initial phase of treatment owing to the acetate efflux along with other tricarboxylic acid (TCA) cycle intermediates (Yang et al., 2008). Other researchers developed a strategy by using syntrophic co-culture of *Klebsiella pneumonia* and *Bacillus subtilis* which demonstrated the collective impact on the reduction of color (82 %), BOD (85 %), COD (79 %), lignin (58 %), and other pollutants from pulp and paper mill wastewater (Yadav & Chandra, 2015). All these illustrated studies show the potential of microbes in bioremediation of waste products.

Number of halophiles, alkaliphiles have been isolated from extreme hypersaline and alkaline environments (Horikoshi, 2004; Oren, 2002). It is well known fact that these microorganisms have high potential for removal of toxic components form the extreme environment (Demirjian et al., 2001; Yang et al., 2010). Halophiles and alkaliphiles are reported in reduction of dye colors (Asad et al., 2007), organic compounds degradation (Alva & Peyton, 2003; Bhatt et al., 2005) and in treatment of wastewater (Woolard & Irvine, 1995). Yang et al, isolated *Halomonas sp*. Y2 and *Halomonas sp.* 19-A from alkaline black liquor and reported to degrade organic compounds as well as production of alkalitolerant hydrolytic enzyme (Yang et al., 2010). The alkaline consortium was constructed and reported to reduce (27.4%) COD, pH and (35.4%) color of wheat straw black liquor (Yang et al., 2008).

2.6. Problem Statement

In this study, the effort was made to use *Halomonas sp.* MCC 2171 as a potential candidate for reduction of organic load in pulp process waste. Strategy was developed to use glycerol as co-substrate to study its effect on growth as well as on polyhydroxybutyrate production. Further the degradation products of carbohydrate and lignin present in press filtrate were identified and their assimilation pattern after growth of *Halomonas sp*. MCC 2171 were determined.

3. MATERIAL AND METHODS

3.1. Microorganism and Culture Condition.

Halomonas sp. (MCC 2171) obtained from Microbial culture collection (MCC), Pune, Maharashtra (India) was used in this study. The strain was initially supplied in stab culture. Table 1 shows the concentration of components required for culturing the microorganism. The optimum temperature for its growth is 30 ^{0}C. The revived culture was stored at 4^0 C and maintained at an interval of 15 days.

Table 1. Media composition for stab culture.

Components	Concentration (g/L)
NaCl	10
Yeast Extract	2.0
Casein	10.0
Agar	15.0
pH	7.5

The components were mixed accordingly and then sterilized by autoclaving for 20 minutes at 120ºC at 15-psig pressure. The media poured aseptically in petri plates and left for solidification. When plates were ready, then streaking was performed and incubated at 30ºC for 24 hours.

3.2. Characterization of Pulp mill process waste.

Press filtrate (PF) and black liquor (BL) were collected from Star Papers Mills Ltd., Saharanpur, India and stored in the refrigerator till further use. The physical characterization of pulp press filtrate and black liquor was carried out with respect to BOD, COD, pH, TSS and elemental analysis. BOD, COD were determined using standard protocol given by APHA (Apha, 1985). These were calculated after achieving the stationary phase and compared with respect to the press filtrate's calculated value. Elemental analysis was done using Inductive Coupled Plasma Mass Spectroscopy (ICP-MS). It done to get the concentration of ions in the wastewater.

3.2.1. Chemical Oxygen Demand (COD) Protocol

Chemical Oxygen Demand was performed as per given by American Public Health Association (APHA) protocol (Apha, 1985). Initially, supernatant was collected from un-inoculated media and from inoculated media (after achieving stationary phase). Sample were diluted approximately, that its color should not be converted into green after adding all the required chemical. For COD measurement added 3.5 ml of diluted sample into the COD tubes and for blank used 3.5 ml of distilled water. Followed by addition of 0.05 g of $HgSO_4$ and 1.5 ml of Potassium Dichromate and mixed. Then, drop wise addition of 3.5 ml of Sulphuric acid reagent was done. Sulphuric acid reagent was prepared by addition of 1 g of $AgSO_4$ into the 1000 ml of H_2SO_4. Tube were then sealed with their caps and inverted several times and then kept in the pre heated COD digester at 150℃ for 2h. After reaction completion, cooled the sample at room temperature. After addition of 1or 2 drops of ferroine indicator, titrated it with (0.1N) Ferrous Ammonium Sulphate (FAS). The endpoint is blue-green to reddish-brown. Noted down the amount of FAS used for distilled water and for the samples.

Calculate the COD (mg/L) by putting the values in equation:

COD (mg/l) = (A- B) x N x 8000 / ml of sample

Where:

A = ml of FAS used for blank

B = ml of FAS used for sample

N = Normality of FAS

8000 = milli equivalent weight of oxygen x 1000 ml/L

3.2.2. Biological Oxygen Demand (BOD) Protocol

Biological Oxygen Demand was performed as per given by American Public Health Association (APHA) protocol (Apha, 1985). In this method, supernatant was collected from un-inoculated media and from inoculated media (after achieving stationary phase). Transferred the sample in the BOD bottles and make up to 300 ml using distilled water. Then, kept the bottle in incubator at 20℃ for 5 days. After 5 days of incubation, added 1 ml of $MnSO_4$ followed by addition of 1 ml of alkali-iodide-azide reagent. Closed the bottle with stopper and let the brow precipitate settle down. It indicates the presence of oxygen. If no brown precipitate

forms that is there was no oxygen present in the bottle. Now, added 1ml of H_2SO_4 and closed the bottle. Mixed it by inverting the bottle several times, till the precipitate dissolves again. Finally, added 2ml of starch and titrate with (0.025N) $Na_2S_2O_3$ solution.

Calculated the BOD (mg/L) as per the equation:

D_1 (mg/L) (un-inoculated sample) = (0.2 x 1000) x (0.025 N) ml of thiosulphate / 200

D_2 (mg/L) (inoculated sample) = (0.2 x 1000) x (0.025 N) ml of thiosulphate / 200

BOD = (D_1 - D_2) x 100 / (% dilution)

3.2.3. Total Suspended Solution (TSS) calculation.

TSS was performed as per the NREL protocol (Sluiter et al., 2008). First step is drying of crucible at 105℃ for 24h and after cooling in the desiccator, note down their weight. Then, 1.2 gram of pulp process waste was added to the crucible and weighed again. Same was done when sample was added to the crucible. Then keep the crucible at 105℃ for 24 hours. Take the crucible out from oven and cool it in desiccator. Record the change in weight and calculate TSS percentage.

Percentage of TSS was calculated as per the equation: $\% TSS = \frac{W3-W1}{W1-W2}$ x 100

Where:

W1 = Weight of empty and dried crucible.

W2 = Weight of sample and crucible.

W3 = Weight of dried sample in crucible.

3.2.4. Total Organic Carbon (TOC) Analysis.

TOC were analyzed to study the amount of carbon found in an organic compound and measure the wastewater quality. TOC was measured using Agilent Analyzer multi N/C 3100. Typically, it provides the Total Carbon (TC) and Total Inorganic Carbon (TIC). So, by subtracting the amount of inorganic carbon from the total carbon gives the Total Organic Carbon (TOC). The three main steps in TOC analysis.

1. Acidification: - Addition of acid and sparging of inert – gas allows carbonates and bicarbonates ions conversion into CO_2.
2. Oxidation: - In this step carbon in the remaining sample oxidized into carbon dioxide and other gases using high temperature combustion.
3. Detection: - Conductivity were used for detection of carbon content in the wastewater.

3.3. Crude Glycerol Preparation

It is also known as the biodiesel waste. It is produced by the transesterification reaction in biodiesel manufacturing process. Crude glycerol was prepared using the soya bean oil. 1.8 gram of Potassium hydroxide was mixed in the 33.5 ml of methanol and then added to the 120 ml soya bean oil (i.e. 3.6:1 (v/v) ratio of oil to methanol). Then mixture was mixed at room temperature for 4 to 5 hours. After mixing it is then left for phase separation in separating funnel. The upper layer was biodiesel and lower layer was the crude glycerol having, methanol, soaps and glycerol in it (Ruhal et al., 2011).

3.4. Pulp process waste medium condition

Press Filtrate medium (PF medium) was used for growth and compositions were as follows. Press filtrate 90%(v/v) and water phase 10%(v/v) containing yeast extract and glycerol and were added in such a way that final concentrations of yeast extract and glycerol in the medium were 2.5 g/l and 0-2%(w/v) respectively. Press filtrate and water phase were autoclaved separately and added in the laminar hood. In case of crude glycerol, it was added in such a way that final concentration of glycerol in PF medium were 0.4 %(w/v) and 0.8 %(w/v). Crude glycerol was not pre-treated and glycerol concentration was determined using free glycerol reagent (Sigma Aldrich). Crude glycerol was synthesized in the laboratory as per method described by Ruhal et al. 2011.

The following steps were performed for growth analysis and polyhydroxybutyrate (PHB) production:

- PHB production was carried out in 250 ml Erlenmeyer flasks containing 50 ml production media. Initial pH of the medium was 8.5.
- The production media was inoculated with freshly grown culture from agar plates and flasks were incubated at 200 rpm at 30^0C in an orbital shaker.

