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Isolation and optimization of Bacillus strains for self healing concrete

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ABSTRACT: Concrete has become one of the most common material in the construction sector worldwide and the demand for the cement is increasing every year but most of the structures are prone to cracking. Cracks in the concrete are inevitable and are one of the weakness of concrete. Water and other salts seep through these cracks and tend to disfigure, destroy the structure and thus reduces the life of concrete. So, there was a need to develop an inherent biomaterial, a self repairing material, which can successfully remediate cracks in concrete. The development of self healing concrete technology has become one of most important aspect in the field of construction.

A total of two thousand seventy colonies were observed and fifty five Bacillus colonies were isolated and identified by Gram staining and different biochemical tests. From the results of optimization, it was observed that Nutrient agar media was the best supportive for the growth of Bacillus strains at optimum temperature 40°C. Among different carbon and nitrogen sources tested, cellulose and ammonium sulphate were utilized as best carbon and nitrogen source. Among metal ions and surfactants studied, ferrous chloride and Tween-80 supported best growth of Bacillus species and selected Bacillus isolates showed good growth in static condition.

KEYWORDS: Bacterial concrete, Self Healing, cracks, optimization, Bacillus

I. INTRODUCTION:

Concrete is a strong durable material composed of cement, aggregate and water is the most used building material in the world. Concrete is the most widely used man made construction material. It has specialty of being cast in any desirable shape but plain concrete, however possesses very low tensile strength, limited ductility and little resistance to cracking (Chahal et. al, 2012). Cracks in the concrete form a major problem which effects the durability of the structure, modifications have been made from time to time to overcome such difficulties, a self repairing bio material has developed that can remediate the cracks and fissures in concrete (Ramchandran et al, 2001). The application of bacteria for the repair or maintenance of various materials is not new. In previous studies the potential of bacteria to clean concrete surfaces (De Greaf et al, 2005) improved the strength of cement, sand and mortar (Dick et al, 2006; Gosh et al, 2005), repair of degraded lime stones and ornament stone surfaces (Rodriguez-Navarro et al, 2003) and crack repair on surfaces of concrete structures was investigated (Holt,2001 ; Bang, 2001). This paper mainly focuses on the optimization of selected bacterial strains which are to be used in the strengthening of concrete. Optimization is the process of finding the greatest or least value of a function for some constraint, which must be true regardless of the solution. Alternatively, it means the best possible solution for a given problem under defined set of constraints. Purpose of optimization is to achieve the "best" design relative to a set of prioritized criteria or constraints.

II. MATERIAL AND METHODS:

All the chemicals used in the present investigations were purchased from Sigma Aldrich and Hi Media companies, Mumbai, India. Soil samples were collected from brick kilns, furnaces, rocks of different areas of Warangal District, Telangana, India and analyzed for the growth of different bacteria by using nutrient agar media (NAM), calcium carbonate precipitating media ($CaCO_3$) and urease agar media (UAM). Four Bacillus colonies thus isolated were morphologically, physiologically and biochemical characterized with the help of keys provided in Bergey's Manual of Systemic Bacteriology (Holt, 2001) and confirmed by precise molecular identification using 16S rRNA sequencing analysis. These sequences were submitted in National Centre for Biotechnology Information (NCBI GenBank Accession Numbers – MN809595, MN849173, MN849426 and MN849881).

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To obtain maximum growth conditions of Bacillus, the following conditions were optimized and the results are presented in Tables 1–8. Optimization studies were used to determine the medium components which optimize their concentrations and cultivation conditions that support the growth of the selected strains. To optimize the conditions for the better growth of four selected strains of Bacillus species were analyzed by changing the following conditions such as media, temperature, pH, carbon, nitrogen sources, metal ions, surfactants, static and shaking conditions. The growth of the strains was calculated in growth units (O.D) by using UV visible spectrophotometer.

III. RESULTS:

Media: For media optimization studies, three different media were taken i.e, nutrient agar media (NAM), calcium carbonate precipitating media ($CaCO_3$) and urease agar media (UAM). Among four isolated strains of Bacillus, Bacillus anthracis showed good growth in NAM and $CaCO_3$ precipitating media where as less growth was recorded in urease agar medium, Bacillus thuringenisis strain showed good growth in NAM but no growth in urease and calcium carbonate precipitating media. Bacillus albus showed growth only in calcium carbonate precipitating media, however Bacillus mycoides showed growth in all the media tried (Table-1).

Table -1: Effect of media on four strains of Bacillus species

STRAIN	NAM	$CaCO_3$ Media	Urease Media
Bacillus thuringenisis	0.988	0.013	0.020
Bacillus albus	0.152	0.813	0.144
Bacillus mycoides	0.997	0.825	0.775
Bacillus anthracis	0.988	0.832	0.012

Temperature: Growth and survival of microorganisms are greatly affected by the temperature of the environment. The temperature required for the good growth of all the four strains was 40˚C i.e., all the selected four strains showed better growth upto the temperature of 50˚C and growth decreased substantially after 50˚C (Table-2).

