INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 15, No. 2, p. 475-489, 2019

RESEARCH PAPER OPEN ACCESS

Cold-adapted halotolerant *Rhodococcus* sp. BGI-11, a potential candidate for biotechnological applications

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Key words: Baturaglacier, Cold-adapted bacteria, Halotolerant, Enzymes, Antibiotic resistance.

http://dx.doi.org/10.12692/ijb/15.2.475-489 Article published on August 30, 2019

Abstract

Rhodococcus sp. BGI-11 was isolated among 7 otherpsychrotrophic bacteria from ice of Batura glacier in the mighty Karakoram Range. Strain BGI-11 was selected among the rest of glacial isolates on the basis of its variable growth characteristics as well as its ability to utilize hydrocarbons as carbon and energy source. Phylogenetic analysis using 16S rRNA gene revealed the strain is most closely related to *Rhodococcuserythropolis* with a similarity index of 99.76%. BGI-11 was able to grow at a temperature range of 4-35°C, pH 4-11 and salinity up to 8% (w/v). Results for carbon utilization revealed the strain is able to use glucose, sucrose, maltose, galactose and lactose sugars. This strain also demonstrated the ability to use glycerol and molasses as growth substrates. The strain demonstrated growth in all the 8 culture media tested including, nutrient rich and the selective media. BGI-11 exhibited antimicrobial activity against the multidrug resistant strains of *Bacillus* sp. and *candida albicans*. BGI-11 also demonstrated positive activity for lipase and DNase enzymes. Antibiotic susceptibility pattern revealed the strain is resistant to methicillin and trimethoprim/sulfamethxazole while sensitive to imipenem, vancomycin, and ofloxacin. The most exciting physiological characteristics exhibited by this strain was its ability to use long chain hydrocarbon hexadecane as a carbon substrate. Thus, the cold-adapted halotolerant*Rhodococcus* sp. BGI-11 can be used as a potential candidate for various biotechnological applications. BGI-11 can further be used for bioremediation of hydrocarbons in deep oceans, an environment also characterized by low temperature and moderate salinity.

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Introduction

Among the extreme environments on the earth, low temperature contributes the majority. Around 85% of the earth is permanently or periodically exposed to temperatures below 5˚C. Deep oceans makes a large part of this cold environments as 71% of the earth is covered by ocean and 90% of its volume is permanently below 5˚C, followed by Arctic and Antarctic polar regions, the high mountains and cave system (Margesin and Miteva, 2011). These extreme environments have been successfully inhibited by bacteria and other microorganisms which not only survive but actively metabolize at such low temperatures. Bacteria able to grow at low temperatures or near the freezing point of water have been known since the nineteenth century. Since major part of the earth is permanently exposed tolow temperatures, therefore it is obvious that cold inhabiting microorganisms play a significant role in global ecology. Morita (1975) defined psychrotrophs as bacteria with wide growth-temperature range, being able to grow near freezing point of water but have an optimal growth temperature above 20˚C. They are thus distinct from true psychrophiles, which grow optimally at 15˚C or lower and are rarely able to grow at temperatures above 20˚C (Morita, 1975). The psychrotrophs are often found in environments with seasonal temperature fluctuations, such as the sub-Arctic regions and are phylogenetically diverse as compared to true psychrophiles (Margesin *et al.*, 2007).

Glacier accounts for 10% of the land surface and is considered as one of the harsh environments on biosphere. Microorganisms inhabiting cold environments encounter some challenges such as extreme low temperatures which may ranges from -56°C to -10°C. Other limitations include low nutrient and water availability, reduced enzymatic reaction rates, darkness and salinity (Margesin and Miteva, 2011).These extreme environments are inhabited by microorganisms either in dormant and vegetative forms and adapted to this extremely cold temperature by evolving different mechanisms for survival(Feller and Gerday, 2003; Fedotova and Dmitrieva, 2016; Olijve *et al.*, 2016; Kralova, 2017).

Psychrophiles have great potential in biotechnological exploration. Thus a strong interest is developing in cold-adapted microorganisms, resulting in isolation and screening for potential novel microbial species and its metabolites. Within the past decade, an increased number of bacteria which also includes novel species from permanently cold environments have been isolated and characterized including, permafrost (Bakermans *et al.*, 2003), glacier ice (Miteva *et al.*, 2004), Arctic sea ice (Junge *et al.*, 2002), and Antarctica cryoconite hole (Christner *et al.*, 2003). Enzymes from the cold-active microorganisms have plentiful applications in the textile, food, detergent, and animal feed industry (Gerday *et al.*, 2000), nevertheless this field is still at its infancy as a small portion of organisms producing these enzymes have been identified and characterized.

