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MICROBIAL ASSIMILATION AND ENZYMATIC DEPOLYMERIZATION OF NATURAL RUBBER BY *MUCOR* SP. ISOLATED FROM RUBBER LATEX CONTAMINATED SOIL

Nayanashree G., Sowmya H. V., Thippeswamy B.*

Dept. of P.G. Studies and Research in Microbiology, Bio-Science Complex, Jnanasahyadri, Kuvempu University, Shankaraghatta-577451, Shivamogga.(Dist.), Karnataka, India.

***Corresponding Author: Thippeswamy B.**

Dept. of P.G. Studies and Research in Microbiology, Bio-Science Complex, Jnanasahyadri, Kuvempu University, Shankaraghatta-577451, Shivamogga.(Dist.), Karnataka, India.

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ABSTRACT

Rubber products are widely used in our daily life these products are mainly made up of Natural rubber (NR) which is obtained from the latex of tree *Hevea brasiliensis* commonly called Rubber tree. After usage of these natural rubber products the disposal of these products are the world wide solid waste problem. Microbial degradation of products can be solution for this problem. In current work different fungal species were isolated from the soil were natural rubber sample was dumped. In all the isolated fungi *Mucor* sp., effectively degraded rubber. Further it was studied that laccase and manganese peroxidase enzyme produce by *Mucor* sp., was responsible for rubber degradation.

KEYWORDS: *Mucor* sp., *Hevea brasiliensis*, vulcanization, polyisoprene, laccase, manganese peroxidase.

INTRODUCTION

Rubber products are widely used in our daily life these products are mainly made up of Natural rubber (NR) or cis-1,4 polyisoprene which is obtained from the latex of tree *Hevea brasiliensis* commonly called Rubber tree. Rubber trees are basically found in tropical & semitropical countries such as Indonesia, Malaysia, Sri Lanka, South America and India have abundant resource of natural rubber. Two main types of polyisoprenoids that differ according to their isomerism are synthesized by plants, the first one is the *cis* isomer natural rubber (NR) (poly(*cis*-1,4-isoprene)) and the second one is the *trans* isomer gutta-percha (GP) (poly(*trans*-1,4-isoprene)). The average composition of the natural rubber latex is 25-30% polyisoprene, 1-1.8% proteins, 1-2% carbohydrates, 0.4-1.1% neutral lipids, 0.5-0.6% polar lipids, 0.4-0.6 inorganic components, 0.4% amino acids etc., and other 50-70% water (Rose *et al.*, 2002).

The global rubber consumption in 2026 was estimated to be 15.6 million metric tons of which 65% were used for tire production and other 35% is used for the production of other rubber products such as rubber balloons, mats, rubber bands, pipes, gaskets, sheets etc.

After usage of these natural rubber products the disposal of these products are the world wide solid waste problem. One of the solutions to reduce this problem is to recycle the used waste rubber. But due to the chemical cross linking formed during vulcanization it is not possible to simply melt and reshape the products as in case of polythene. So other alternatives such as microbial degradation of the product should be developed. Microbial degradation is mainly carried out by various microorganisms such as bacteria and fungi (Lions *et al.*, 2000).

MATERIALS AND METHODS

For the isolation of rubber degrading fungi, latex contaminated soil and natural rubber sheet were collected from rubber processing unit and then it was brought to

the laboratory and preserved in the refrigerator for further use.

Isolation of natural rubber degrading fungi by Warcup method

For the isolation of fungi 0.05g of air dried soil sample contaminated with rubber latex, was weighed and added to the centre of the petriplate, then melted and cooled Czapek Dox agar was poured to the petriplate was rotated clockwise and anticlockwise for the isolation of fungi. After plating plates were incubated at 27±2°C for 7 days (Warcup, 1951).

Plate assay for the screening of fungi capable of degrading natural rubber

For the screening of natural rubber degrading fungi pure culture of isolate was directly inoculated on the sterilized, pre weighed natural rubber disc and then kept for incubation for 2 months. After a time interval of 2 months natural rubber sample inoculated with organism was washed thoroughly, dried at 50°C in hot air oven for 24 hours and final weight was recorded (Borel *et al.*,1981).

Screening of natural rubber degradation by using Mineral salt medium (MSM)

Natural rubber degrading ability of the fungi was checked in the laboratory conditions by growth experiment in mineral salt medium (MSM) (Pan *et al.*, 2009), where natural rubber was used as sole carbon source. Previously isolated fungi was inoculated to conical flask containing MSM and kept for incubation for 2 months on rotary shaker. Flasks were incubated at 27±2°C, triplicates were maintained. After incubation period natural rubber discs were removed and observed for the growth of fungi. Then natural rubber discs were washed dried at 50°C in hot air oven for 24 hours and weight loss was checked (Tsuchii and Tokiwa, 2001).

