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Screening of Glycolic Acid Producing Chemolithotroph from Neyveli and Kanjamalai Mines in India

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ABSTRACT

In the present study chemolithotrophs producing glycolic acid were isolated from unexplored low grade iron ore (magnetite quartzite) and Lignite mining sites mines of Kanjamalai hill and Neyveli respectively in India. A total of twenty nine soil and three rock samples were taken from different location and screened for sulfur and iron oxidizing chemolithotrophs. The soil samples were inoculated into medium containing ferrous sulphate at pH 2.0 and incubated for 3 days at 30°C. Thirty three isolates were screened to utilize ferrous sulphate. Among the thirty three isolates, thirteen isolates were Gram positive short rods and twenty were of pleomorphic forms. All the thirty three isolates were found to be spore-formers and non-motile. A rapid and simple spectrophotometric assay was done to screen the production of glycolic acid by these isolates. It was observed that all the thirty three isolates produced glycolic acid and a maximum of 0.11mg/mL was produced by KM13 isolate.

1.Introduction

Glycolic acid, the simplest alpha hydroxy acid, is one of the most important fine chemicals. Due to its unique properties it has wide applications in adhesives, metal cleaning, textiles, leather processing, biodegradable polymers, and cosmetic products [28]. The chemical synthesis of glycolic acid has certain disadvantages such as production of undesirable toxic byproducts. Alternatively the biological process is more advantageous than chemical process as it does not produce any byproducts. The biological production of glycolic acid includes biotransformation and production of glycolic acid using microbes. Currently several research on biotransformation using microorganisms has been focused. Bacteria such as *Gluconobacter oxydans* DSM, *Acidovorax facilis* 72, *Brevibacterium casei*, *Gordona*, *Rhodococcus rhodochrous* and *Alcaligenes sp*. ECU0401 were used for this purpose. However the main limitation of this process is the end-product inhibition which results in low production yield [11, 27]. Therefore screening of efficient glycolic acid producing bacteria is a suitable choice for biological production. Recently Ivan Ñancucheo and Barrie Johnson 2010 [15] reported that glycolic acid was produced by three chemolithotrophic bacteria (*L. ferriphilum*, *At. ferrooxidans*, and *At. caldus*) as exudates from actively growing cells.

Chemolithotrophs include bacteria that are capable of oxidizing ferrous iron and sulfur that have been isolated from different mining environment. There has been a great increase in our knowledge in understanding the diversity of the microbiology of biomining environments, and it's potential. The diversity of such microorganisms is far more diverse than that was recognized even a couple of decades ago [18]. India is a country which is rich in minerals and the microbial communities are yet to be exploited in many mining areas. Therefore attempts were made to isolate chemolithotrophs from different mining sites in India. In coal, sulfur occurs both in organic form (thiol, sulfide and thiophene compounds) and in inorganic form, (pyrite and elemental sulfur). The sulfur content in coal varies widely from 0.5% to 11% that are sufficient to support microbial population [16]. Iron is one of the abundant element in the earth's crust and are present in the form of oxides such as Magnetite – Fe3O4 (72.4%Fe and 27.7%O); Haematite-Fe2O3 (69.4% Fe, 31.06% O) and pyrite-FeS2 (46.6% Fe and 53.4% S) [13].

Chemolithotrophic bacteria present in such environments differ in morphology, nutrition (metabolism of inorganic and organic substrates) and growth characteristics [4, 25, 14, 22].. However, their important feature is the ability to oxidize Fe^{2+}, S^{o} and sulfide minerals. Sulfur-oxidizing bacteria such as *Acidithiobacillus thiooxidans*, *Thiobacillus thioparus*, *Sulfobacillus* and iron-oxidizing bacteria such as *Acidithiobacillus ferrooxidans* have been detected in acid sulfate soils rich in pyrite [26, 19, 9]. A number of reports suggested the prevalence of *Sulfobacillus* in coal spoil heaps, acidic geothermal springs and sulfide mine heaps and dumps [1, 6]. Gram-positive sulfur-oxidizing bacteria, including *S. acidophilus* and *S. thermosulfidooxidans* can grow autotrophically (exclusively using inorganic carbon), heterotrophically (using only organic carbon) or mixotrophically (simultaneously using organic and inorganic carbon) [29, 2]. The main goal of this study is isolation of chemolithotrophs from lignite and magnetite mines of Neyveli and Kanjamalai of India. Later the chemolithotrophs were screened for glycolic acid production using a simple spectrophotometric method.