Table -2: Effect of temperature on four strains of Bacillus species

Organism	20˚C	30˚C	40˚C	50˚C	60˚C	70˚C
Bacillus thuringenisis	1.092	0.900	1.637	1.192	0.049	0.024
Bacillus albus	1.127	1.074	1.755	1.649	0.783	0.484
Bacillus mycoides	1.092	0.694	1.510	1.293	0.056	0.048
Bacillus anthracis	1.729	1.642	2.041	1.168	0.058	0.029

In general the growth rate was high in 40˚C and was considered to be optimum for the growth of Bacillus species. Bacillus anthracis recorded maximum growth (2.041) at 40˚C followed by Bacillus albus (1.755).

pH : Bacillus species showed their diversity in growth with variation in pH. Too acidic or too alkaline pH are not supported for good growth of Bacillus strains .The maximum growth rates ranges in between pH 6-10. Bacillus thuringenisis showed its maximum growth at pH 7 (1.279) while Bacillus albus recoded maximum growth at pH 10 (0.895). Strain Bacillus mycoides showed its maximum growth at pH 8 (1.218) and Bacillus anthracis showed its maximum growth at pH 7 (1.696), (Table -3).

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Table-3: Effect of pH on four strains of Bacillus species

Organism	pH										
	3	4	5	6	7	8	9	10	11	12	13
Bacillus thuringenisis	0.418	0.433	0.478	1.278	1.279	0.875	0.811	0.716	0.683	0.661	0.542
Bacillus albus	0.227	0.292	0.320	0.452	0.455	0.891	0.893	0.895	0.544	0.322	0.058
Bacillus mycoides	0.030	0.039	1.086	1.113	1.215	1.218	1.221	0.994	0.778	0.477	0.312
Bacillus anthracis	0.033	0.055	1.590	1.681	1.696	1.646	1.582	0.895	0.222	0.176	0.026

Carbon sources: Six types of carbon sources were optimized for the selected four strains. Cellulose, Glucose, Lactose, Mannitol, Sucrose and Maltose are the carbon sources used for optimization studies. From the table it was evident that the growth rate of Bacillus species varied substantially with carbon sources. Mannitol was the carbon source supported for maximum growth of Bacillus thuringenisis (0.602), while Bacillus albus and Bacillus anthracis recorded their maximum growth with lactose and showed their growth rates 0.612 and 1.392 respectively (Table-4).

Table-4: Influence of carbon sources on the growth of four strains of Bacillus species

Organism	Cellulose	Glucose	Sucrose	Lactose	Mannitol	Maltose
Bacillus thuringenisis	0.242	0.121	0.168	0.067	0.602	0.013
Bacillus albus	0.282	0.146	0.149	0.162	0.125	0.149
Bacillus mycoides	0.352	0.407	0.023	0.763	0.769	0.272
Bacillus anthracis	0.591	0.301	0.218	1.392	1.291	1.182

Nitrogen sources: Six types of nitrogen sources i.e, Ammonium sulphate, Gelatin, Yeast extract, Sodium nitrate, Potassium nitrate, and Casein were used for the optimization studies. From the table it was evident that the growth rate of Bacillus species varied substantially with nitrogen sources. Casien was the nitrogen source supported for maximum growth of Bacillus thuringenisis (0.800) and Bacillus albus (0.742), while Bacillus mycoides recorded its growth rate at 0.860 with Ammonium sulphate ,the maximum growth rate of Bacillus anthracis was noticed with Gelatin as a carbon source (0.497) (Table-5).

Table-5: Influence of nitrogen sources on the growth of four strains of Bacillus species

Organism	Ammonium sulphate	Gelatin	Yeast extract	Sodium nitrate	Potassium nitrate	Casein
Bacillus thuringenisis	0.380	0.415	0.445	0.074	0.063	0.800
Bacillus albus	0.072	0.509	0.325	0.058	0.128	0.742
Bacillus mycoides	0.860	0.097	0.146	0.192	0.699	0.698
Bacillus anthracis	0.398	0.497	0.125	0.136	0.176	0.182

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Metal ions : Different metals ions like Al, Ba, Co, Fe, Zn and K were tested for their effect on the growth of four Bacillus strains. All these metals were taken in the chloride form. From the table it was evident that growth rate of Bacillus thuringenisis (0.913), Bacillus albus (1.127) was best supported with ferrous chloride and Bacillus mycoides (0.309) recorded maximum growth with barium chloride while Bacillus anthracis (0.518) recorded maximum growth rate with zinc chloride (Table -6).

Table-6: Influence of metal ions on the growth of four strains of Bacillus species

Organism	Ammonium chloride	Barium chloride	Cobalt chloride	Ferrous chloride	Zinc chloride	Potassium chloride
Bacillus thuringenisis	0.385	0.426	0.453	0.913	0.285	0.162
Bacillus albus	0.611	0.046	0.197	1.127	0.011	0.084
Bacillus mycoides	0.204	0.309	0.148	0.079	0.204	0.189
Bacillus anthracis	0.316	0.328	0.262	0.093	0.518	0.383

Surfactants : To determine the effect of surfactants supplement such as Tween-20 , Tween -80 and Trition -100 in the media for the growth of different strains were analyzed and the results are presented in Table-7. Bacillus thuringenisis (1.102), Bacillus albus (1.579) showed better growth after utilizing Tween-80. Bacillus mycoides (0.665) and Bacillus anthracis (0.693) were best reactive for Tween 20.