Confirmation of natural rubber degradation by staining with Schiff's reagent

Evidence for degradation and mineralization of *cis*-1,4-polyisoprene rubber hydrocarbon chain was obtained by staining treated natural rubber discs with Schiff's reagent. In a tightly stopper bottle, 10 ml of fuchsin reagent was added to a sample and kept for incubation for 10-30 minutes at room temperature. After 10-30 minutes excess amount of the reagent was discarded and 10ml of the sulfite solution was added in order to suppress nonspecific reaction of untreated sample (Berekka *et al.*, 2000).

Confirmation of natural rubber degradation by Scanning Electron Microscopy (SEM)

Evidence for degradation and mineralization of *cis*-1,4-polyisoprene natural rubber hydrocarbon chain was obtained by observing the natural rubber discs under SEM. For the observation natural rubber discs inoculated with organism and present in the MSM, which were subjected for degradation were observed under field emission-scanning electron microscopy (FEI-SIRION, Eindhoven, Netherland) (Lions *et al.*, 2000).

Confirmation of natural rubber degradation by Fourier Transform Infrared Spectroscopy (FTIR)

Chemical changes that arose directly on the natural rubber surface as result of the degradation process were determined using FTIR spectroscopy. NICOLET 380 FTIR spectrophotometer from Thermo Fisher Scientific, France was used which gives transmittance spectra in IR range 4000 to 400 nm. (Roy *et al.*, 2005).

Characterization of enzymes responsible for biodegradation of natural rubber

It was studied that laccase and manganese peroxidase enzymes were responsible for the natural rubber degradation.

Screening for Laccase and Manganese peroxidase enzyme production by fungi

Screening for laccase enzyme produced by *Mucor* sp., was done on plates containing following composition (g/l): 3.0 peptone, 10.0 glucose, 0.6 KH_2PO_4, 0.001 $ZnSO_4$, 0.4 K_2HPO_4, 0.0005 $FeSO_4$, 0.05 $MnSO_4$, 0.5 $MgSO_4$, 20.0 Agar (pH-6) supplemented with 0.02% guaiacol. *Mucor* sp. was inoculated into this plate and the plate was incubated at 30°C for 7 days. Laccase activity was visualized on plates containing 0.02% guaiacol, since laccase catalyzes the oxidative polymerization of guaiacol to form reddish brown zones in the medium (Viswanath *et. al*, 2008).

For the screening of manganese peroxidase enzyme producing organisms H_2O_2 was added to the laccase screening media.

Mass production of enzyme by submerged fermentation

Pure cultures of *Mucor* sp., was inoculated to submerged state fermentation medium for the production of extracellular enzymes by using MSM media and was maintained at the incubation temperature of 27±2ºC for 3 months (Shraddha *et al.*, 2011).

Determination of Laccase and Manganese peroxidase enzyme activity by using Spectrophotometer

Guaiacol (2mM) in sodium acetate buffer (10mM pH 5.0) was used as substrate. The reaction mixture contained 3ml 10mM acetate buffer of pH 5, 1ml guaiacol and 1ml enzyme source and enzyme blank contained 1ml of distilled water instead of enzyme source. The mixture was incubated at 30ºC for 15minutes and absorbance was read at 450nm blank using UV spectrophotometer (Papinutti *et al.*, 2006). Manganese peroxidase enzyme activity was calculated by following laccase enzyme activity determination procedure, but for the reaction mixture 1 ml of H_2O_2 was added and incubated.

RESULTS

Isolation of natural rubber degrading fungi by Warcup method

In isolation of rubber degrading fungi by Warcup method different fungi were isolated and recorded. In the isolated organism *Mucor* sp., was pre-dominant and commonly isolated. Thus it was screened to test natural rubber degrading ability (fig. 1).

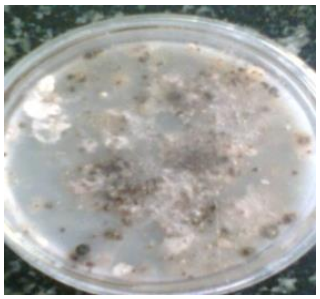


Fig. 1: Isolation of natural rubber degrading fungi by Warcup method.