2. Materials and methods

2.1 Sample collection

Samples were collected at a depth of 1cm square from different location in sterile polythene bags labeled properly and transferred to the laboratory for further processing. A total of twenty soil samples and one rock were taken from mining

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sites of kanjamalai hill (Latitude 11° North 39 minutes and Longitude 78° East 10 minutes). This region is made of low grade iron ore (magnetite quartzite) which is of metasedimentary origin. Nine soils and two rock samples were collected from lignite mines of Neyveli (Latitude of 13˚North 12 minutes and Longitude 79˚ East 54 minutes, India. Lignite is the younger offspring of the coal family belonging to the Miocene age (25 million years) and popularly known as Brown Coal.

2.2 Screening and isolation of ferrous sulphate oxidizing chemolithotrophs

Samples (1gm) were suspended in 10 ml of 0.9% (w/v) sterile NaCl solution and allowed to settle for 10 min. 2ml of the suspension was then transferred to a flask containing 30 ml of sterilized growth medium. The composition of the growth media was varied depending on the nutritional requirements of the isolates. A common minimal salt medium supplemented with yeast extract but without glucose (K_2HPO_4 3.0 g/l, KH_2PO_4 3.0g/l, $(NH_4)HPO_4$ 1.2g/l, $MgSO_4$ 0.24g/l, Fe_2SO_4 1.6 g/l, Yeast extract 0.02% (w/v), pH 2.2) was used for isolation of heterotrophic organisms [20]. Mixotrophic organisms were isolated on a similar medium of pH 2.5 but supplemented with glucose (5mM) and Yeast extract (0.001%). Isolation in 9k medium **(Solution A:** $FeSO_4$.7H2O 33 g/l, pH 2.5, distilled water 300 ml; **Solution B:** KH_2PO_4 0. 4 g/l, $CaCl_2$ 0.2 g/l, $MgSO_4$ 0.4 g/l, $(NH4)_2SO_4$ 0.4 g/l, pH 3, distilled water-700 ml) was also performed [23]. For preparation of 9K medium solution B was prepared separately and sterilized, the filter sterilized solution A was added aseptically. The pH was adjusted to 1.5- 2.0 using 10% sulphuric acid. The above enrichment procedure was repeated three times in order to make the isolates to adapt to the environment.

2.3 Characterization of chemolithotrophic bacteria

Conventional phenotypic characterization assays for each isolates (Gram stain, spore stain, catalase and oxidase) were carried out as described in Bergey's Manual of Systematic Bacteriology. The growth rate of the isolates was studied by observing the growth pattern in minimal medium up to 72 hours. About 0.1 ml of the sample was withdrawn every 2 hours and counted with the help of a Neubauer chamber.

2.4 Estimation of glycolic acid

The sample was centrifuged at 6000rpm for 20 minutes and the supernatant was collected. To 15µl of the supernatant 1mL of freshly prepared naphthol (100 mg of β-naphthol was dissolved in 92.5% sulfuric acid) was added. It was then incubated in boiling water bath for 20 min until a marked yellow- green color developed [12]. Then 4ml of distilled water was added to each sample with mild vortexing and the optical density of the samples was measured at 473 nm using a double beam Systronics spectrophotometer. All the experiment in this work was carried out in triplicates and the mean±standard deviation was reported.