- Growth was monitored every 6 h interval by taking Optical density of cell suspension at 600 nm.
- Cell suspensions was obtained after centrifugation of broth followed by washing with 3% NaCl and finally suspending in same solution.
- At the stationary phase, remaining fermentation broth was centrifuged at 4-8^{0}C and 8000-10000 rpm followed by washing with 3% NaCl solution and the pellet was dried at 70^{0}C overnight.
- Dried pellet was stored at refrigerator till further analysis. The supernatant was stored at refrigerator till further use.

3.5. Lignin Separation

200g of black liquor was taken in a reagent bottle and placed at 45 °C incubator until the required temperature reached. 6M Sulphuric acid was added till pH of 1-2 was attained. Then the bottle was placed in incubator shaker at 45 °C for1 hour. The precipitate was separated and liquor left were stored at 4 °C (Zhu et al., 2014).

3.6. Polyhydroxybutyrate (PHB) Analysis

3.6.1. GC-MS Method

The PHB content was determined by Gas-Mass spectroscopy (GC-MS) according to the (Braunegg et al., 1978) method. Dried pellet and PHB standard were trans-esterified in 2ml each of acidified methanol (3% v/v sulphuric acid) and chloroform at 100^{0}C for 210 min. (0.05 % Benzoic acid was used as internal standard) under reflux conditions. After esterification, 1ml of distilled H_2O was added and reaction mixture was vortexed, kept the mixture for 3-4 h for phase separation followed by the organic phase separation. This organic layer was separated and filtered through 0.22 µm nylon filter and used for GC-MS analysis.

GC-MS analysis was performed on Agilent GC-MS (7890A GC, 5975 C inert XLEI/ CI MSD & 7693 auto sampler 7693) system equipped with DB-5MS column (J&W 122 - 5533:2504.94574 column dimension 300m*250µm*1µm, 325^{0}C). Two microliter (2µl) of sample was introduced by split injector with helium as a carrier gas (1.3 ml/min). Injector and detector temperatures were set to 220^0 C and 260^0 C and oven temperature was set using a temperature program: 80^{0}C for 1 min, ramp of 10^0 C min^{-1} up to 120^0 C: 35^0 C min^{-1} rise up to

250^0 C and hold for 2 min. Standard plot of PHB was used for quantification of PHB in sample. The confirmation of PHB in sample was performed by comparing the retention time of 3-HB methyl ester of standard with retention time of 3-HB methyl ester in sample. From chromatogram area of 3-HB of sample and standard plot, percentage PHB content of cell was determined. The PHB production in g/l was determined by multiplying PHB content of pellet with corresponding pellet weight obtained from fermentation broth.

3.6.2. Gravimetric Analysis

Gravimetric analysis of PHB was carried out by extraction of dried pellet powder (80-85 mg) with chloroform (10 ml) at reflux conditions for 3-4 hour. The chloroform solution was filtered and further concentrated by room temperature evaporation to 1 ml followed by precipitation with ten volume of cold methanol and repeated three times (Burniol-Figols et al., 2018). The precipitate was separated and dried at 70^0C and dried powder was weighed for determining PHB content.

3.7. Characterization of PHB

3.7.1. Fourier Transform Infrared (FTIR) Spectroscopic Analysis.

The confirmation of chemical nature of PHB was confirmed by using FTIR. For this method PHB was extracted from the grown cell of *Halomonas sp.* MCC 2171 in press filtrate medium. PHB extraction from the cells were done using Burniol et al protocol (Burniol-Figols et al., 2018). In this protocol cells were initially heated at 100 ℃ in chloroform under reflux condition for 3.5 hour. Then the solution was filtered and concentrated upto 1 ml followed by PHB precipitation with 10 volumes of chilled methanol. The content then dried and weighed and stored. Then the PHB polymer were subjected to the FTIR spectrophotometer. The spectral range was from 400 – 4000 cm^{-1}.

3.7.2. Thermogravimetric Analysis (TGA)

Thermal analysis was done to check the thermal stability of PHB, when subjected to increasing temperature condition. In this method the PHB was initially extracted from then dried into powder form using Burniol et al method (Burniol-Figols et al., 2018). PHB powder then placed on alumina pan and subjected increasing temperature form 50 ℃ to 400 ℃ with rise of 20 ℃ min^{-1}.

3.8. GC-MS of Low Molecular Weight Aromatic Compounds

Uninoculated (control) and bacterial inoculated samples (50 ml) were centrifuged (8000g for 15 min) to remove biomass. Supernatants were acidified to pH 1–2 with concentrated HCl and then thoroughly extracted with three volumes of ethyl acetate. The organic layer was collected, dewatered over anhydrous Na_2SO_4 and filtered through Whatman no. 54 filter paper (Raj et al., 2007). The residues were dried under a stream of nitrogen gas. The ethyl acetate extracts residues were analyzed as trimethyl silyl (TMS) derivatives (Lundquist & Kirk, 1971). In this method, 100 ml dioxane and 10 ml pyridine were added in samples followed by silylation with 50 ml trimethyl silyl [BSTFA (N, O-bis (trimethylsilyl) trifluoroacetamide) and TMCS (trimethylchlorosilane)]. The mixture was heated at 60^0C for 30 min with periodic shaking to dissolve residues.

GC-MS analysis was performed on Agilent GC-MS (7890A GC, 5975 C inert XLEI/ CI MSD & 7693 auto sampler 7693) system equipped with DB-5MS column (J&W 122 - 5533:2504.94574 column dimension 300m*250µm*1µm, 325^0C). Two microliter (2µl) of sample was introduced by split injector with helium as a carrier gas (1 ml/min). The column temperature was 50 ℃ (5 min); 50 – 160 ℃ (20 ℃/min, hold 3 min); 160 – 300 ℃ (8 ℃/ min, hold 3 min). The ion source and transfer line temperatures were maintained at 250 ℃ and 200 ℃. Peaks of the different compounds then obtained at different retention times in the Total Ion Count. For the identification the compounds, the mass spectra obtained in the GC – MS then analyzed by comparing it with the National Institute of Standard & Research (NIST) library available in the instrument. This library provided the probability of presence particular compounds at particular retention time along with the percentage similarity.

4. RESULTS AND DISCUSSION

4.1. Selection of Type of Pulp Mill Waste

First step was type of pulp mill waste selection according to their known properties. General types of waste that are produced by the paper mill industries are:

- Black Liquor: - It is rich in lignin content because it is generated during the conversion of wood chips into pulp. It is high in pH (11 – 13) (Yang et al., 2008). Because of presence of chromophores in lignin it is dark in color.
- Press Filtrate: - It is generated in dewatering stage of washed pulp. It is the diluted form of black liquor containing all the components at much lower concentration. Press filtrate is generally recycled back to washing of pulp in a counter current washer which finally ends up as a black liquor.
- Chlorine Water: - It is produced during the bleaching stage. Bleaching is done to increase the brightness of pulp using chlorides, hypo-chlorides. Hence wastewater release from this stage is rich in chlorine content and highly toxic in nature. Due to low pH, it is also unsuitable for microbial growth.
- Primary Wastewater: - It is produced at the end after the completion of all the stages i.e. pulping, screening, bleaching, washing, dewatering, drying. It consists of all type of waste present in the pulp mill and preferably used for the microbial growth. Industry generally treat the wastewater at this stage only. Wastewater of above stages if leaks directly to environment then may cause various problems.

As it is well known that primary wastewater is generally used for the microbial growth and industries also used to it treat it. So work was done using press filtrate and black liquor. Initially *Halomonas sp.* MCC 2171 doesn't able to grow in chlorine water so it was avoided in all future experiments. Now the press filtrate and black liquor were used as a medium for microbial growth.

4.2. Characterization of Press filtrate and Black liquor

The press-filtrate and black liquor was obtained from Star Paper Mill Ltd India and it was used without any further pre-treatment. The physical characterization of pulp press filtrate was carried out with respect to BOD, COD, pH, TSS and elemental analysis (Table 2). BOD, COD

were determined using standard protocol given by APHA (Apha, 1985). It was observed that press filtrate had BOD of 1237 mg/L and COD of 3360 mg/L with pH of 9.7. Elemental analysis was done using Inductive Coupled Plasma Mass Spectroscopy (ICP-MS). Elemental characterization of press filtrate shows that Sodium content was highest (36.35 g/l) followed by Ca (15.26 g/L), K (4.8 g/L) and Mg (4.2 g/L) (Table 1). Similarly, characterization of black liquor was carried out with respect to COD, pH, TSS and elemental analysis. Thus it seems halophilic bacteria like *Halomonas* sp. MCC 2171 can be a suitable bacterium for growth on press filtrate. BOD of black liquor was not determined because of presence of chromophores of lignin. These chromophoric groups are generally resistant or require large amount of time for the microbial degradation hence, increasing the BOD incubation needs to be increased to 20 to 100 days. So, BOD of black liquor cannot be measured using 5-day BOD method (Bajpai, 2013).

Table 2. Characterization of Press Filtrate and Black liquor.