Plate assay for the screening of microorganisms capable of degrading natural rubber

In *Mucor* sp., inoculated natural rubber discs initial weight of the rubber disc was 10g and final weight was 8.95g and there was decrease in 1.05±0.020 weight and percentage of weight loss was 10.5% (fig. 2).

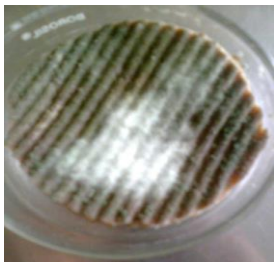


Fig. 2: Growth of *Mucor* sp., on rubber sheet.

Screening of natural rubber degradation by using Mineral salt medium (MSM)

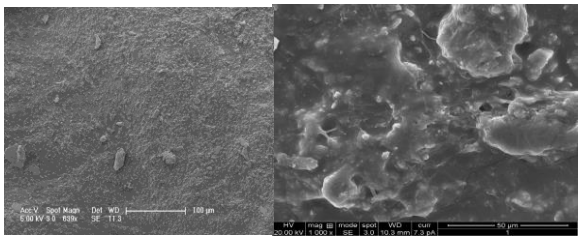
Growth experiment was conducted by using mineral salt medium weight loss was observed and growth of fungi was observed on the natural rubber discs. Initial weight of *Mucor* sp., inoculated sample was 3g and final weight was 2.15g and there was a weight loss of 0.85±0.026 g and percentage of weight loss 28.3% degradation.

Confirmation of Natural rubber Degradation by Staining with Schiff's Reagent

Rubber sheets which were inoculated with microorganism turned to purple colour and there was no colour formation in the blank. Formation of purple colour in the treated sample is due to the presence of aldehyde and ketone group which is produced as a result of degradation of cis-1,4-polyisoprene units.

Confirmation of natural rubber degradation by Scanning Electron Microscopy (SEM)

Natural rubber discs were observed under SEM, bio-film formation, complete disintegration and formation of cavities on the natural rubber discs was observed (Fig 3).

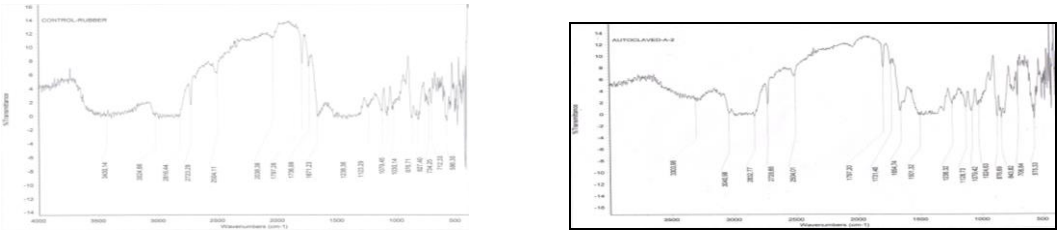


***Mucor* sp. treated** **Control**

Fig. 3: SEM images of natural rubber showing degradation.

Confirmation of natural rubber degradation by Fourier Transform Infrared Spectroscopy (FTIR)

Natural rubber disc, which was treated by *Mucor* sp. was subjected to FTIR, peaks at the wave 2728.66cm^{-1} and 1654.74cm^{-1} was found, which indicates the release of aldehyde and ketone group as a result of degradation. Presence of aromatics (3040.98cm-1), carboxylic acid (2509.49cm^{-1}), carbonyl (1742.41cm^{-1}) and alcohol (1090.41cm^{-1}) groups were observed, which confirms rubber degradation (fig. 4).



Control ***Mucor* sp. treated**

Fig. 4: Confirmation of natural rubber degradation by FTIR.

Enzymatic studies of natural rubber degradation

It was studied that laccase and manganese peroxidase enzymes were responsible for the rubber degradation.

Screening of Laccase and Manganese peroxidae enzyme producing organisms

Mucor sp., was inoculated on the laccase and manganese peroxidase medium there was a formation of reddish brown colour around the colonies since laccase and manganese peroxidase catalyzes the oxidative

polymerization of guaiacol to form reddish brown zone. *Mucor* sp., which showed positive result for rubber degradation showed positive result for laccase and manganese peroxidase enzyme screening.

Spectrophotometrical analysis of laccase and manganese peroxidase enzyme activity

Mucor sp., showed maximum activity of both laccase and manganese peroxidase in 10th week (Table 1. and Fig. 5)

Table 1: Showing laccase and manganese peroxidase activity.

