International Journal of Botany Studies
www.botanyjournals.com
ISSN: 2455-541X, Impact Factor: RJIF 5.12
Received: 28-02-2021, Accepted: 02-04-2021, Published: 10-04-2021
Volume 6, Issue 2, 2021, Page No. 485-489

Isolation of cement degrading filamentous fungi and screening of their efficacy for biocementation

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Abstract
Cement dust is usually grey powder before mixed with other materials and water. It is one of the major source of environmental pollution. The purpose of the present study highlighted that degradation ability of filamentous fungi and efficacy of biocementation. Soil samples was collected from cement dumping sites in around Tiruvarur District, Tamilnadu, India. Soil samples are subjected to serial dilution and identification by Lactophenol Cotton blue mounting technique and standard manuals. Identified filamentous species are *Aspergillus Niger* and *Fusarium species.* Then it was allowed to grown in different cement concentration (1g, 2g, 3g, 4g, 5g) using silicate basal medium with optimized parameters such as pH, temperature, and inoculum concentration. Gravimetric estimation for weight reduction also preferred. Compared to *Fusarium species* urease positive *Aspergillus Niger* was able to produce gluconic acid at pH 7 and Biocementation production was also noticed. Gluconic acid production was characterized by paper chromatography (colorspot) and HPLC.

Keywords: cement, *Aspergillus Niger*, *Fusarium sp*, biocement

Introduction

Now-a-days cement and concrete industries are huge. These industries create many environmental and health issues. Hence the ultimate goal of this research work was planned to overcome problem of cement degradation and biocement production using environmental friendly and cost effective approach. Microbes play a vital role in this innovative degradation process and require interdisciplinary approach. Microorganisms bacteria, cyanobacteria, fungi, algae, and lichens-are liable to grow on building materials. Biological activity contributes to deterioration of building material and its interaction with physic-chemical mechanisms is responsible for long term deterioration [1]. Fungi are among the most harmful organisms associated to biodeterioration of organic and inorganic materials [2]. Cements used in construction are usually inorganic, often lime or calcium silicate based, and can be characterized as either hydraulic or non-hydraulic, depending on the ability of the cement to set in the presence of water (see hydraulic and non-hydraulic lime plaster). The fungi play a important role in the deterioration of concrete. Under humid condition the fungi biofilm was encouraged to grow on the surface of the normal concrete specimens and allowed to slowly degrade the concrete because of its interaction with the products of microbial metabolism [3]. Acid producing fungus also plays a big role in concrete biodeterioration[4] isolated a fungus from concrete samples and identified as *Fusarium* species and observed both weight loss and release of calcium when concrete was exposed to the isolate.Microorganisms can produce acids able to bring about the degradation of cement. Two acid producing alkalophilic fungal strains have been isolated from soil, a strain of *Aspergillus niger* which produce gluconic acid and oxalic acid and of *Mycelia sterila* which produces gluconic acid and malic acid. Biocementation is a process to produce binding material (biocement) based on microbial induced carbonate precipitation (MICP). Based on that the selected of microorganism should meet the criteria such as: i) have a high urease enzyme activity (for bacteria) ii) ammonium and calcium ion tolerable iii) not pathogenic [5]. Biocement is a product innovation from developing bioprocess technology called biocementation.

Materials and Methods

Sample Collection

Sample was collected from cement dumping area site in around Tiruvarur District, Taminadu, India.

Isolation of Filamentous Fungi

The soil samples were serially diluted and plated on Rose Bengal agar medium (pH 7.2) and Potato dextrose agar medium (pH5.6)

Identification of Filamentous Fungi

The filamentous fungi was identified by Lactophenol cotton blue staining technique and standard manuals.

Growth of Filamentous Fungi Isolate Cement as Substrate

The filamentous fungal growth rates are determined and inoculated into silicate fungi medium with different substrate.