Parameters	**Press filtrate**	**Black liquor**
BOD	1237 mg/L ± 37.5	-
COD	3360 mg/L ± 160	160000 ± 10000 mg/L
pH	9.70 ± 0.23	12.45 ± 0.35
TSS	2.94 ± 0.002	15.06 ± 0.30
Na+	36.35 g/L	58.07
Ca2+	15.26 g/L	6.19
K+	4.8 g/L	0.13
Mg2+	4.171 g/L	3.32
Mn	0.087 g/L	0.01
Fe	1.3 g/L	0.245

4.3. Growth Characteristics of *Halomonas sp.* MCC *2171* in Pulp Process Waste

Initial growth experiment was carried out in PF medium (containing 90% press filtrate + 10% water containing 2.5 g/l of yeast extract concentration). From the growth profile, it can be observed that within 12-18 h, stationary phase was achieved with maximum optical density (at 600 nm) of 6.6 at 18 h (Fig 1). Thus it confirms that press filtrate is a suitable medium for growth of *Halomonas sp*. MCC 2171. Further analysis of COD and BOD of cell free fermentation broth indicates that there were 29.7% and 33.3% reductions respectively with respect to press filtrate. While, there was no growth observed in black liquor medium (containing 90% black liquor + 10% water containing 2.5 g/l of yeast extract concentration). The reason might be the presence of high salt concentration as shown in table 2, or presence of inhibitory compounds resulted in no growth of *Halomonas sp.*

An attempt was made to explore addition of other suitable waste as a co-substrate in the medium for further improvement of biological treatment efficiency. Singhal & Thakur, 2009 employed dextrose and tryptone as a co-substrate in pulp mill effluent and not only achieved significant growth but also enhanced de-colorization and detoxification of pulp mill effluent (Singhal & Thakur, 2009). In this regard, glycerol was chosen as this is the main constituent of crude glycerol obtained in biodiesel synthesis. Further, glycerol with high degree of reductance can improve the biomass growth which can leads to higher reductions in BOD and COD of waste. Besides, glycerol is reported not only to serve as a carbon source for growth, but also for PHB production in many *Halomonas sp.* (Kawata et al., 2012; Quillaguamán et al., 2008; Quillaguamán et al., 2010).

4.4. Effect of Glycerol Supplementation

The PF medium was supplemented with pure glycerol at final concentrations varied from 0.4 %(w/v) to 2 %(w/v) and growth was monitored by determining optical density of biomass suspension in saline solution. From the growth profile (Figure 4) it can be concluded that up to a glycerol concentration of 0.8% (w/v) there was a significant increase in optical density of fermentation broth and thereafter there was marginal increase with further increase in glycerol

concentration up to 2% (w/v). Thus, presumption of higher growth due to the addition of highly reduced glycerol in the PF medium was found to be valid. Further, it can be assumed that increase in glycerol concentration beyond 0.8% (w/v) didn't improved the growth significantly as biodegradable carbon content of press filtrate was not sufficient to support higher growth when supplemented with higher glycerol concentration.

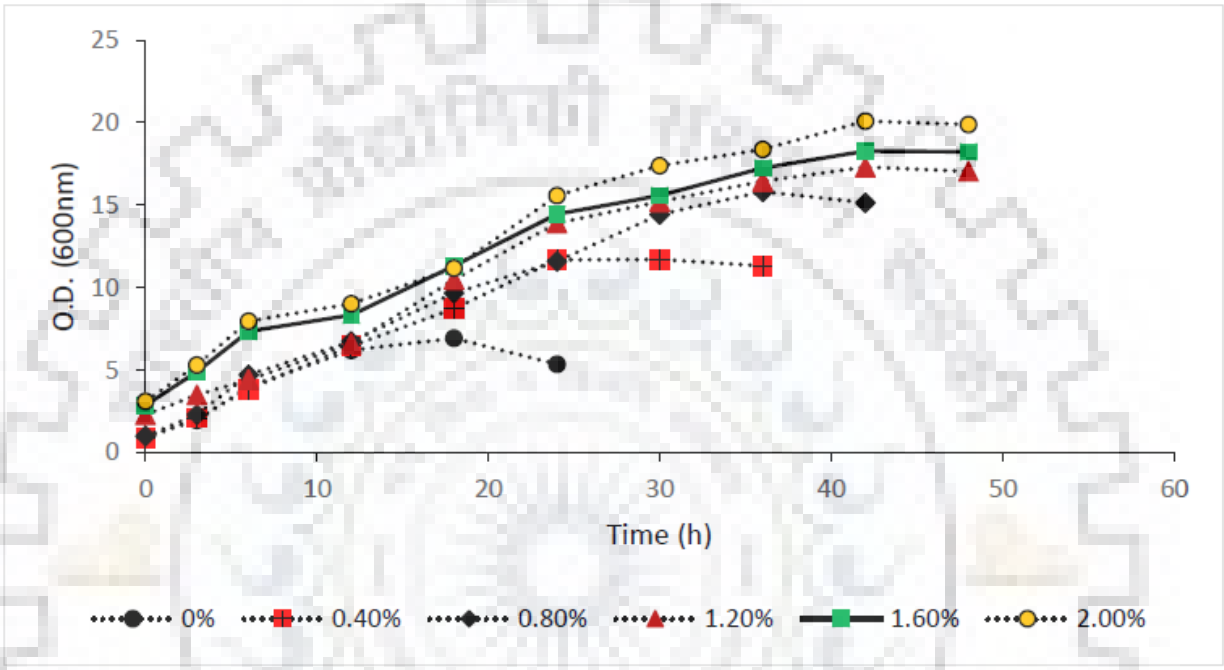

Figure 4. Effect of supplemented glycerol concentration on growth of *Halomonas sp*. MCC 2171 in Press Filtrate medium.

4.4.1. Determination of BOD and COD Reductions

Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD) of cell free supernatant that was collected after 40 – 48 hours (depending of the stationary phase of culture) were recorded (Figure 5 and Figure 6**)**. The percentage reductions of BOD and COD increased with increase in glycerol concentration. BOD and COD reductions profiles of fermentation broth (with respect to press filtrate) indicates that there were maximum reductions of 73.8 and 71.92% at 1.2% glycerol. Further increase in glycerol concentrations didn't yield any further improvement of BOD and COD reductions. Almost two times more reductions of BOD and

COD were achieved at 0.4% glycerol as compared to 0% glycerol. This further confirms the presumed benefit of glycerol addition in PF medium.

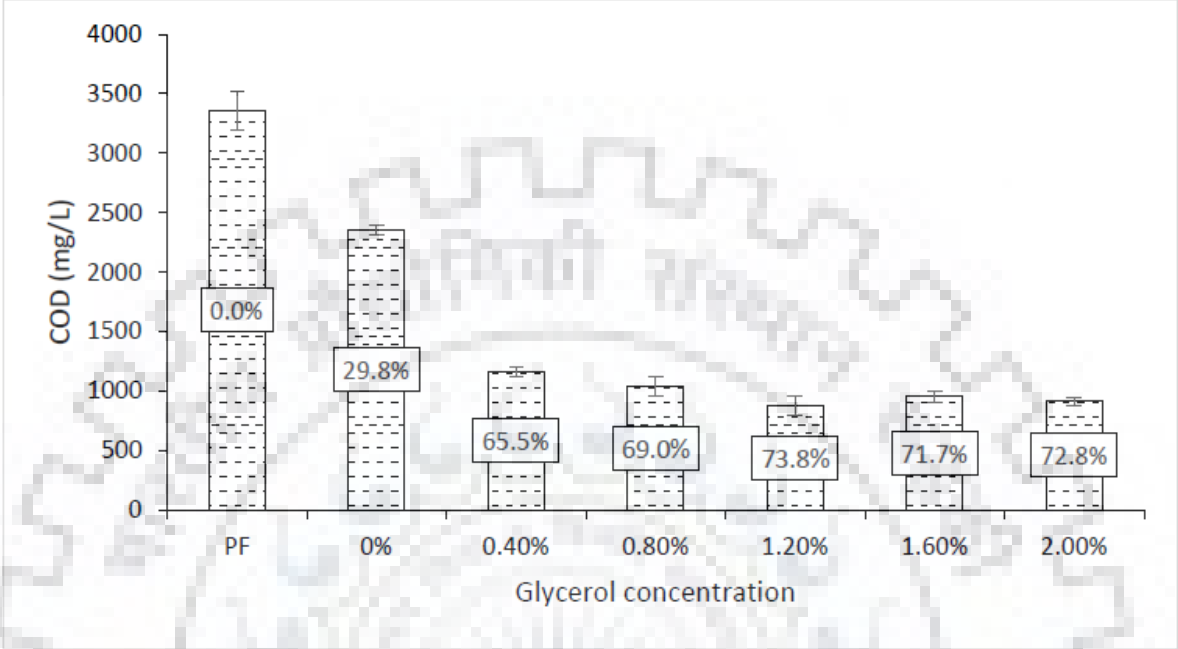

Figure 5. Effect of changing glycerol concentration on COD and percentage COD reductions in PF medium due to growth of *Halomonas* sp. MCC2171.