3. Result and discussion

3.1 Isolation of chemolithotrophic acidophiles

Chemolithotrophs are mainly seen in mining environment which is rich in inorganic compounds and acidic condition in addition facilitates dissolution of elements present in ores. India is one of the few countries that are endowed with vast reserves of minerals such as iron ores, manganese, chromite, lignite etc. However the most challenging issues in India's mining sector is the lack of assessment of these natural resources [21]. Similarly the microbial diversities in areas such as the Kanjamalai and Neyveli mining sites in Tamilnadu still remain unexplored. The role of chemolithotrophic bacteria in such environment are yet to be emphasized. In this regard chemolithotrophs were isolated from lignite and magnetite mines which are rich in organic and inorganic carbon, nitrogen, potassium, phosphorus and sulfur. In the present study twenty isolates were obtained from Kanjamalai and designated as KM 1, KM 2…KM20, for soil and one rock sample as KMR 1. Similarly ten isolates were named as NVL1, NVL 2…NVL 10 for soil NVR 1 and NVR 2 for two rock samples from Neyveli after enrichment culture. After transferring the samples to the laboratory the color and pH of the soil samples were noted. The color varied from black, brown, grey, red, orange to pale yellow, light brown and pale white. The pH of the samples was mostly 6.0 indicating slight acidic nature of the soil.

Table 1. Cultivation of acidophiles in media with different specification.

SAMPLE NAME	9K MEDIUM	MINIMAL SALT MEDIUM	
		GLUCOSE +YEAST EXTRACT	WITHOUT GLUCOSE+ YEAST EXTRACT
NV-1	+	+++	+
NV-2	+	+	+
NV-3	+	+	+
NV-4	+	+	+
NV-5	+	++	+
NV-6	+	+	+
NV-7	+	+	+
NV-8	+	+	+
NV-9	+	+++	+
NV-10	+	+	+
NVR-1	+	+	+
NVR-2	+	+	+
KMR-1	+	+	+
KM-1	+	+	+
KM-2	+	+	+
KM-3	+	+++	+
KM-4	+	+	+
KM-5	+	+	+
KM-6	+	+	+
KM-7	+	+	+
KM-8	+	++	+
KM-9	+	+	+
KM-10	+	+	+
KM-11	+	+	+
KM-12	+	+	+
KM-13	+	+++	+
KM-14	+	+	+
KM-15	+	+	+
KM-16	+	+	+
KM-17	+	+	+
KM-18	+	+	+
KM-19	+	++	+
KM-20	+	+	+

Key: (+) - 1.1×10^2 to 1.9×10^3 cells; (++) - 2.4×10^4 to 3.1×10^5 ; cells (+++) - 3.4×10^5 to 4.1×10^6 cells

Table 2. Presumptive identification of chemolithotrophic acidophiles based on their phenotypic characteristics.