Effects of Various Parameters on Growth of Cement Degrading Filamentous Fungi

Effect of pH-

Determine the p H level for filamentous fungal growth rate (2-8)

Effect of Temperature

Determine the optimum temperature for filamentous fungal growth rate. (25°c-40°c)

Effect of Inoculums Concentration
Determine the inoculums concentration for filamentous fungal growth rate. (0.5%-3%)

Gravimetric Determination of Cement
The cement cubes were first immersed in distilled water for 24 hours with fungal cultures. The samples were dried in an oven at 80°c for 3days and then weighed. Cement cubes were removed from the cultures for determination of weight changes.

Bioweathering of Cement Silicates
Determination of Silicate in Sample [6]
Mixing 0.2ml of sample followed by 5ml of ammonium molybdate. The sample is measured with UV spectrophotometer.

Production of Gluconic Acid by Filamentous Fungi [7]
Isolate filamentous fungi able to produce gluconate from glucose. The filamentous fungi is test in glucose containing basal medium. (pH 7).

Characterization of Gluconic Acid By Paper Chromatography [8] **and Hplc Analysis** [9]
The sample was employed n-butanol –formic acid- water. The paper were irrigated with 7-8hours at 25°c and sprayed with 0.04% bromeresol green.Acids appeared as yellow spot on blue background. The paper were sprayed again with 0.1% orthophenylenediamine and heated at 100°c for 5minutes. The gluconic acid appeared as yellowish green spot. The gluconic acid was determined in culture supernatant obtained after centrifugation.The crude supernatant were analysed by HPLC. Among the sample extracts HPLC for this system consisted of the following components: A pump (Lachrom Hitachi, Mumbai, Model no. L-7400, pump no. L-7110), a rheodyne injector (Cotati, CA, USA, Model 7725i), a kromasil C18 column (250 × 4.6 mm i.d., 5 µm particle size). The column was operated in isocratic mode (50:50 MeOH: H2O) at a flow rate of 0.5 ml/min. Unless otherwise specified, the UV detector was set at 210 nm for all operations.

Urease Activity
The fungal mycelia were filtered by filter paper. Mycelia recovered by filtration were washed three times with 0.1M potassium phosphate buffer (pH 7) and resulting suspension was used for urease activity.

Phenol Hyphochlorite Assay Method [10]
The urease positive isolate filamentous fungi was further tested for urease activity. This was determined in the media according to the phenol hypochlorite assay method. Ammonium chloride (50-100µM) was used as standard.

Screening of Biocementation [11]
500ml water samples was taken as the volume of 100ml filtered urea and 100ml inoculumswere added and incubated for 1week at 37°c.After incubation, the calcium carbonate was filtered. After filtration the paper is kept in hot air oven at 35°c for 6hours.

Results

Isolation of Cement Degrading Filamentous Fungi from Cement Dumped Area Site
Cement dumping soil samples were taken from Tiruvarur district, Tamil nadu for this study. From the soil samples different fungal species were isolated and identified.

Isolation of Filamentous Fungi
The soil samples were serially diluted and plated on Rose Bengal agar medium and potato dextrose agar medium and then plates were incubated.

Identification of Filamentous Fungi
On the Rose Bengal agar medium, the organism was identified as *Aspergillus niger*.On the potato dextrose agar medium, the organism was identified as *Fusarium species.* (Table 1)

Growth of Filamentous Fungi Isolate Cement as Substrate
Determine the optimum medium cement substrate concentration for *Aspergillus niger* and *Fusarium species* growth rate. *In Aspergillus niger* maximum growth rate was observed substrate concentration 3g attained at the OD of 0.78. In *Fusarium species* maximum growth rate was observed in substrate concentration 3g attained at the OD of 0.72. (Table 2)

Effect of Various Parameters on the Growth of Cement Degrading Filamentous Fungi
Effect of pH
Determine the optimum medium pH for *Aspergillus niger* and *Fusarium* species**.**Growth rates were recorded. In *Aspergillus niger* pH 6 attained at the OD of 0.69. In *Fusarium* species pH 6 attained at the OD of 0.78. (Table 3)