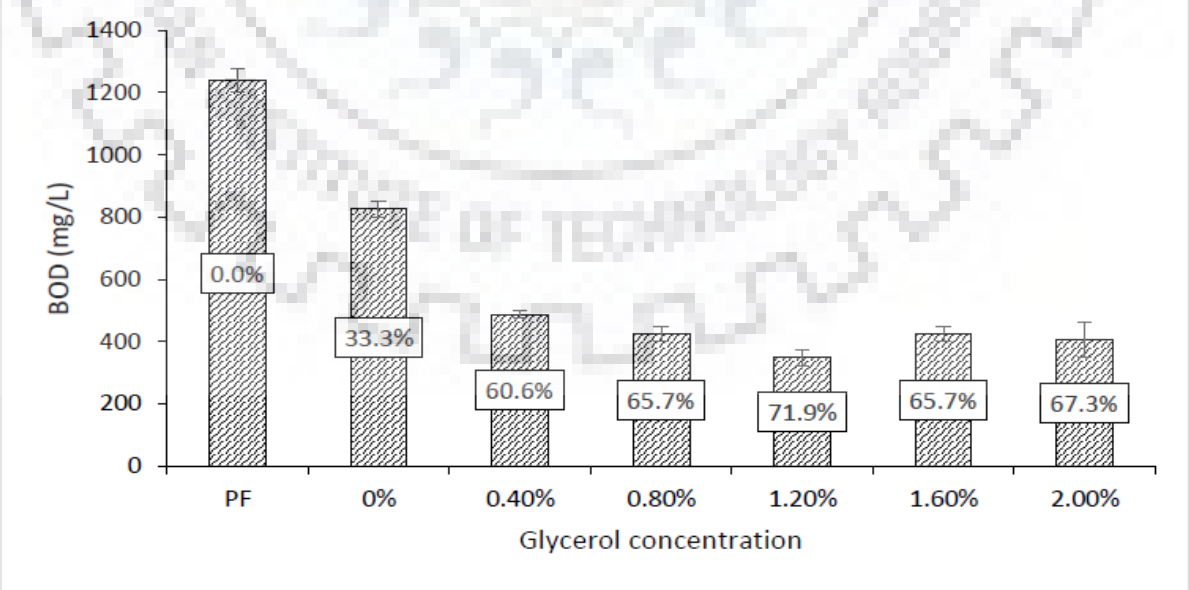

Figure 6. Effect of supplementation of glycerol on BOD and percentage BOD reductions in PF medium due to growth of *Halomonas* sp. MCC 2171.

4.4.2. Total Organic Carbon (TOC) Analysis

Further analysis of total organic carbon indicates that there was 35% reduction (with respect to PF) in case of 0.4% glycerol and this was marginally higher than that achieved at 0% glycerol. Further increase in glycerol concentration resulted in lower reductions in TOC (Table 3). In case of 0.8% glycerol, there was only 2.61 % reduction (as compared to PF) in TOC and thereafter there was no reduction of TOC with respect to TOC of PF. In other word, it confirms that biodegradable carbon content of PF was not sufficient to support higher growth and TOC reductions when supplemented with glycerol concentration higher than 0.8%. Thus this study established that addition of glycerol up to a 0.4-0.8% glycerol in PF medium, improves the biological treatment efficiency with *Halomonas sp*. MCC2171.

Table 3. Illustration of Total Organic Carbon (TOC) in the Press Filtrate medium with varying pure glycerol concentration.

Sample	Concentration (g/L)
PF	7.63±0.01
0%	5.12 ± 0.22
0.4%	4.93 ± 0.28
0.8%	7.43 ± 0.02
1.2%	7.98 ± 0.07
1.6%	8.43 ± 0.33

4.4.3. PHB Analysis by GC-MS Method & Gravimetric Method

Halomonas sp. MCC 2171 was known to produce polyhydroxybutyrate (PHB) (Bera et al., 2015), so an analysis of PHB in dried pellet (obtained after centrifugation of fermented broth) was carried out. Samples were also analysed using GC-MS method (Braunegg et al., 1978). In this method dried cells were first heated at 100^0C in presence of 2ml of chloroform and 2ml of mixture of methanol – benzoic acid (0.05% w/v) – sulphuric acid (3% v/v) under reflux condition for 210 minutes. After esterification reaction, sample then transferred to glass tubes

followed by addition of 1ml of distilled water and vortexed and left for 3-4 hour for phase separation. The organic layer was separated and filtered through 0.22 µm nylon filter and stored in -20^{0}C for further use. The Total Ion Chromatograph (TIC) of methanolysis product of polymer obtained (Figure 8, 9) are authenticated by comparing with the standard PHB graph (Figure 7) as well as the comparison with the NIST library available with the instrument and found to be 3-hydroxy butyrate methyl ester and benzoic acid at ~4 min and ~7 min retention time. NIST library also provided peak area, abundance as well as the percentage quality of peak. The standard PHB with varying known amount (in mg) were prepared and analysed in GC-MS to draw the standard graph of the PHB (mg) with respect to the abundance, so that amount of PHB present in cell could be known using standard PHB graph. Dividing the area of 3-Hydroxybutyrate methyl ester with Benzoic acid peaks, gives the PHB area and which further dividing by the slope form the PHB standard graph provides the amount of polymer present in the cell. Furthermore, dividing by the amount of cell used for PHB analysis will give the polymer percentage present in the cell (Table 4).

Table 4. Percentage of PHB in dried pellets when extracted and analyzed using GC-MS

Glycerol Concentration	PHB Content
0.0%	0%
0.40%	12% ± 2.6%
0.80%	32% ± 3.2%
1.20%	22% ± 2.9%
1.60%	17% ± 14.6%
2.00%	4% ± 1.4%

Figure 7 is the obtained using PHB standard, a commercial powder bought from Sigma-Aldrich. The data then compared with the NIST library. TIC of the methanolysis of PHB produced the 3-Hydroxybutyrate methyl (R_t ~4 minutes) and Benzoic acid (R_t ~~7 minutes) and has given the 99% similarity to respected mass spectra in NIST library. Benzoic is used as

internal standard i.e. will come in TIC whether the 3-HB methyl ester is forming or not. The medium with no supplementation with glycerol has not shown the 3-HB methyl ester in TIC (Figure 8) hence, it is proved that there was no polymer production in absence of glycerol. While cells grown in press filtrate supplemented with glycerol were able to produce the PHB hence, there was formation of 3-HB methyl ester and is reported in the figure 9.

However, it is worth noting that dried pellet not only contains cell but also some precipitated lignin as the color of dried pellet was greyish in color. The PHB content analysis of dried pellet showed significant variation which is probably due to nonhomogeneous nature of dried powder (presence of lignin, cell and other impurities). So to overcome these inconsistencies of PHB analysis, alternative analysis was carried out where PHB was first extracted from dried pellet powder using hot chloroform followed by precipitation with cold methanol. The dried precipitated powder was used for gravimetric analysis of PHB.

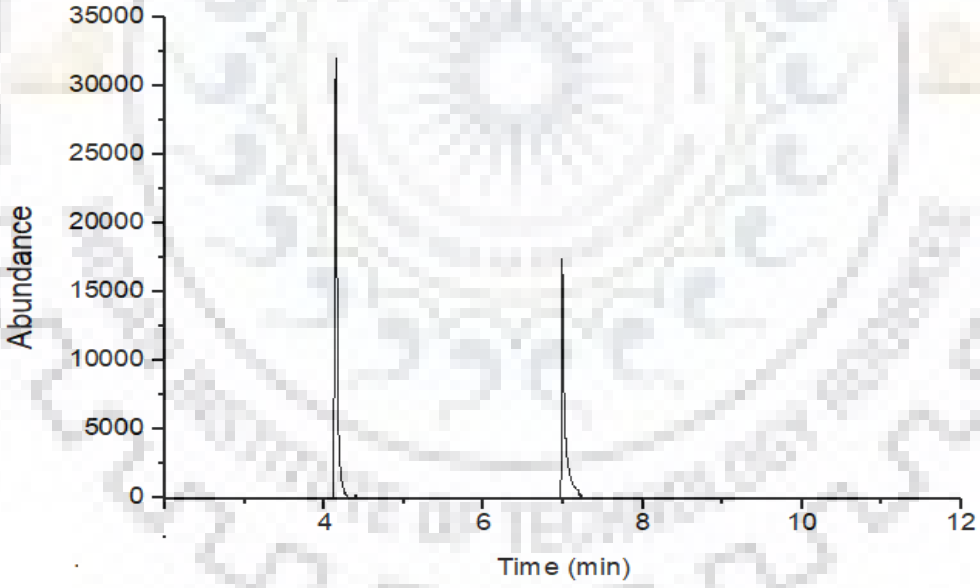

Figure 7. Total Ion Chromatograph of the methanolysis product of the PHB.

