ijlpr 2021; doi 10.22376/ijpbs/lpr.2021.11.2.L91-99

International Journal of Life science and Pharma Research

Research Article

Biotechnology for prospective medical science

Biodegradation of sago effluent by white- rot fungus *Phanerochaete chrysosporium*

***K.Vivekanandhan[1], Dr.M. P. Ayyappadas[1], Dr.S. K. Gangai Abirami[2], R.Renugadevi[1], M. Flory Shobana[1], Dr.V.Subha Priya[1] and Dr.V. Manon Mani[1]**

[1]*Department of Biotechnology, Rathnavel Subramaniam College of Arts and Science (Autonomous), Coimbatore, Tamil nadu, India*

[2]*PG & Research Department of Biotechnology, Nandha Arts and Science College, Erode, Tamilnadu, India*

Abstract: *Phanerochaete chrysosporium* was isolated by serial dilution from sago effluent taken from Attur Sago and Starch Treatment Plant Company Ltd, Tirupur, Tamilnadu, India.The NTG (N-Methyl N-Nitrosoguanidine) treated organisms with high concentration of glucose showed considerable increase in the sago effluent degradation. The mutated organisms were effective in degrading phenolic compounds and cyanide to a greater extent. In the absence of glucose, wild type isolates produced the least amount of enzyme. However, the concentration of glucose on wild type *Phanerochaete chrysosporium* could be increased in enzyme production. NTG was used to improve the efficiency of strain for better biodegradation. The efficiency of biodegraded sago effluent increases the rate of seed germination. The isolated *Phanerochaete chrysosporium* is therefore able to grow using diluted sago effluent as sole carbon source and also reduces the colour of the effluent, decreases chlorides, phenolic contents, COD, BOD and cyanides, thus providing an eco-friendly relation to the society. The effect of sago effluent to inhibit the growth of seeds was overcome by the treatment of the effluent with *Phanerochaete chrysosporium*. Sago effluent was chosen for the project, since it pollutes the ground water, after air pollution. Sago factories release large amount of effluent containing solid and liquid wastes. The heavy load of organic content and lower pH affects the soil. The aim of the present study was to analyse the degradation of effluent by *Phanerochaete chrysosporium*. A comparative analysis was done with biodegraded effluent and untreated effluents.

Keywords: *Phanerochaete chrysosporium,* Biodegradation, N-Methyl N-Nitrosoguanidine (NTG), fungi, bacterial isolation, effluent.

***Corresponding Author**

K.Vivekanandhan , Department of Biotechnology, Rathnavel Subramaniam College of Arts and Science (Autonomous), Coimbatore, Tamil nadu, India

Recieved On 21 February 2020
Revised On 01 February 2021
Accepted On 06 February 2021
Published On 02 March 2021

Funding This research did not receive any specific grant from any funding agencies in the public, commercial or not for profit sectors.

Citation K.Vivekanandhan, Dr.M.P. Ayyappadas, Dr.S.K.Gangai Abirami, R.Renugadevi, M.Flory Shobana and Dr.V.Subha Priya, Dr.V. Manon Mani , Biodegradation of sago effluent by white- rot fungus Phanerochaete chrysosporium.(2021).Int. J. Life Sci. Pharma Res.11(2), L91-99 http://dx.doi.org/10.22376/ijpbs/lpr.2021.11.2.L91-99