SAMPLE NAME	GRAM'S STAINING	MORPHOLOGY	CATALASE	OXIDASE	MOTILITY	SPORE STAINING
NV-1	+	STRAIGHT	+	+	-	+
		RODS				
NV-2	+	STRAIGHT	+	+	-	+
		RODS				
NV-3	+	STRAIGHT	+	+	-	+
		RODS				
NV-4	+	STRAIGHT	+	+	-	+
		RODS				
NV-5	+	PLEOMORPHIC	-	+	-	+
NV-6	+	PLEOMORPHIC	+	+	-	+
NV-7	+	PLEOMORPHIC	+	+	-	+
NV-8	+	PLEOMORPHIC	+	+	-	+
NV-9	+	PLEOMORPHIC	-	+	-	+
NV-10	+	PLEOMORPHIC	+	+	-	+
NVR-1	+	PLEOMORPHIC	+	+	-	+
NVR-2	+	PLEOMORPHIC	+	+	-	+
KMR-1	+	STRAIGHT	+	+	-	+
		RODS				
KM-1	+	PLEOMORPHIC	+	+	-	+
KM-2	+	PLEOMORPHIC	+	+	-	+
KM-3	+	PLEOMORPHIC	-	+	-	+
KM-4	+	STRAIGHT	+	+	-	+
		RODS				
KM-5	+	STRAIGHT	+	+	-	+
		RODS				
KM-6	+	STRAIGHT	-	-	-	+
		RODS				
KM-7	+	PLEOMORPHIC	+	+	-	+
KM-8	+	PLEOMORPHIC	+	+	-	+
KM-9	+	PLEOMORPHIC	+	+	-	+
KM-10	+	STRAIGHT	+	+	-	+
		RODS				
KM-11	+	PLEUMORPHIC	-	+	-	+
KM-12	+	STRAIGHT	+	+	-	+
		RODS				
KM-13	+	PLEOMORPHIC	+	+	-	+
KM-14	+	STRAIGHT	+	+	-	+
		RODS				
KM-15	+	STRAIGHT	+	+	-	+
		RODS				
KM-16	+	PLEOMORPHIC	+	+	-	+
KM-17	+	STRAIGHT	+	+	-	+
		RODS				
KM-18	+	PLEOMORPHIC	+	+	-	+
KM-19	+	PLEOMORPHIC	+	+	-	+
KM-20	+	PLEOMORPHIC	+	+	-	+

Key (+) positive, (-) negative

3.2 Screening of ferrous sulphate oxidizing chemolithotrophs

All the isolates were able to grow in ferrous sulphate supplemented medium with pH 2.0, 2.2 and 2.5. But luxuriant growth was seen in media supplemented with yeast extract (0.02%) when added as growth factor. The growth of the chemolithotrophic bacteria was observed in terms of cell count [Table 1].

4.5 Characterization of chemolithotrophic bacteria

Only a few acidophiles such as *Acidithiobacillus ferrooxidans*, the *Sulfobacillus* species, *Thiomonas*, *Alicyclobacillus* species and some archaea can utilize a broad range of energy substrates, including iron (II), sulfur and minerals in sulfides environments [24, 10]. In this study all the isolates were observed to be Gram positive, spore formers and non-motile and twenty nine isolates showed both catalase and oxidase positive (Table 2). Therefore these characteristics correlate with the genus *Sulfobacillus* sp. however; molecular biological methods are the key factor in the complete identification of bacteria. The capability to utilize substrates, and oxidize iron (II) and sulfur at acidic condition indicates that they may be of ecological significance in bioleaching environment [7]. The growth pattern of each isolates showed maximum growth at 48 hours *(data not shown). The capacity to form endospores indicates that it can adapt better to survive in adverse condition [3].

4.7 Estimation of glycolic acid

The measured absorbance of the standardized solutions was plotted against the known concentrations of glycolic acid in the range of 0.5–2.5µg.The slope and intercept value for the linear relationship of the standard graph was determined and it was found that relationship between *x* and *y* variables is linear and the *coefficient of determination* $r^2 = 0.994$ indicates very strong relationship as the correlation greater than 0.8 are considered strong. The unknown concentration of the sample was determined by extrapolating against the known concentration of glycolic acid. Glycolic acid was detected in all the isolates after 48 hours, out of thirty three isolate KM 13 produced glycolic acid at high concentration of 0.11mg/mL as represented in Table 3.

Table 3. Estimation of glycolic acid using spectrophotometric assay.

Isolate	Mean (Optical density)	Standard deviation
NV 9	1.610667	0.016921
NVR 2	0.300333	0.002082
KM 3	0.202	0.002
KM 12	1.601	0.020881
KM 13	1.816	0.013077

Mean value calculated for the triplicates

The error associated with the spectrophotometer determination of values was determined using one-way ANOVA (Table 4-5), regression (Table 6) and rank correlation to check the mean values, standard deviation and 95 % confidence intervals.

Table 4. Anova Single Factor Absorbance versus sample Summary.