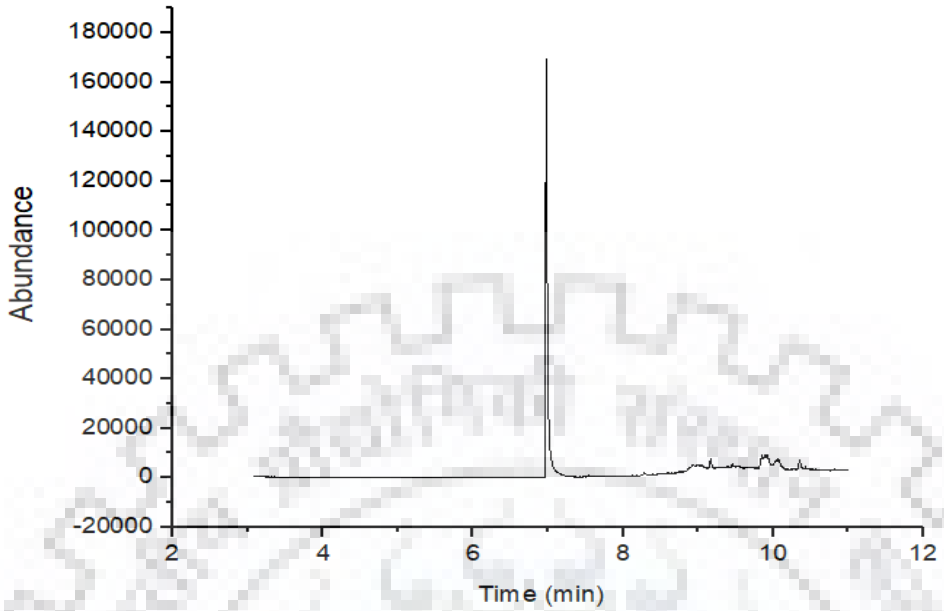

Figure 8. Total Ion Chromatograph of the methanolysis product of the extract from the cells when grown in PF without any supplementation of glycerol. It confirmed that there is no PHB production occurs.

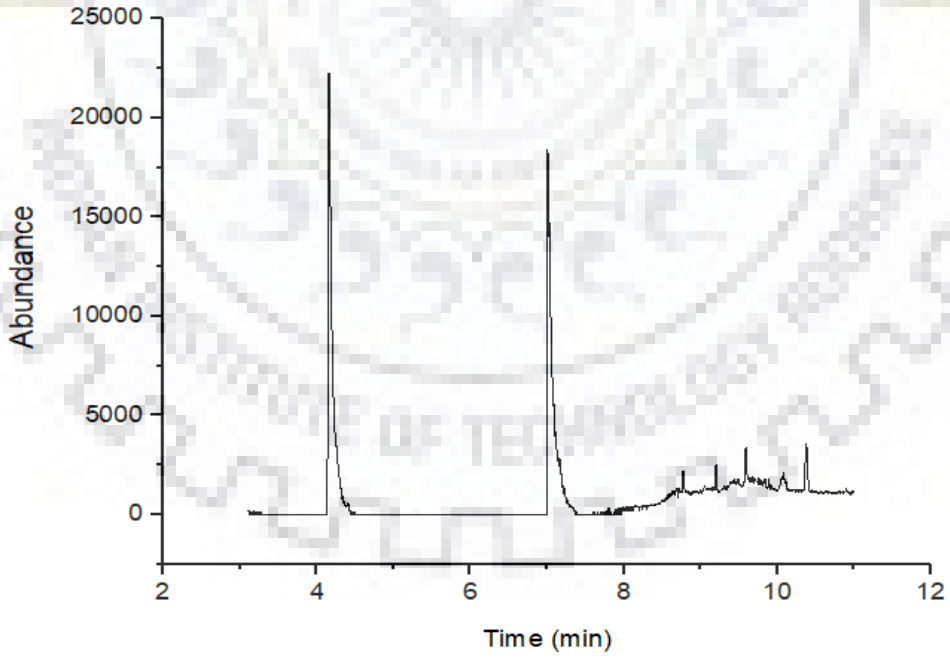

Figure 9. Total Ion Chromatograph of the methanolysis product of the polymer from the cells, when grown under supplementation of glycerol.

At 0% glycerol, there was no PHB production and maximum content of 36% was achieved at 1.2% glycerol in the dried pellet (Table 5). Calculated (based on PHB content and dried pellet weight) PHB titre of 2.2 g/l was achieved at 1.2% glycerol. Thus it indicates that addition of glycerol not only improved the BOD and COD reductions but it also induced PHB synthesis. This is probably the first study which showed that addition of glycerol in pulp process wastewater treatment, not only improved the biological treatment efficiency but also recover some of carbon degradation products in the form of polyhydroxybutyrate.

Table 5. Percentage of PHB in dried pellets when cultured in PF medium with different pure glycerol concentrations.

Glycerol Concentration	PHB Content
0.40%	18% ± 3.1%
0.80%	33% ± 0.4%
1.20%	36% ± 2.3%
1.60%	19% ± 10.96%
2.00%	15% ± 0.1%

4.4.4. Total Suspended Solid Analysis

TSS was determined to study the effect of growth of *Halomonas sp.* MCC 2171 on the reduction of solid content in the media. It is correlated with TSS in press filtrate. In press filtrate TSS was measured to be 4.50% and maximum reduction is achieved in the media supplemented with 0.4% followed by 0% and 0.8% glycerol (Table 6).

Table 6. Reduction in TSS percentage with respect to the TSS in Press Filtrate.

Sample	TSS
PF	4.50%
0.0%	3.07%
0.4%	2.97%
0.80%	3.08%
1.20%	3.28%
1.60%	4.02%
2.00%	3.72%

Glycerol supplementation

4.5. Growth Analysis of *Halomonas sp.* MCC 2171 in Black Liquor

As PF medium usually contains less biodegradable organic carbon as compared to black liquor so an effort was made to use black liquor instead of PF. But there was no growth absorbed in black liquor (Figure 10). So lignin was removed from the black liquor using Zhu et al protocol (Zhu et al., 2014). Then the growth was checked for *Halomonas sp.* (Figure 11). There was no growth seen in the black liquor medium (containing 90% black liquor + 10% water containing 2.5 g/l of yeast extract concentration). No visible growth was achieved with *Halomonas sp*. MCC2171**.** This is probably due to presence of high content of inhibitory compounds in black liquor. Similar observations were also made by Claudia et al., 2018 with synthetic black liquor (SBL) supplemented with glucose at 100%(v/v) SBL concentration using *P. pastoris* pox 1 B. However, 50% reduction of COD was achieved with *P. ostreatus* with 100% SBL. Thus maximum SBL concentrations of 10 %(v/v) and 5 %(v/v) were used for *P. pastoris* and *P. ostreatus* for detoxification of black liquor as it resulted in more than 90% reduction of COD.

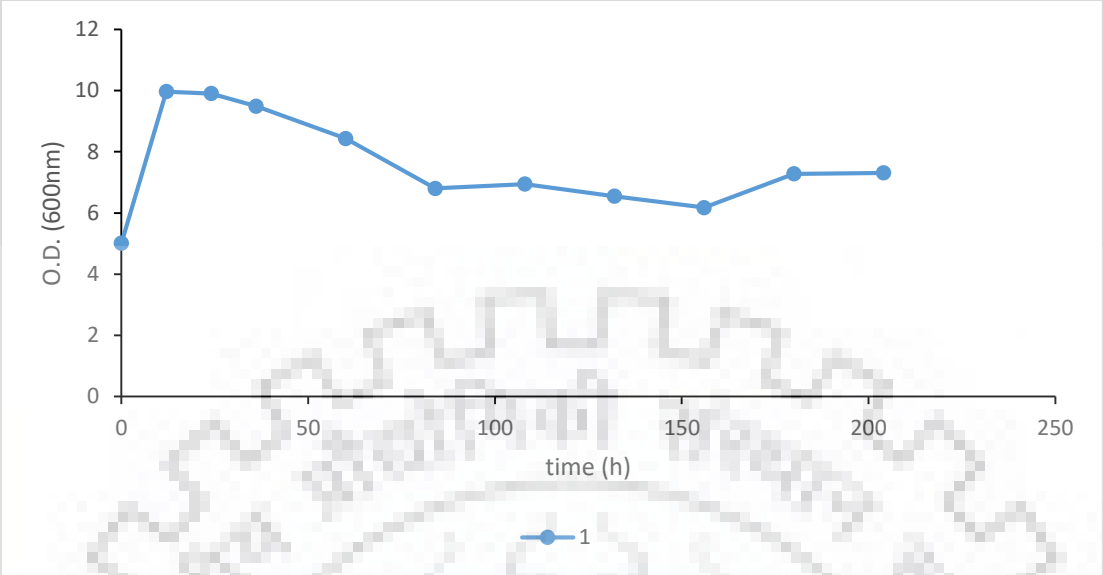

Figure 10. Illustration of growth of *Halomonas sp*. MCC 2171 in black liquor.