Table-7: Influence of surfactants on the growth of four strains of Bacillus species

Organism	TWEEN-20	TWEEN-80	TRITION-100
Bacillus thuringenisis	0.377	1.102	0.301
Bacillus albus	0.507	1.579	0.176
Bacillus mycoides	0.665	0.403	0.182
Bacillus anthracis	0.693	0.500	0.176

Static and Shaking conditions : After observing the growth of selected strains in both static and shaking conditions, Bacillus mycoides (1.579) and Bacillus albus (1.199), showed best growth in static conditions, whereas Bacillus thuringenisis (1.847) and Bacillus anthracis (1.579) showed best growth in shaking conditions at 120 rpm, 37˚C (Table-8).

Table-8: Influence of static and shaking conditions on four strains of Bacillus species

Organism	STATIC	SHAKING
Bacillus thuringenisis	0.949	1.847
Bacillus albus	1.919	1.618
Bacillus mycoides	1.579	1.278
Bacillus anthracis	1.574	1.579

IV. DISCUSSION:

The genus Bacillus has been mostly used for the biological development of calcium carbonate based minerals as, which is considered as an ureolytic bacteria. The formation of calcium carbonate by using this type of bacteria is because of the hydrolysis of urea to carbon dioxide and ammonia. Addition of urea is extremely recommended because bacteria are acknowledged to hydrolyze urea by urease to increase the general pH (Burne and Marquis 2000), exploit it as a nitrogen source (Burne and Chen, 2001) and consume it as a reserve of energy (Mobley and Hausinger, 1989).

The isolated bacteria were screened quantitatively by gram staining, endospore staining and for the urease test. All the isolated bacteria of the present study were identified as Bacillus and most of the calcifying bacteria belong to the Bacillus genera. The bacterium was studied for calcification and Bacillus species was used for the concrete applications (Ercole et al, 2007). In this investigation culturing conditions such as growth media, pH, temperature and different sources are focused. Media optimization was done by three media i.e, calcium carbonate precipitating media, nutrient agar media and urease agar media. Nutrient agar media was considered as the best and most common culture media for the bacteria and yeast extract was the best media which was used as a nitrogen source for maximum mass cell production (Whiffin, 2004; Al-Thawadi, 2008; Henriques, 2011).

pH is an important factor which influences calcite precipitation. Urea hydrolysis will happen, once urease enzyme will attain specific pH values which would result in Calcite precipitation, which is subjective to pH. Many researchers have stated that the optimum pH in favor of urease is 8.0, beyond which the activity of enzyme dwindles (Fischer et al, 1999; Gorospe et al, 2013). An increased pH is essential for ammonia via urea hydrolysis. Cell breathing allows aerobic bacteria to release CO_2 , which is complemented by a boost in pH owing to ammonia creation (Ng et al, 2012). The carbonate is liable to liquefy than to precipitate if the pH levels reduce (Loewenthal and Marais, 1978). pH of the selected strains varied in different ranges .The optimum pH of the growth medium for the bacterium was 7.0, although it was found that the bacteria can grow well at pH 11. The pH of the environment affects microorganism and microbial enzymes and also influence the dissociation and solubility of many molecules that indirectly influence microorganism (Black., 2015). The pH range of several isolates which were active and stable in alkaline environments from pH 9-11 (Fischer et al, 1999). The optimum pH of the growth medium for the bacterium was 7.5, although it was found that the bacterium can grow well at pH 11, above this pH the bacterial growth was inhibited to some extent (Gosh and Mandal, 2006). The bacterial isolates which were used for the biocementation were showed growth in pH range 7 to 9 (Jagadeesh kumar et al, 2013).

Temperature plays an important role in catalysis process of urea by means of urease. The most favorable temperature stretches from 20 to 37 ^{0} C (Okwadha and Li, 2010). Urease is totally stable and steady at 35^0 C (Dhami et al, 2014), however beyond 55 ^{0} C there is 47% decline in enzyme activity. Bacteria have a characteristic optimal growth temperature at which it exhibits the highest growth and reproduction rate. At 40-50˚C temperature the isolates showed better growth rate and found to be alive at -3˚C low temperature to 70˚C high temperature (Maniknandan and Padmavathi, 2015).

V. CONCLUSION:

From the results it was evident that from the optimization studies, cellulose is considered as the best carbon source, Ammonium sulphate as nitrogen source, Ferrous chloride as the metal ion source showed better growth for utilization and Tween-80 as surfactant source and almost all the isolates showed better growth in static conditions where as some of the isolates showed growth even in shaking conditions also.

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