1. INTRODUCTION

Sago industries in Tamilnadu, of about 700–1000 units are engaged in Tapioca processing, under small scale industries (SSI) sector. Sago is a product, prepared from the milk of "Tapioca Root". Its Botanical name is "Manihot esculenta Crantz Syn Utilissima" Tapioca (*Manihot esculenta Crantz*) which was introduced in India during the later part of the 18th century. In Tamil Nadu, Sago was first produced in Salem in 1943-1944. Sago production started on a cottage scale basis in India by pulping the tapioca roots. Tapioca Sago is generally known as "SAGO" (sabudana in hindi or javvarisi in tamil) in India. About 500 million people eat tapioca in the world. It contains 98% carbohydrates, calcium and vitamin. The manufacturing process consists of washing, peeling, crushing, slurring, settling, sizing, roasting, drying and polishing. But the negative impact on groundwater quality needs to be investigated. Sago effluent irrigated lands highly affect the soil structure, texture, pore size and soil microflora and it leads to a decrease in the moisture content of soil, increase hardness of soil, decrease water holding capacity and also prevents the air circulation factories[1,2]The effluent is acidic in nature, it is highly organic nature it leads to high BOD, COD and solids. There are inorganic constituents like phosphate, sulfate, chloride and nitrate. The effluent includes starch, phenolic compound and cyanide that are biodegradable. This wastewater if allowed to stagnate, gives rise to the generation of foul odor, when raw wastewater is discharged into water bodies (lakes, rivers, streams, etc.), it will deplete the dissolved oxygen content (DO) of water. The oxygen requiring aquatic life including flora and fauna is likely to be affected. Groundwater affected nears the sago processing industry. *Phanerochaete chrysosporium* play a role in the degradation of complex carbon compounds such as starch, cellulose, pectin, lignin, lignocelluloses, etc .*Phanerochaete chrysosporium* is emerging as the model system for bioremediation[3]. Lignin peroxidases plays a vital role in the plant cell wall by biodegradation of lignin. The enzymes were included under the family of oxidoreductases, which was first discovered in white-rot fungus *Phanerochaete chrysosporium*. Morphologically, the genus Phanerochaete was divided to several sections and subgenera. White rot fungi efficiently degrade lignin, a complex aromatic polymer in wood that is among the most abundant natural materials on earth.[4,5]The species of Phanerochaete are characterized by the membranaceous, resupinate basidiocarps, a monomitichyphal system, simple-septate generative hyphae (single or multiple clamps may be present in subiculum), clavate basidia and smooth, thin-walled, inamyloid, hyaline, cylindrical to ellipsoid basidiospores and by causing white rot on both conifers and hardwood. [6]These fungi use extracellular oxidative enzymes that are also able to transform related aromatic compounds found in explosive contaminants, pesticides and toxic waste. It is an extracellular hemeprotein that is H_2O_2-dependent, with unusually high redox potential and low optimum pH. It is capable of oxidizing a variety of reducing substrates, including polymeric substrates. [7]It has the distinction of being able to oxidize methoxylated aromatic rings without a free phenolic group, which generates cation radicals that can react further by a variety of pathways, including ring-opening, demethylation, and phenol dimerization. In contrast with laccases, LiP does not require mediators to degrade high redox-potential compounds, but it needs H_2O_2 to initiate catalysis. It has been observed that extracellular enzymes of white-rot fungi play an important role in the deconstruction of lignin in lignocellulosic biomass. Consequently, lignin degradation of crop residues by the laccases of Schizophyllum commune has been studied.[8] The present study was done to check the efficacy of the biodegradation effect of the isolated fungi from sago effluent. *Phanerochaete chrysosporium* play an important role in the degradation of complex carbon compounds such as starch, cellulose, pectin, lignin, lignocelluloses, etc. *Phanerochaete chrysosporium* is emerging as the model system for bioremediation. White rot fungi produced various iso-forms of extracellular oxidize and peroxidase, which is involved in the degradation of the organic and inorganic compounds like phosphate, sulfate, chloride, phenolic compound and cyanide NTG (N-Methyl N-Nitrosoguanidine) belongs to the class of mutagens called alkylating agents. The organism is treated with the addition of alkyl group (methyl, ethyl) to the ring nitrogenase as well as exocyclic oxygens of purines and pyrimidines of the DNA, lead to the formation of phosphodiester in nucleic acid. A mutated organism leads to the overproduction of enzymes such as Lignin peroxidase. Manganese peroxides increase the degradation of effluent.[9-10] Sago effluent was chosen for the project, since it pollutes the ground water, after air pollution. Sago factories release large amount of effluent containing solid and liquid wastes.[11] Due to the heavy load of organic content and lower pH are affect the soil.The sago waste water is toxic to plant cultivation; the present study will be effluent degrading by *Phanerochaete chrysosporium*.The white-rot fungus *Phanerochaete chrysosporium* has the ability to degrade a wide variety of structurally diverse organic compounds, including a number of environmentally persistent organopollutants. The unique biodegradative abilities of this fungus appears to be dependent upon its lignin-degrading system.[13]The non-specific and partially extracellular nature of this system suggests that it may be useful as a supplementary means to treat organo chemical wastes. The efficiency of biodegradable industrial effluent was tested. Comparative analysis with biodegraded effluent and untreated effluents. Sago effluent was chosen for the project, since it pollutes the groundwater, after air pollution. Sago factories release a large amount of effluent containing solid and liquid wastes. The heavy load of organic content and lower pH affects the soil.[14]The sago wastewater is toxic to plant cultivation, The main objective of the present study will be the degradation of effluent by *Phanerochaete chrysosporium.* The efficiency of biodegradable industrial effluent was tested. Comparative analysis was done with biodegraded effluent and untreated effluents.