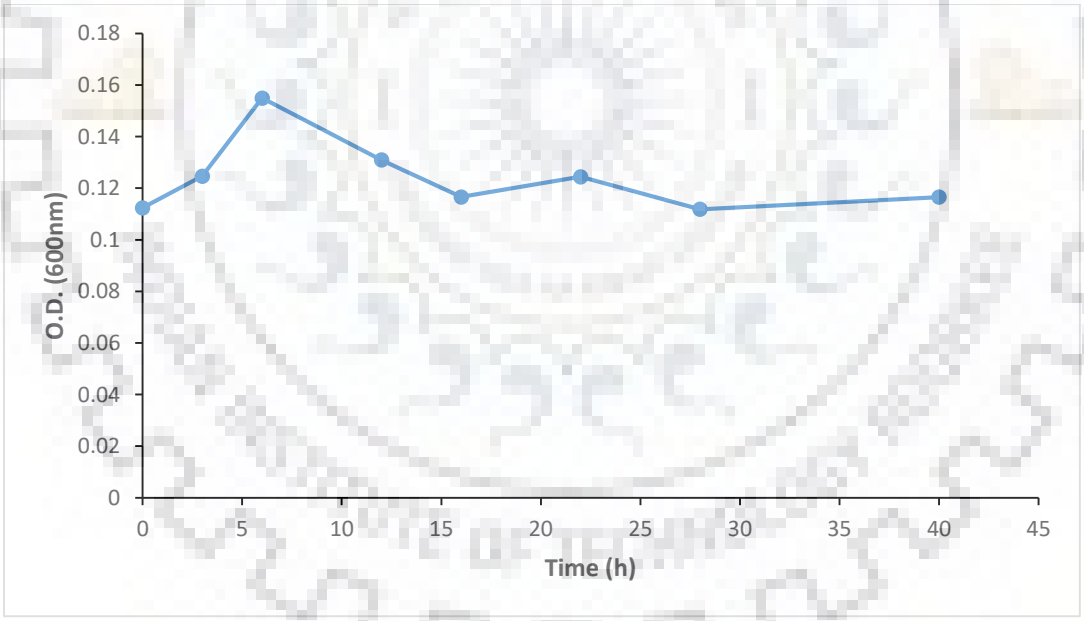

Figure 11. Growth of *Halomonas sp*. MCC 2171 in black liquor after removal of lignin.

4.6. Effect of Crude Glycerol and Black Liquor Supplementation

To improve the PHB production and biological treatment efficiency of Press Filtrate medium, new sets of experiment was carried out where instead of pure glycerol, crude glycerol

was used at glycerol concentration of 0.4% and 0.8% along with Press filtrate. Further, press filtrate was supplemented with black liquor at 10% (v/v) along with 2.5 g/l of yeast extract and 0.4% and 0.8% glycerol equivalent of crude glycerol (Figure 12). Addition of black liquor resulted in precipitation of lignin compounds hence, optical density (at 600nm) of medium at zero hour was higher. Growth in crude glycerol supplemented PF medium had followed the same pattern as it was in pure glycerol supplemented PF medium.

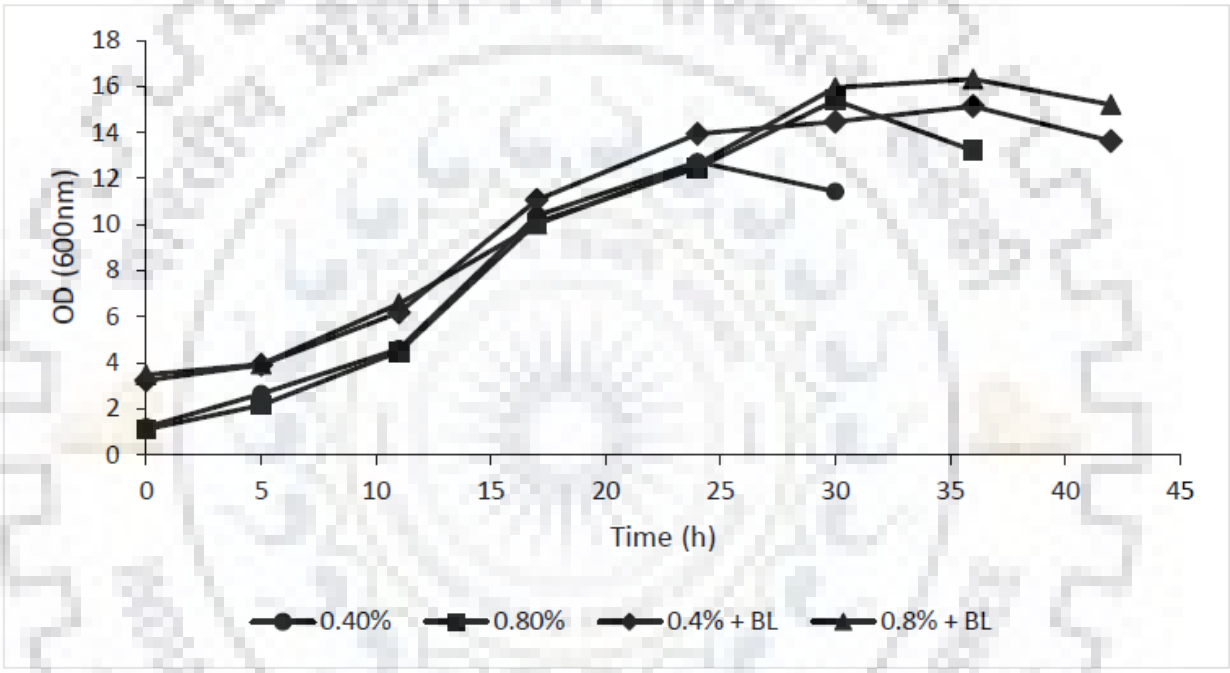

Figure 12. Illustration of *Halomonas sp*. MCC 2171 growth in (**1**) – 0.4% glycerol equivalent of crude glycerol in press filtrate medium, (**2**) – 0.8% glycerol equivalent of crude glycerol in press filtrate medium, (**3**) – 0.4% + BL crude glycerol in press filtrate medium with 10% black liquor and (**4**) – 0.8% + BL crude glycerol in press filtrate medium with 10% black liquor.

PHB production was higher in crude glycerol supplemented PF medium than in pure glycerol supplemented PF medium at 0.4% glycerol concentration but marginally less at 0.8% glycerol (Table 7). The maximum PHB production of 0.94 g/l was achieved in crude glycerol supplemented PF medium at 0.8% glycerol. BOD and COD reductions (with respect to zero-

hour BOD and COD values of medium) were 64.86 %; 68.96%; and 60.8%; 65.3% for crude glycerol supplemented medium at 0.4% and 0.8% glycerol respectively (Figure 13 & Figure 14). However, in comparison to PF, COD reductions were 56 and 61% whereas 61.6 and 63.6% reductions in BOD were achieved in crude glycerol supplemented medium at 0.4% and 0.8% glycerol respectively.

Table 7. Percentage of PHB in dried pellets when cultured in PF medium supplemented with crude glycerol and black liquor (BL).

Concentration of glycerol and black liquor	**PHB content**	**PHB (g/l)**
0.40%	37% ± 0.74%	0.74±0.01
0.80%	26% ± 0.59%	0.94±0.21
0.40% + BL	24% ± 1.4%	0.56±0.24
0.80% + BL	17% ± 0.24%	0.72±0.03

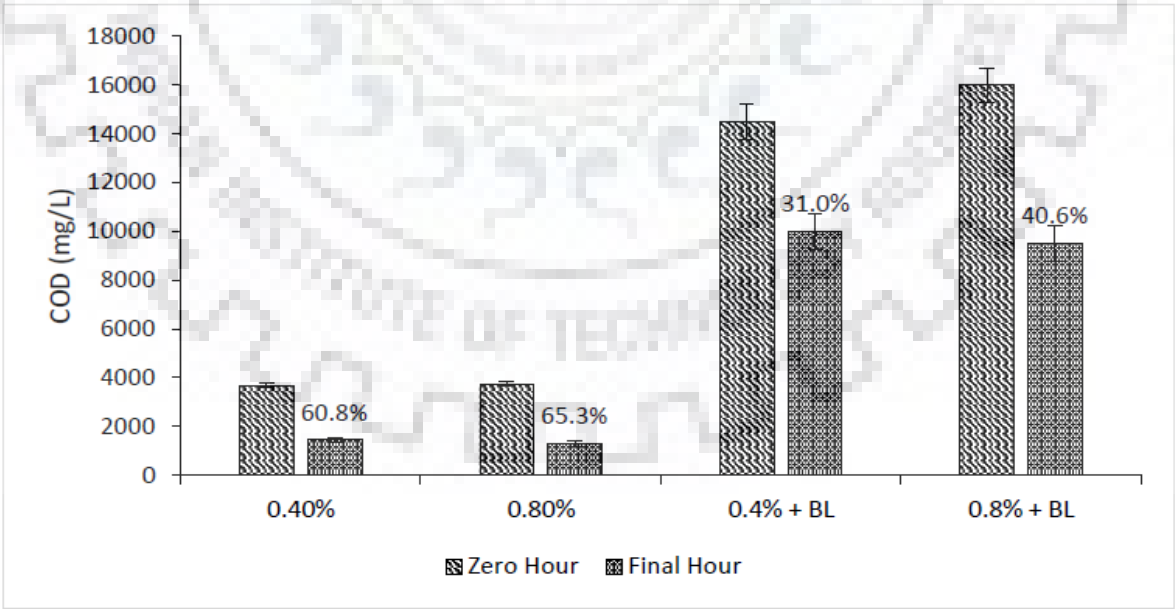

Figure 13. Effects of supplementation of crude glycerol and black liquor on COD and Percentage reduction of COD due to growth of *Halomonas sp*. MCC 2171 in (**1**) 0.4% glycerol equivalent of crude glycerol in press filtrate medium, (**2**) 0.8% glycerol equivalent of crude glycerol in press filtrate medium, (**3**) 0.4% +BL crude glycerol in press filtrate medium with 10% black liquor and (**4**) 0.8%+BL crude glycerol in press filtrate medium with 10% black liquor. Zero hour – Sample taken before inoculation and Final hour – Sample taken after achieving stationary phase of the culture.