Effect of Temperature
Determine the optimum medium temperature for *Aspergillus niger* and *Fusarium* species growth rates were recorded. In *Aspergillus niger* temperature 25°c attained at the OD of 0.71. In *Fusarium species t*emperature 25°c attained at the OD of 0.75. (Table 4)

Effect of Inoculum Concentration
Determine the optimum medium inoculums concentration for *Aspergillus niger* and *Fusarium species* growth rates were recorded. In *Aspergillus niger* inoculums concentration at 2% attained at the OD of 0.62. In *Fusarium species* inoculums concentrations 2% attained at the OD of 0.59. (Table 5)

Gravimetric Determination of Cement
The cement cubes were first immersed in distilled water for 24 hours so that drying procedure would start with water – saturated samples. The samples were dried in an oven at 80°c for 3days and then weighed. At various times during incubation with the fungal cultures, cement cubes were removed from the cultures for determination of weight changes. *Aspergillus niger* degrade the cement and reduce the weight more than *Fusarium species.* (Table 6)

Determination of Silicate in the Sample
The ammonium molybdate yellow analysis was performed by mixing 0.2ml of sample (*Aspergillus niger, Fusarium*

sp). H_2SO_4 followed by 0.3M ammonium molybdate. The sample was allowed to react with 5minutes and then it was measured at 400nm with UV spectrophotometer.

Production of Gluconic Acid by *Aspergillus Niger*

Aspergillus Niger were able to produce gluconate from glucose.The fungi was to be tested in glucose containing basal medium. It was found that dissolution of silicates in these cases resulted from the complexation of the cationic components of the silicates by gluconate. The growth activity appears on glucose basal medium. Gluconic acid is characterized by paper chromatography and HPLC.

Biocement Formation

Phenol Hypho Chlorite Assay Method

The urease positive isolates were further tested for the urease activity. Optical density was measured at 626 nm and one unit of urease is defined as the amount of enzyme hydrolyzing 1µmol urea/min. The growth profile studied up to 24, 48, 72, 96, 120 hours.It was observed from graph that in *Aspergillus niger* the optical density has increased up till 48h which is 0.67 respectively which keep on decreasing up till 120hours which is 0.49.(Table 7)

Crystal Nucleation Site Development at Biocement

500ml of water samples was taken as the volume of 100ml (3M) concentrations of filtered urea and 100ml inoculums were added and incubated for one week at 37ºc

. After incubation the deposits of calcium carbonate was filtered using normal filter paper. After filtration the paper containing the deposits is kept inside the hot air oven at 35ºc for 6 hours. The *Aspergillus niger* precipitated at similar rates but produced whitish and transparent crystal aggregates.

Table 1: Morphological characteristics of isolated filamentous fungi

Species	Morphological characteristics
Aspergillus niger	Growth begins initially as a yellow colony that developed black into a dotted surface as conidia were produced with age, the colony become color. Long conidiospores support spherical vesicles of brown rough walled conidia.
Fusarium sp	Colonies are usually fast growing pale or bright colored with or without cottony aerial mycelium. The color of the thallus varies from whitish to yellow, pink, red or purple shades.

Table 2: Effect of substrate

S. No	Substrate concentration (g)	Optical density (450nm)	
		Aspergillus niger	***Fusarium sp***
1.	1	0.73	0.71
2.	2	0.74	0.66
3.	3	0.78	0.72
4.	4	0.77	0.69
5.	5	0.76	0.67

Table 3: Effect of pH

S.NO	pH	Optical density (450nm)	
		Aspergillus niger	***Fusarium sp***
1.	2	0.49	0.59
2.	3	0.52	0.64
3.	4	0.58	0.69
4.	5	0.64	0.75
5.	6	0.69	0.78
6.	7	0.65	0.76
7.	8	0.63	0.74