2. MATERIALS AND METHODS

2.1 *Collection of Sago Effluent*

The sago industry effluent was collected directly from the effluent industry at the point of discharge from the factories. 3 samples of effluent were collected from Attur Sago and Starch Treatment Plant Company Ltd, Tirupur, Tamilnadu, in a five-liter plastic can. Before collecting the samples in the plastic can, they were rinsed two or three times with the water to free from preservative or dechlorinating agents. After collection, it was brought to the laboratory and maintained in the refrigerator at 60 °C[15].

2.2 *Microbial Count on Sago Effluent*

2.2.1 *Plating Techniques*

2.2.1.1 Pour plating

Each diluted 1ml of the sample was transferred to sterile petri plates. Sterilized nutrient agar and Sabouraud's Dextrose Agar (SDA) were poured into the petri plates and mixed thoroughly.[16]

2.2.1.2 Spread plate

The sterilized medium was poured onto the petri plate and allowed to solidify. 0.1ml of the sample was poured onto the surface of the medium from each dilution.[17]

2.3 *Culturing of microorganisms*

2.3.1 Malt Extract Agar

Malt extract agar provides all the nutrients required by the microorganisms. The media was used to grow pure culture of *Phanerochaete chrysosporium*. Malt Extract Agar contains Maltose 12 g/l, Dextrin2.5 g/l, Glycerol 1.0 g/l, Pepto complex 2.6 g/l, Agar 17 g/l[18]

2.3.2 Malt Extract Broth

Malt extract broth is a liquid media generally used to culture microorganisms. Malt Extract contains maltose, glucose and yeast extract. The inoculated medium was incubated at 37 ^{0}C for 48 – 91 h on the shaker. *Phanerochaete chrysosporium* was maintained at 26°C by sub-culturing at two-week intervals on Malt extract agar. Amended with 1% agar stocks were stored as plugs of mycelia frozen at –80 ° C in 15% glycerol (w/v).[19]

2.4 *Isolation of Fungi from Sago Effluent*

The technique requires a previously diluted sample for inoculation, the cells were spread over the surface of a solid agar medium with a sterile L-shaped bend rod while the petri dish was spin on a lazy-Susan turntable. This will spread the cellulose over the agar surface. All the petri plates were incubated in an inverted position for 48 to 72 h at 25°C. The isolated fungi culture was identified by performing lactophenol cotton blue staining and observed under light microscope (Olympus, Gurgaon, India) to determine the morphology of mycelia.[20]

2.5 *Enzyme Assay*

Phanerochaete chrysosporuim produces three types of extracellular enzymes. Manganese dependent peroxidase (MnP), Lignin peroxidase (LiP) and Laccase.MnP and LiP are involved in the degradation process and were assayed by the amount of enzyme production in the degradation medium.[21]

2.6 *Effect of NTG on Enzyme Production*

20 ml log phase culture was taken and centrifuged at 4000 rpm for 10 minutes. The obtained pellet was suspended in 5ml malt extract broth and 0.05 ml (0.025%) of NTG was added to the mixture. After keeping the above mixture for 45 minutes, the pellet was washed with phosphate buffer (pH 7.4). The pellet was incubated with malt extract broth overnight. 1ml of malt extract broth 1ml was taken and serial dilution was made from 10^{-1} to 10^{-8} dilution. The serial dilution (0.1 ml) from each test tube was performed and plated on malt extract agar. The plates were incubated at 28 ° C for 48 to 91 h. After 48 h the growth was observed on the plates. The growth of colonies was observed and inoculated into the medium and incubated at 28°C for 48 to 91 h. The enzyme was estimated after the incubation period.[22-23]

2.7 *Preparation of Phanerochaete chrysosporium Crude Enzyme Extract (partial purification)*