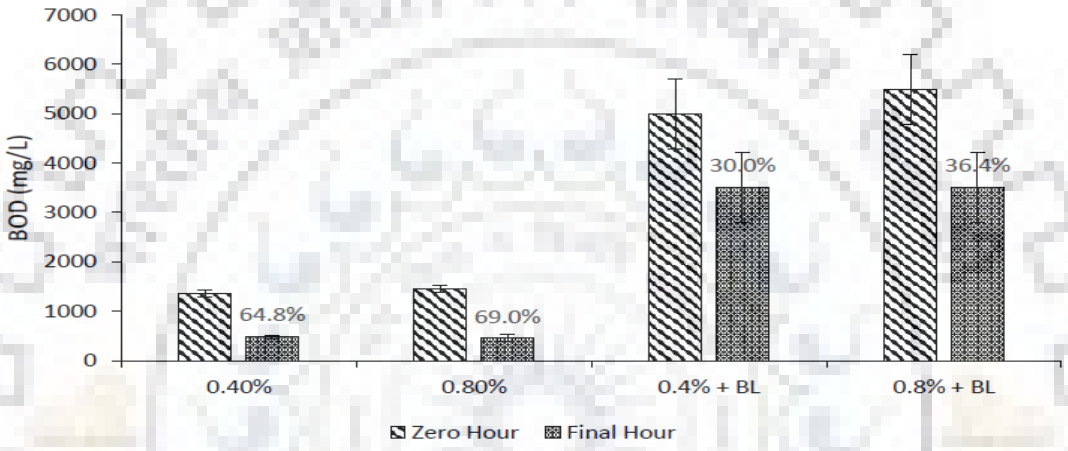

Figure 14. Effects of supplementation of crude glycerol and black liquor on BOD and Percentage reduction of BOD due to growth of *Halomonas sp*. MCC 2171 in (**1**) – 0.4% glycerol equivalent of crude glycerol in press filtrate medium, (**2**) – 0.8% glycerol equivalent of crude glycerol in press filtrate medium, (**3**) – 0.4% crude glycerol in press filtrate medium with 10% black liquor and (**4**) – 0.8% crude glycerol in press filtrate medium with 10% black liquor. Zero hour – Sample taken before inoculation and Final hour – Sample taken after achieving stationary phase of the culture.

TOC reductions was 32.8% in crude glycerol supplemented medium at 0.4% glycerol whereas corresponding reduction was 31.7% at 0.8% glycerol (Table 8). Thus in crude glycerol supplemented medium at 0.8% glycerol, TOC reduction was significantly improved as compared to 0.8% pure glycerol supplemented medium. This can be presumed to due to presence of other minor biodegradable impurities like fatty acid or soap in crude glycerol.

Table 8. TOC of cell free fermentation broth in PF medium supplemented with crude glycerol and black liquor.

Sample	Zero Hour TOC (g/L)	Final Hour TOC (g/L)
0.40%	-	5.12
0.80%	-	5.21 ± 0.465
0.40% + BL	15.21	9.73 ± 1.72
0.80% + BL	15.53	9.62 ± 0.53

4.7. Characterization of PHB

4.7.1. FTIR Analysis

Fourier Transform Infrared Spectroscopy is used to analyze the PHB extracted from the cells. Figure 15 shows the absorbance of the chemical components that are present in the PHB. The result showed that alcoholic O-H shows absorbance at ~3433, stretching of =O at 1678, =C-O comes at ~1119. These are in accordance with Gangurde et al and Ramsay et al (Gangurde et al., 2013; Ramsay et al., 1990). In the IR spectrum absorbance at 1400 give the presence of =C-H. Absorbance of C=O stretching shows at ~1678 because addition of monomeric unit, i.e. methyl group as it is expected for the C=O valence vibration of a thioester bond (Colthup, 2012; Shah, 2012). It also represents the molecular chain of highly ordered structure of PHB, because of absorbance of carboxyl group lies in 1655 – 1760 cm^{-1} (Lathwal et al., 2018). Hence it exhibits the presence of polymeric PHB

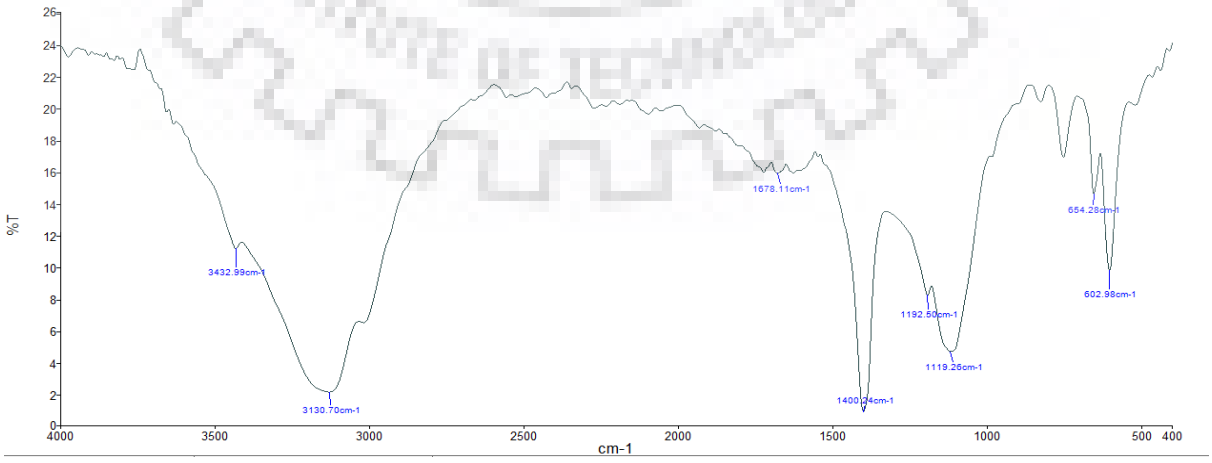

Figure 15. Spectrum of FTIR of PHB extract from *Halomonas sp*. MCC 2171.

4.7.2. TGA Analysis

Thermogravimetric analysis (TGA) of PHB was done to determine its thermal stability. It generally focuses on the 5% weight loss temperature. Around 10 mg of PHB was taken and placed on alumina pan and subjected to heating rate of 20 ℃ min^{-1} from 50 to 600 ℃ temperature (Figure 16). The weight loss of PHB started at 270 ℃ (98.9 % available polymer) and completely decompose at 315 ℃ (0.8% polymer left) and at 600 ℃, PHB decomposes completely i.e. proving that it is not having any inorganic material that might present in press filtrate (Venkateswar Reddy et al., 2015).

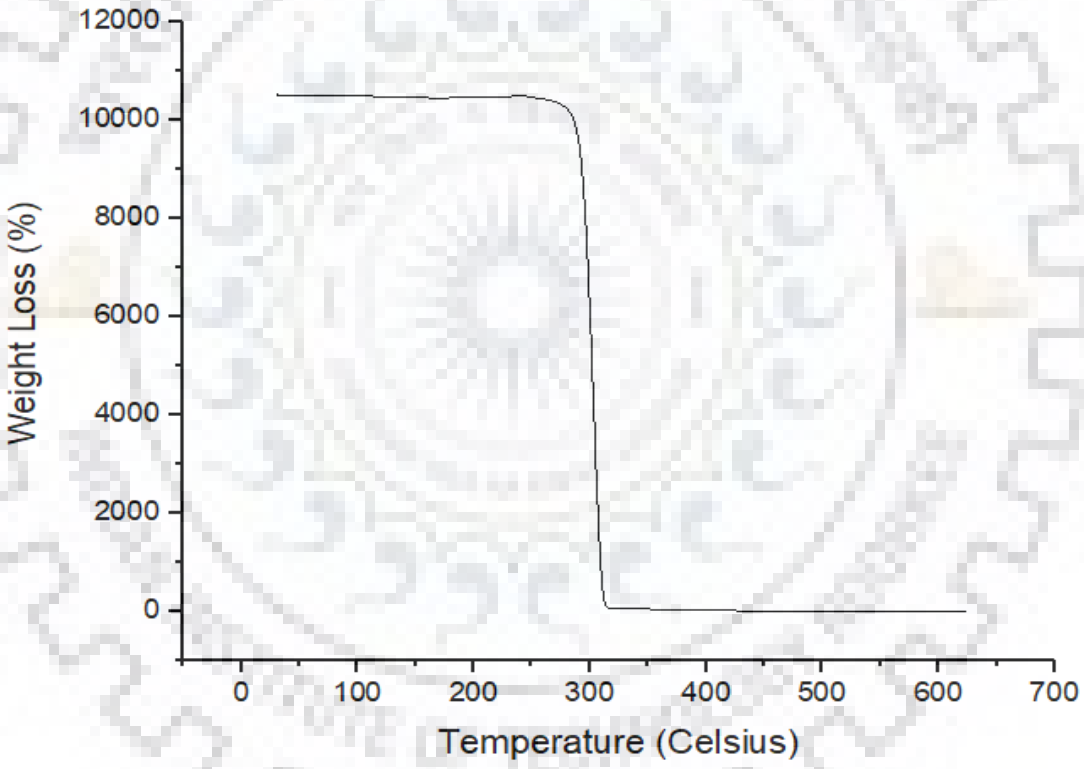

Figure 16. Thermogravimetric analysis (TGA) of PHB extracted from *Halomonas sp*. MCC 2171.