Petroleum hydrocarbons are one of the major environmental pollutants which contaminate terrestrial and freshwater through accidental spills or shipping activities (Arulazhagan *et al.*, 2010). Petroleum mainly consists of alkanes while n-hexadecane is a major component of alkanes. Alkanes are highly inflammable but one of the least reactive among organic compounds. The successful bioremediation of alkanes such as hexadecane is dependent on accessibility of this compound to the degrading microorganisms, inherent biodegradability of the pollutant and optimum condition for the degradation process (Kebria *et al.*, 2009). Low molecular weight alkanes are degraded rapidly as compared to long chain and multiple branched alkanes (Stroud *et al.*, 2007).

The indigenous cold-adapted microorganisms play a key role in biodegradation of petroleum products and other pollutants in low temperature environments. This low temperature favors the growth of psychrophiles/psychrotrophs and hence the degradation activity. The aerobic biodegradation of a large number of petroleum components at low

temperatures has been reported in Arctic, Antarctic and alpine regions. These component includes n-alkanes, aromatic and polycyclic aromatic hydrocarbons (Margesin and Schinner, 2001). Cold inhabiting microorganisms have a tremendous potential for applications in bioremediation processes as they harbors enzymes which maintains high catalytic activity and unusual specificity at low temperatures. Introduction of specific cold-adapted microorganisms in mixed cultures in contaminated environments is expected to improve biodegradation of organic pollutants.

The northern area of Pakistan consists of tangled mountains including the western Himalayas, Karakoram and Hindukush rangesand considered as world's most heavily glaciated area outside of the Polar Regions. These ranges in Pakistani geographical boundary host more than 5000 glaciers and feed water to the Indus River System (Rasul *et al*., 2011). Batura glacier with a length of~59 km (Hewitt, 2013) is one of the longest glacier outside the polar region. The glacier is located in Passu village of Hunza valley, in the Karakorum Range of Pakistan. Glaciers in the Karakoram Range of Pakistan are least explored in terms of their microbial diversity and potential for industrial applications.

The aim of this study was to explore some of the physiological characteristics of the cold-adaptedstrain BGI-11, with potential industrial applications. BGI-11 was selected for further studies among the 8 glacial isolates based on its ability to use hexadecane as carbon source.Also initial screening on plates demonstrated the strain could grow well at low temperature, moderate salinity, and extremes of pH.The ability to utilize long chain hydrocarbon (hexadecane) as carbon source and the polyextremophilic nature of this strain resulted in its further study. Working with psychrophilic bacteria or their enzyme saves energy as they maintain high catalytic activity at low temperatures which negates expensive heating processes required to work with their mesophilic counterparts. The strain also demonstrated activities for enzymes and antimicrobial compounds. All these characteristics makes BGI-11 a potential candidate for biotechnological applications and bioremediations of petroleum hydrocarbons.

Materials and methods

Enrichmentof ice sample and isolation of BGI-11

BGI-11 was isolated among 7 other bacteria from the ice of Batura glacier through sample enrichment method (unpublished). Plating of direct or diluted sample did not yield any colonies even after prolong incubations for several months at 4°C and 15°C.For enrichment of the sample, 10 ml melted ice was added to 90 ml of R2A, TSB and LB broth and incubated at 4°C and 15°C in a shaker incubator. Once the broth media turned turbid, 100 µl samples from each inoculating media were plated on their respective agar medium (unpublished). Morphologically different colonies observed were streaked on separate agar plates containing same medium to obtain their pure culture,which also includedisolate BGI-11.

Identification of isolate BGI-11

Primary identification of isolate BGI-11 was done on the basis of colony morphology, cell morphology, pigmentation, and biochemical characteristics. Isolate BGI-11 was later identified through 16S rRNAgene sequencing.

DNA extraction, 16S rRNA sequencing and phylogenetic analysis

DNA was extracted using UltraClean microbial DNA isolation kit (MO BIO Laboratories), according to the manufacturer instructions. Amplification of 16S rRNA gene was performed through PCR using universal primers 27F and 1492R. The alignment was thoroughly analyzed and the ambiguous aligned regions were removed from the sequence using Chromas (version 2.6.6). 16S rRNA gene sequence of BGI-11 and the most similar sequences were identified through BLAST in the EzBiocloud database (Yoon *et al*., 2017). 16S rRNA gene sequence of BGI-11 was deposited in the NCBI GenBank database with the accession number MK522045. Finally, phylogenetic tree was constructed for BGI-11 using

neighbor joining method with bootstrap values in the MEGA 7 software (Saitou and Nei, 1987).

Physiological characterization of Rhodococcus sp. BGI-11

Growth at different temperatures

Temperature growth range of the *Rhodococcus* sp.BGI-11 was checked at 5-45°Cwith an increment of 5 on R2A agar plate in duplicates. Result interpretation was done after incubating plates at their respective temperatures for one week (Miteva *et al.*, 2004; Zhang *et al.*, 2013). Growth was also checked in liquid culture at 5, 15, 25, 35 and 45˚C. Overnight culture of BGI-11 in exponential phase of growth was used for inoculation and flasks were incubated in shaker incubators at 150rpm. Optical density (OD_{600}) was used daily to monitor the growth for 6 days in a microplate reader (SpectraMax M5).