2.7.1 Ammonium Sulphate Precipitation

The charges on protein in the solution can be neutralized by the addition of salts and this has been used in the purification of protein. The supernatants of production medium cultured were treated with solid Ammonium sulphate (pH) at 20, 30, 40, 50, 60, 70, 80, 90 and 100% saturations and held for hours at 4 $^{\circ}$C with stirring. The mixture was centrifuged at 2000 rpm for 1 hour (4 ° C). The precipitates were recovered and suspended in 5ml of 50 mM sodium phosphate buffer at pH 7. The precipitated protein sample was subjected to salting out procedure.[24 -25]

2.7.2 Desalting of Proteins by Dialysis

Dialysis is commonly used for removing salts from proteins. The presence of salts in protein interferes in many ways. Hence semi-permeable membranes (0.4µm) called dialysis tubes are used which allow compounds with small molecular weight to pass through them and not those with high molecular weight as proteins are held back. The protein solution to be desalted was taken inside a dialysis bag and the two ends were secured tightly to prevent leakage. The bag was now suspended in a large vessel containing about 100 fold excess water preferably diluted in condition. Buffer containing 50 mM of Phosphate buffer (pH) was used for suspending the dialysis tubes. The salt molecules pass freely and get diluted by large volumes of fluid in the external medium. Repeated changes of dialysis fluid help in reducing the salt concentration inside the bags to negligible levels. To concentrate the protein, the sample in the dialysis bag was suspended in a sucrose solution where the water inside the sucrose being impermeable remains in the solution. The bag now contains desalted partially purified protein.[26]

2.8 *Enzyme Assay*

2.8.1 Lignin Peroxidase Assay

LiP proteins in the crude samples were precipitated by the addition of (NH_4) SO_4followed by centrifugation at 1000 rpm for 15 min. The pellet was dissolved in twice the original volume with demineralized water. The Lip activity was measured spectrophotometrically (310 nm, 30°C) by monitoring the production of Indole (9.300 M-1 CM-1)[25]. The reaction mixture (0.5 ml) contained 50 mM Sodium tartrate (pH 3.0), 2mM Tryptophan and a 50 l sample. The Lip activity was corrected for interferences before to the addition of H_2O_2 (0.5 ml) which was initiating the Lip assay. The lip activity is expressed in units per litre (1mmol of VAD min-1). Standard graph was prepared using iodine.[28]

2.8.2 Manganese Peroxidase Assay

Manganese peroxidase activity was determined using $MnSO_4$ as substrate. The assay mixture comprises 0.5mM $MnSO_4$ and 0.5 mM H_2O_2 in 50 mM Sodium malonate buffer pH 4.5.

Oxidation of Mn^{2+} to N^{3+} was followed by measuring the absorbance at 270nm,due to the formation of Mn^{3+}malonate (270=11,590 M-1 CM-1) Enzyme activity was expressed in one unit.[29]

2.9 *Separation of Protein by SDS-Polyacrylamide Gel*

Polyacrylamide gel electrophoresis is an efficient technique for the separation of proteins.[30]

2.9.1 Materials Required

PAGE system, Vaseline, brown tips, test tube, standard flask, measuring cylinder, pipette, tubes, micropipette.

2.9.2 Reagent preparation

10% Ammonium persulphate (APS) prepared freshly before use. Always use fresh APS for quick polymerization freshly taken from refrigerator SDS stored at temperature. The glass plates were cleaned by soaking overnight in detergent and washed thoroughly with water and dried.[31]Threeplexi spacers were placed beside the two side edges and across the bottom edge of the dry plate. Assembled the two glass plates and the three spacers tightly with clamps. The plates were placed vertically on the flat surface. For making gels, the gel mix was prepared by following composition- Table 1[32]

Table 1: Preparation of Separating Gel

Name of the Reagent	4	7.5	10	12.5	15
Solution A (ml)	1.3	2.5	3.3	4.1	8.0
Solution B (ml)	2.5	2.5	2.5	2.5	2.5
Distilled H_2O (ml)	6.2	5.0	4.2	3.4	2.5
APS (ml)	80	80	80	50	50
TEMED (ml)	5	5	5	5	5