4.8. Identification of assimilated carbohydrate and lignin degradation products

The effect of growth of *Halomonas sp.* MCC 2171 on assimilation of degradation products of lignin and carbohydrate present in press filtrate. The low molecular weight compounds were analyzed by TMS derivative method (Raj et al., 2007). Firstly, supernatant after removing the biomass using centrifugation, were acidified (pH 1-2) using HCl and then compounds were extracted using three volumes of ethyl acetate. Then it was dried under the nitrogen stream and analyzed using TMS derivative method (Lundquist & Kirk, 1971). Figure 17 showed that there was assimilation of organic compounds with respect to compounds presents in press filtrate. Table 9 shows the compounds that were identified in press filtrate and inoculated media when compared with the NIST library. Some compounds such as Apocynin is generally used as immune booster and as an antioxidant in humans cells (Heumüller et al., 2008) and it is produced after growth of *Halomonas sp.* MCC 2171. Ethanone is small size non-toxic compound and also reported to use as silica gel derivative (Sharma et al., 2013). From Table 9 it can be observed that degradation products like organic acids are mostly assimilated during growth of *Halomonas sp. MCC 2171.* Hence this culture is not only reducing organic content but also reducing the organic compounds present in press filtrate along with co-production of polyhydroxybutyrate.

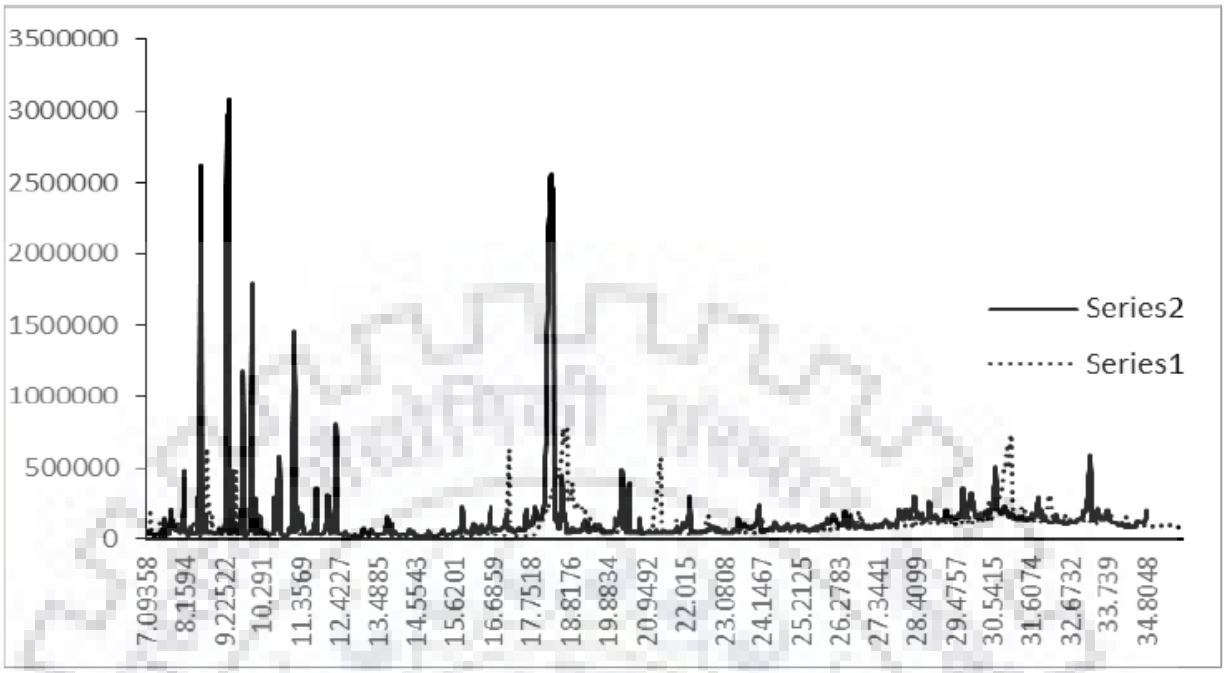

Figure 17. Total Ion Count of TMS derivatives of compounds extracted with ethyl acetate in Press Filtrate (PF Series 2) and Inoculated medium (Series 1) supplemented 0.8% Crude Glycerol.

Table 9. Compounds identified in control and inoculated media

Retention time(min)	Identified Compound	Press filtrate	Inoculated
8.8	1-[(Trimethylsilyl)oxy]propan-2-ol	+	+
9.6	Silane	+	+
10.9	4-Methyl-2-hexen-1-ol	+	-
11.2	Pentanoic acid	+	-
17.2	Apocynin	-	+
17.6	Bis(trimethylsilyl)bromosuccinate	+	-
19.6	(3,4-Dihydroxyphenyl)acetic acid	+	-
21.4	Ethanone	-	+
29.8	Abietic acid	+	-
30.7	Bis(2-ethylhexyl) phthalate	-	+
32.8	Docosanoic acid	+	-

Supplementation of black liquor, didn't improved the biomass growth significantly at 0.8% glycerol whereas at 0.4% glycerol, growth was marginally higher (Figure 12). This may be due to higher sodium content in black liquor as compared to PF. In terms of BOD and COD reductions in black liquor and crude glycerol supplemented medium, 30%, 36.36% and 31%, 40.6% were achieved at 0.4% and 0.8% glycerol concentration respectively. Total organic carbon content reductions were 35.9% and 38% in black liquor supplemented medium with 0.4% and 0.8% glycerol concentration respectively. In these case, comparisons of BOD, COD and TOC reductions were carried out with respect to un-inoculated medium to account for the contribution of black liquor. As the black liquor crude glycerol supplemented medium has higher BOD and COD loads, thus in terms of absolute reductions of BOD and COD it was higher than that achieved with 0.4 and 0.8% crude glycerol supplemented PF medium. In terms of PHB production, black liquor supplementation didn't result in any improvement. Further improvement in PHB production and growth are expected in black liquor, crude glycerol supplemented medium by using lower volume percentage of black liquor.

Zheng et al, reported 64.8%, 50.5%, 53.2% reductions in COD, color and lignin using bio-augmentation technique with special mixed microorganisms, when supplied in black liquor having 10g COD/L day (Zheng et al., 2013). *Rhizopus oryzae* and *Pleurotus sajor caju* reported to reduce the COD by about 74- 81 % of bleached Kraft Eucalyptus globulus (Freitas et al., 2009). However, fungal systems are not applicable for scale up at industrial level, because of pH and oxygen limitations as black liquor are turbid and alkaline. Additionally, mycelia may also choke the plant lines resulting in bio sludge bulking. Many bacterial species have also been studied for their potential in pulp and paper wastewater treatment. Raj et al, (2014) reported the 83% and 78% BOD and COD reductions using *Paenibacillus sp.* strain LD1 (Raj et al., 2014). *Serratia liquefaciens* was found to effectively reduce 85% COD of pulp mill wastewater after 144 hour treatment (Haq et al., 2016). Gupta et al, reported the 73% reduction of COD using *Aeromonas formicans* (Gupta et al., 2001). None of these earlier studies reported any production of value added products. In the present work, *Halomoans sp.* MCC 2171 was able to reduce BOD and COD of pulp process waste and it was also able to produce the PHB by co-feeding of crude glycerol.

5. CONCLUSION

The addition of glycerol resulted in accelerated growth with simultaneous enhanced reductions in BOD and COD of medium. PHB analysis of dried pellet obtained after centrifugation of fermentation broth indicates that without the presence of glycerol there was no PHB production. Organic content is converted into the valuable product. While presence of 1.2% pure glycerol in PF medium resulted in maximum PHB accumulation of 36% and 0.4% glycerol in crude glycerol supplemented PF medium had resulted in 37% PHB accumulation. Also, the assimilation of low molecular compounds after the growth of *Halomonas sp.* MCC 2171 shows the potential of this strain in degrading the toxic components of the pulp mill waste. Treated pulp process waste with lower BOD, COD loads and lower TOC can also be recycled back to pulp washing stage for improved washing efficiency.

6. FUTURE ASPECTS

- Study needs to be done by increasing the concentration of black liquor and crude glycerol in the press filtrate media.
- Further study of identification of any co-polymer forming.
- Needs to identify, if any enzyme is involved degradation of lignin components
- To study the assimilation of compounds and their function in after growth of *Halomonas sp.* in Black liquor.

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