Table 4: Effect of Temperature

S.NO	Temperature(ºc)	Optical density (450nm)	
		Aspergillus niger	***Fusarium sp***
1.	25	0.71	0.75
2.	30	0.69	0.68
3.	35	0.65	0.64
4.	40	0.60	0.59

Table 5: Effect of Inoculum Concentration

S.NO	Inoculum concentration (%)	Optical density (450nm)	
		Aspergillus niger	***Fusarium sp***
1.	0.5	0.55	0.51
2.	1	0.58	0.52
3.	1.5	0.61	0.58
4.	2	0.62	0.59
5.	2.5	0.57	0.56
6.	3	0.53	0.54

Table 6: Weight Reduction of cement cubes

S.NO	Weight Reduction (hours)	*Aspergillus niger* (g)	*Fusarium sp* (g)
1.	Control	2	2
2.	24	1.9	1.7
3.	48	1.7	1.5
4.	72	1.5	1.3

Table 7: Phenol hyphochlorite assay method

S.NO	Hours	Optical density(626nm)
		Aspergillus niger
1.	24	0.59
2.	48	0.67
3.	72	0.63
4.	96	0.53
5.	120	0.49

Discussion

In the present investigation suggested that the cement degradation by fungi. Fungi are a natural part of our environment and play an important in decomposition of organic matter. They can grow on almost any building material if there is enough moisture available and cause damage to the structure [12]. *A.niger* which produce gluconic acid and oxalic acid. *Fusarium* which produce gluconic acid.Among the fungal cultures tested, *A. Niger* demonstrated the highest capacity for concrete deterioration. Electron microscopic studies of concrete exposed to *A.niger* over one year showed spalling years covered with a mycelial net, cracking and the formation of abundant crystals on the concrete surfaces and encrusting fungal hyphae. The fungus *Fusarium sp* is equally capable of degrading concrete. A wide range of acids are produced by fungi, including acetic, oxalic, glucuronic acids [13]. In addition to forming insoluble calcium complexes, these acids can be utilized by the microorganisms. They are capable of etching and extending the fungal hyphae into the interior of the concrete, resulting in enlargement of the damaged area and an increase in porosity. These observations coupled with the calcium release and weight loss suggest that organic acids produced by fungi may be responsible for the deterioration. Weight loss, after removal of precipitates, indicates that more calcium complexes were formed in the presence of fungi. The results are correlated was to evaluate the potentiality of urease production by *Aspergillus Niger* strains to select the best urease producer among them also we compared a simple and reliable screening method for urease production with the conventional methods. Our findings similar to isolation of cement degrading bacteria and screening of their efficacy for biocementation by [14] *Bacillus species* which grow in different cement concentrations as a sole nutrient source the maximum growth rate was observed at 3g supplementation of cement. In our Results highlighted that *Aspergillus Niger* has the best cement degradation capacity than *Fusarium species*.

Conclusion

In our study highlighted that efficient urease positive filamentous *Aspergillus niger* has the best cement degradation capacity than *Fusarium species*.In conclusion the involvement of filamentous fungi in concrete degradation was investigated. Maximum growth rate was observed as 3g substrate and OD values (0.78) p H level 6, Temperature 25°c, Inoculum at 2% concentration was attained for *Aspergillus Niger*. Weight reduction also greater in *Aspergillus Niger* compared to *Fusarium species.* Urease positive ability of *Aspergillus niger* produce more gluconic acid and characterized by paper chromatography in terms of colorspot detection and crude supernatant was analysed by HPLC with a retention time (peak). Bio cementation was also noticed as whitish and transparent crystal aggregates. Our study suggested that organic acid produced by *Aspergillus Niger* and *Fusarium species* and also form soluble calcium complexes with the concrete, resulting in dissolution. Further research is to be needed to identify indigenous fungi for degradation and overcome the limitations faced in this research.

Acknowledgement

The authors are thankful to Tamilnadu State Council for Science and Technology,(TNSCST), Chennai for Funding.

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