Mucor sp.	1st week	2nd week	3rd week	4th week	5th week	6th week	7th Week	8th week	9th week	10th week	11th week	12th week
Laccase	0	0	0.0011 ±0.0001	0.0018 ±0.0004	0.0027±0.0001	0.0039 ±0.0003	0.0058 ±0.0001	0.0079 ±0.0002	0.0104 ±0.0003	0.0121±0.0001	0.0094 ±0.0001	0.0077 ±0.0003
Mangenese peroxidase	0	0	0.0014 ±0.0001	0.0026 ±0.0004	0.0038 ±0.0002	0.0057 ±0.0001	0.0072 ±0.0001	0.0091 ±0.0001	0.0119 ±0.0001	0.0135 ±0.0001	0.0104 ±0.0001	0.0077 ±0.0001

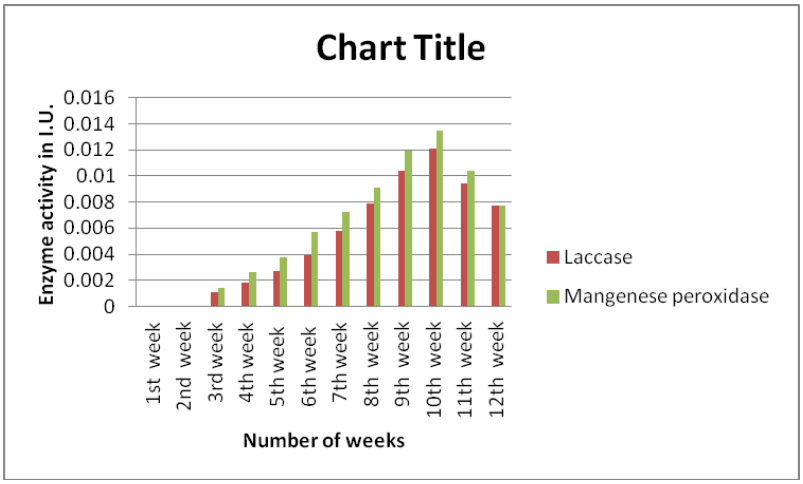


Fig. 5: laccase and manganese peroxidase activity.

DISCUSSION

Present study was carried out to isolate natural rubber degrading fungi. It was studied that *Mucor* sp., is capable of degrading natural rubber. Degradation of natural rubber was studied by carrying out growth experiment in MSM, and degradation was confirmed by staining, SEM, FTIR studies. Further enzyme responsible for degradation was studied. Laccase and manganese peroxidase were the enzymes responsible for degradation.

Similar attempts were made by several other scientists to degrade rubber by using microorganisms.

Roy *et al.*, (2005) made an attempt to study on natural rubber (NR) biodegradation through solid-state fermentation (SSF) and submerged fermentation (SMF) has been carried out for both bacterial as well as fungal species. There was a change in the organic carbon content along with the average molecular weight of the treated rubber samples indicated rubber hydrocarbon utilization and its degradation.

Berekaa *et al.*, (2000) conducted similar work and tested the biodegrading ability of different bacteria belonging to the genera *Gordonia* (strains Kb2, Kd2 and VH2), *Mycobacterium*, *Micromonospora* and *Pseudomonas*. All strains were able to use natural rubber (NR) as well as NR latex gloves as sole carbon source.

Similar study was carried out by Tokiwa *et al.*, (1999) in his study he showed that forty-seven percent of a tire tread strip with a natural rubber content of 100 phr (parts per hundred of rubber) was completely mineralized by a mutant strain, Rc, of the rubber-degrading organism, *Nocardia* sp. Strain 835A.

CONCLUSION

Rubber products are widely used in our daily life. These products are made up of natural vulcanized rubber and other chemical additives. Due to vulcanization of the natural rubber these rubber are very resistant to high temperature and persist in environment for very long time. Rubber materials have been increasingly used now a days in different area after usage its disposal is a very big solid waste problem. It can not be easily recycled due to the sulphur cross linking formed during vulcanization. If they are burnt they release enormous amount of carbon-di-oxide and some other gases which causes environmental pollution and contribute to the global warming. Rubber products such as balloon which are disposed in the natural environment are considered to be dangerous to wild animals if they are consumed by animals.

So, one of the alternative way to solve these problems is to subject these product to biodegradation. During the present study rubber discs were dumped in the soil were removed at regular interval of time and then plated on

the media to isolate the organism. In the isolated organism and *Mucor* sp., effectively degraded the rubber sample. The present study has showed that, it is possible to use *Mucor* sp., to degrade natural rubber effectively. Along with this, enzymes responsible for natural rubber degradation was also characterized.

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