After adding APS and TEMED to the gel solution, they were mixed well and poured the solution in between the glass plate of the performed chamber. The mixture is over layered with a thin layer of 0.1 SDS and was allowed to polymerize. After polymerization the SDS solution was decanted and the comb was placed and filled the space with a 3% slashing gel mixture. The stacking gel was allowed to polymerize for 30 minutes. The clamps and spacer at the bottom were removed and the comb was also removed carefully by sliding vertically upwards. The slab gel was assembled and electrophoresis buffer was added to lower and upper chambers. The samples were prepared and placed in boiling water bath for three minutes. The samples were loaded by using micropipettes, using a syringe with a bend needle and air bubbles were removed from the bottom of the gel. After completion, the gel plate was removed from the apparatus and the gel was washed with distilled water and stained with Coommassie blue for about two hours, after sufficient staining. The gel was destained using acetone to a desired period and was viewed under white light to identify the protein.[33]

2.10 *Purification of Treated Effluent* [7]

Treated effluent was detected with some waste debris. Small quantity of alum was added and all the wastes got settled in the bottom and the supernatant was collected with diluted water.[34]

2.11 *Seed Germination Ability of Biodegraded Sago Effluent*

2.11.1 Seed Germination on plate

The black gram (*Phaseolus mungo*) seeds were surface-sterilized in a 1% hypochlorite solution and washed three times in sterile distilled water. Fifty seeds were placed in sterilized glass Petri dishes of uniform size lined with two filter paper discs. These filter discs were then moistened with 5ml of distilled water for control and with the same quantity of various concentrations of the sago effluent (25%, 50%, 75% and 100%) in distilled water. Seeds were maintained in a controlled temperature incubator at 24°C and were kept moist as needed with 3-5ml of the appropriate treatment. Three replicates were carried out for each treatment and the germination studies were repeated three times. The seeds that germinated were counted and removed from the petri dish at the time of the first count on each day until there was no further germination.()[35-36]

2.11.2 Seed Germination on pot culture study

The black gram seeds (*Phaseolus mungo*) were surface sterilized with 1% hypochlorite solution and washed three times in distilled water. Then the seeds were soaked with four different concentrations of (25%, 50%, 75% and 100%) of sago effluent. Seeds were inoculated in the pot and added respective dilution of effluent and maintained moisture content of the soil. After 5 to 7 days seed germination percentage was calculated and compared with control.[37]

3. STATISTICAL ANALYSIS

All statistical analyses were calculated using Microsoft excel version 2007. The data was analyzed by germination value, calculated by using peak value multiplied with germination percentage. The peak value was determined by cumulative percentage germination on each day divided by no of days elapsed since initial inhibitions.

4. RESULTS AND DISCUSSION

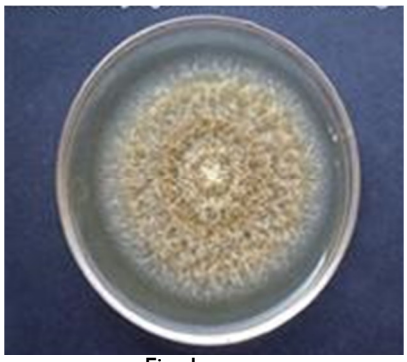

Fig. 1

Fig. 2

Fig 1:*P. chrysosporium*in Malt Extract Agar (MEA) growth medium;
Fig2: *P. chrysosporium*in Malt Extract Broth (MEB).

Figure No.1 & 2 Phanerochaete chrysosporium fungus was isolated from sago effluent by using Malt agar.

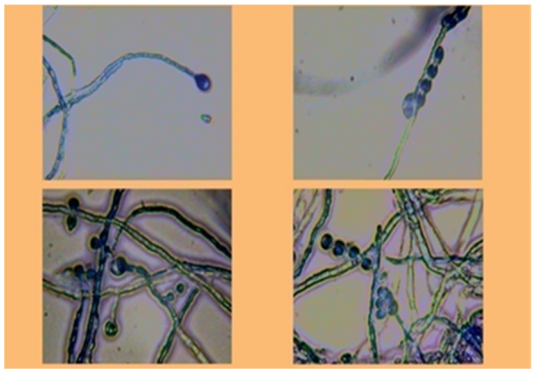

Fig. 3: Microscopic examination of white-rot fungus[38]

The morphological and enzyme production characteristics of isolates Figure. No: 3[39]