Groups	Count	Sum	Average	Variance
Sample	5	15	3	2.5
Absorb	5	0.68	0.136	0.00088

Anova

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	20.50624	1	20.50624	16.39922	0.003686	5.317655
Within Groups	10.00352	8	1.25044			
Total	30.50976	9				

Table 5. Anova Single Factor Concentration versus sample summary.

Groups	Count	Sum	Average	Variance
Sam	5	15	3	2.5
conc1	5	5.575	1.115	0.631438

ANOVA

Source of Variation	SS	Df	MS	F	P-value	F crit
Between Groups	8.883063	1	8.883063	5.673473	0.044413	5.317655
Within Groups	12.52575	8	1.565719			
Total	21.40881	9				

Table 6. Regression analysis: Absorbance versus concentration.

Regression Statistics	
R Square	0.963429
Adjusted R Square	0.951239
Standard Error	0.006551
Observations	5

ANOVA								
					Significance F			
	Df	SS	MS	F				
			0.00339	79.0327				
Regression	1	0.003391	1	4	0.003001			
Residual	3	0.000129	4.29E-05					
Total	4	0.00352						
	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%	Lower 95.0%	Upper 95.0%
Intercept	0.095144	0.00545	17.4573	0.00041	0.077799	0.11248	0.07779	0.11248
x axis	0.036643	0.004122	8.89003	0.00300	0.023525	0.04976	0.02352	0.04976

Residual Output

Observation	Predicted y axis	Residuals
1	0.154688	-0.00469
2	0.106136	0.003864
3	0.102472	-0.00247
4	0.154688	-0.00469
5	0.162016	0.007984

Pearson correlation of concentration and absorbance was determined and the value 0.981544 obtained suggested that strong relationship exist between the concentration and absorbance. A further statiscal analysis was carried out using regression which also indicated there is a relationship between absorbance and concentration. Standard deviation is the statistical method used in determining the accuracy of a spectrophotometer. From the statistical values obtained it was evident that as $p < 0.05$ it was significant [8]. In addition 95% confidence interval also attributed that the values obtained from spectrophotometer was accurate. The use of spectrophotometer method offers a few advantages over the conventional chromatographic analysis. This assay can be easily performed and less time consuming as immersion in a boiling water bath enhances color change rapidly from nearly colorless to yellow-green in the initial 5 minutes.

In general chemolithotrophic bacteria use Calvin-Benson-Bassham (CBB) pathways or reductive tricarboxylic acid (rTCA) cycle for assimilating carbon dioxide. Genes involved in the CBB cycle are reported to be present in *Sulfobacillus acidophilus* [5, 17]. A key enzyme present in the CBB cycle is ribulose bisphosphate carboxylase/ oxygenase (RuBisCO). This enzyme generally combines with ribulose bisphosphate (RUBP) in the presence of carbon dioxide. In addition it can also oxidize RUBP to phosphoglyceric acid and phosphoglycolate. Enzymatic hydrolysis of the latter produces glycolate, which is exported out of actively-growing cells. This may be the possible reason for detection of glycolic acid in such chemolithotrophs.

4. Conclusions

Acidophilic chemolithotrophic bacteria lose significant amounts of carbon when they fix carbon dioxide as exudates during active growth as well due to lysis of cells. In this present study, glycolic acid was obtained from actively growing chemolithotrophic bacteria isolated from lignite and magnetite quartzite mines in India. A simple and rapid screening method was followed based on a spectrophotometer reaction of glycolic acid produced by isolates with β-naphthol in sulfuric acid solution. This technique was claimed to be free from interference from commonly-encountered low molecular weight organic acids. As per our knowledge this is the first report on chemolithotrophs isolated from low grade iron ore (magnetite quartzite) mines of Kanjamalai hill, in India. Such fundamental knowledge on microbial diversity will assist in understanding the microbial behavior in mining sites. Further primary screening of ideal glycolic acid producing acidophiles will be more useful because biological process is more effective than chemical process.

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