Lignin peroxidase and Manganese peroxidase are of importance since of extracellular enzyme that fungi excrete into the environment and the organisms may be useful in elucidating the control of exo-enzyme synthesis. In liquid state fermentation highest production of enzymes was achieved during 7 days to 40 days growth of *Phanerochaete chrysosporium*. The optimum concentration of Glucose for Lignin peroxidase and Manganese peroxidase enzyme production was found to be 6%. [40](Fig 4:). The separation of protein in SDS- PAGE was assessed and the result was exhibited in (Fig 5) When the NTG treated culture was grown on glucose medium, the amount of lignin peroxidase and manganese peroxidase showed a four-fold increase, partial purification of lignin peroxidase and manganese peroxidise was done by solvent precipitation absorption method and SDS page method. The precipitate while running in SDS-PAGE along with molecular weight marker, the precipitate gels separate as lignin peroxidase and Manganese peroxidase from the culture filtrate of *Phanerochaete chrysosporium*. The SDS-PAGE revealed distinct bands of various molecular weights (LiP molecular mass of 40 Kda, MnP molecular mass of 45 Kda.[41]The Lignin peroxidase increases at about 7th day and then decreases. On the other hand, Manganese peroxidase activity was almost undetectable before 7th day and then increased from 7th day onwards. (Fig 6)

Fig. 4: Optimization of glucose on the growth of *P. Chrysosporium* in MEA and MEB

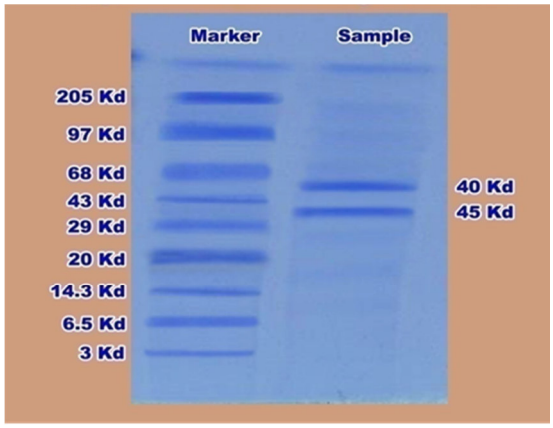

Fig 5: SDS PAGE analysis of crude protein from *Phanerochaete chrysosporium*

To investigate the performance of mutated *Phanerochaete chrysosporium* various concentrations of sago effluent were used[42] (25%, 50%, 75%, and 100%)

Fig 6: Biodegradation of sago effluent by *P. chrysosporium*

The decrease in all parameters was lower than in the case of inhibition performed in shaken flasks at 28°C. The *Phaseolus mungo* crop species differ widely in response to different concentrations of sago effluent with respect to germination percentage and Rate of Germination, and germination value. A lower concentration of the sago effluent didn't inhibit seed germination in *Phaseolus mungo.* In general, the germination percentage decreased with an increase in the concentration of the effluent. The germination was inhibited in all the seeds of *Phaselous mungo* studied with concentration exceeding 50% [43] (Fig. 7).

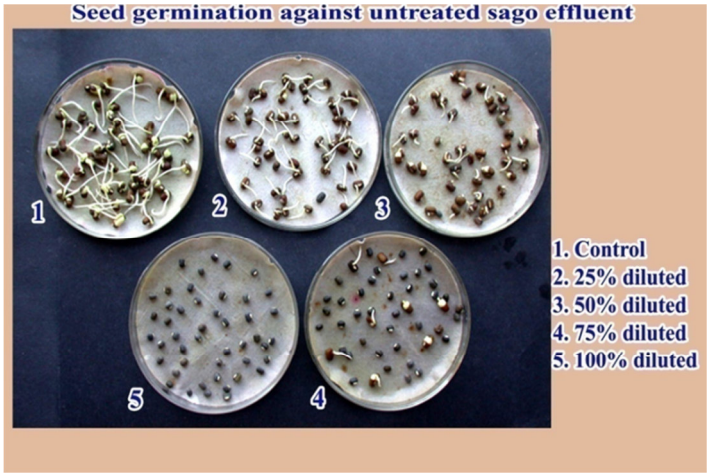

Fig 7: Seed Germination in sago effluent

The sago effluents used in the present study had a pH of around 4.5 high phenolic compounds and high cyanide irrespective of the degree of dilutions. Our results are consistent with findings of earlier work which observed a decrease in seed germination with increase of concentration of effluent. Interestingly, in 25% sago effluent source, the rate of germination, peak value, and germination percentage was more than untreated sago effluent (Table 2, 3).

Table 2: Effect of Different Concentration (v/v) of Treated Sago Effluent on Rate of Germination, Peak value and Germination Rate

Types of cultures	Control			25% Effluent			50% Effluent			75% Effeluent			100% Effluent		
	S	P	G	S	P	G	S	P	G	S	P	G	S	P	G
Wild type without Glucose	15.6	11.75	552.25	6.8	9.1	248.7	6.6	8.7	233.9	6.5	8.6	225.3	6.3	8.3	209.6
Wild type with Glucose	15.6	11.75	552.25	7.5	10	300	7.3	9.7	286.4	7.2	9.5	273.2	7	9.3	261.3
Mutant type with Glucose	15.6	11.75	552.25	8.1	10.8	354.9	8	10.6	341	7.8	10.4	326	7.6	10.2	312.2

Table 3: Effect of Untreated Sago Effluent on Seed Germination

Concentration	Rate of germination	Peak value	Germination Rate (mg/ l)
Control	15.6	11.75	552.25
25%	6.3	8.4	192.2
50%	1.5	2	12
75%	0.00	0.00	0.00
100%	0.00	0.00	0.00

50%, 75% and 100% treated sago effluent seeds showed marginal increase in their rate of germination and germination percentage more than untreated effluent (Fig. 8)

Fig 8: Seed germination in biodegradated sago effluent

The increase of germination and germination percentage in 25%, 50%, 75% and 100% treated effluent may be due to the loss of phenolic compounds, cyanides and inorganic compounds. The high surface-to-cell ratio of filamentous fungi makes them better degrade under certain niches like contaminated soils.

5. CONCLUSION

The majority of NTG-treated species with high glucose concentrations among the enzymes analyzed showed a substantial increase in sago effluent degradation. *Phanerochaete chrysosporium* was isolated and used for the treatment of sago effluent. The least amount of enzyme was produced by wild types in the absence of glucose. The concentration of glucose from the wild type of *Phanerochaete chrysosporium* could however increase the production of enzymes slightly .NTG has been used to increase the strain productivity for better biodegradation. The majority of NTG-treated species with high glucose concentrations among the enzymes analyzed showed a substantial increase in sago effluent degradation. The mutated species were to a greater degree efficient in destroying phenolic compounds and cyanide. The productivity of the biodegraded sago effluent increased the germination rate of seeds. Therefore the isolated *Phanerochaete chrysosporium* strain is able to grow using diluted sago effluent as its sole source of carbon and to reduce colour, chloride, phenolic material, COD, BOD and cyanide in particular, making it a good candidate for the effective treatment of this waste water. It is concluded that seed growth is inhibited by the effects of sago effluent. The effect of sago effluent was overcome by treatment with *Phanerochaete chrysosporium* effluent treatment. Owing to their higher organic loading, Sago processing industries discharge wastewater, which poses a danger to water bodies. In the global scenario, worries about pollution-related issues are convincing all production sectors to follow cleaner manufacturing practices. Whenever bioremediation statistics are debated, bacterial agents are concentrated and fungi are much less studied. However because of their aggressive growth, great production of biomass and extensive hyphae rich in soil, one should realise the greater potential of fungi. Biotechnological aspects will be more focused on future work. The highly efficient degradative enzyme generating genes can be cloned into bacteria and associated fungi and on the other hand, bacterial genes can be transferred to appropriate fungi. This research was therefore aimed at developing a cost-effective and environmentally-friendly treatment plant based on microbial enzymes to efficiently treat the effluent of the sago factory in order to reuse the water for industrial purposes. Fungi have been shown to solubilize coal, a highly polymeric material that is more complex than lignin. As a result, fungi are increasingly being used in future environmental bioremediation work.

6. AUTHORS CONTRIBUTION STATEMENT

Mr.K.Vivekanandhan gathered the data with regard to this work. Dr.Ayyappadas,M.P, Mrs.R.Renugadevi, Dr.S.K.Gangai Abirami, Dr.V.Subhapriya and Mrs.M.FloryShobana analyzed these data and suggestions were given towards the designing of the manuscript. Mr.K.Vivekanandhan and Dr.V.Manonmani have supervised the findings of this work. All authors discussed the methodology and results and contributed to the final manuscript.

7. CONFLICT OF INTEREST

Conflict of interest declared none.

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