Growth at different pH

Growth of the *Rhodococcus* sp.BGI-11 was checked at different pH ranging from 3 to 13 with an increment of 1 on R2A agar plates in duplicates. Result interpretation was done after incubating plates at 15˚C for one week (Shivaji *et al.*, 2013; Zhang *et al.*, 2013).pH optimization was also performed in the broth culture.

Overnight culture of BGI-11 was inoculated in R2A broth with pH ranging from 4 – 11. All the flasks were incubated at 15˚C in a refrigerated shaker incubator at 150 rpm. Optical density (OD_{600}) was used every day for 6 days to monitor the growth and readings were taken in a microplate reader.

Salt tolerance

Rhodococcus sp.BGI-11 was checked for its ability to grow under halophilic conditions. Both broth culture and plate techniques were used. For plate streaking method, the *Rhodococcus* sp.BGI-11 wasstreaked on R2A agar plates supplemented with 2, 4, 6, 8, and 10% NaCl (w/v) and incubated at 15°C. Growth was checked after an incubation time of one week (Shivaji *et al.*, 2013; Zhang *et al.*, 2013). Isolate BGI-11 was also grown in broth culture supplemented with different concentration of salt up to 10% (w/v). Flasks were kept in shaker incubator at 15˚C and optical density was measured in a microplate reader daily up to 7 days.

Growth on different media

Growth on different media were checked using nutrient rich, oligotrophic and selective media. The media included R2A (Difco), tryptic soya agar (Oxoid), nutrient agar (Oxoid), Luria Bertani agar (Oxoid), SaboraudDextrose agar (Oxoid), mannitol saltagar (Oxoid), MacConkey agar (Oxoid) and Müeller Hinton agar (Oxoid). Growth on all media were assessed by streaking *Rhodococcus* sp.BGI-11 on the respective agar plates and were incubated at 15˚C for one week (Zhang *et al.*, 2013).

Utilization of carbon sources

The ability of *Rhodococcus* sp.BGI-11 to use different sugars as carbon source was assessed in R2A broth. The sugars used were glucose, galactose, sucrose, maltose and lactose. R2A broth was prepared without adding dextrose and instead all these sugars at a concentration 2% (w/v) were added. *Rhodococcus* sp.BGI-11 culture in log phase was used as inoculum and flasks were incubated at 15˚C in shaker incubator (150 rpm) for one week. Optical density (OD_{600}) was used every day to monitor growth in microplate reader. Ability of *Rhodococcus* sp.BGI-11 to use molasses and glycerol as carbon substrates was also checked in a similar method discussed for sugars utilization.

Antibiotic resistance/Antibiotic susceptibility testing

Disc diffusion method (Bauer *et al.*, 1966) was used for the susceptibility test of *Rhodococcus* sp.BGI-11 to antibiotics using Mueller-Hinton agar plate.BGI-11 inoculumwas standardized to 10^8 CFU/ml McFarland standard. Two narrow spectrum antibiotics including methicillin (10 µg) and vancomycin (5 µg); 3 broad spectrum antibiotics including,imipenem (10 µg), ofloxacin (5 µg), and trimethoprim sulfamethaxazole (30 µg) were used. Plates were incubated at 15˚C and results were interpreted after 24 to 48 hours. The susceptibility of *Rhodococcus* sp.BGI-11 to each

antibiotic was determined from measurement of the zone of inhibition.

Enzyme activity

Rhodococcus sp.BGI-11 was screened for its ability to produce extracellular enzymes. Lipase activity was checked by streaking *Rhodococcus* sp.BGI-11 on R2A supplemented with 1% Tween-40 along with 0.001% rhodamine B and 0.01% $CaCl_2$ (Booth, 1971). Formation of opaque hazy zone of calcium soap crystals around the growth indicated hydrolysis of the Tweens. For amylase production, starch hydrolysis test was performed by the method used preciously (Priest, 1977). Briefly, *Rhodococcus* sp.BGI-11 was streaked on R2A agar surface supplemented with 1% starch. Plates were incubated at 15˚C for 05 days. Lugol's iodine solution was flooded on the surface of the plates and observed for change in color.

The areas of the medium containing unhydrolysed starch was stained dark purple while hydrolyzedzones around growth were clear. Carboxymethylcellulose (CMC) (0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.2% carboxymethylcellulose sodium salt, 0.02% peptone and 1.7% agar) agar plates were used to test cellulase activity (Kasana *et al.*, 2008). *Rhodococcus* sp.BGI-11was spot inoculated on CMC agar and plates were incubated at 15˚C for 05 days.Plates were later flooded with Gram's iodine for 3 to 5 minutes. Zone of clearance around the growth indicates positive for cellulase production. Protease activity was checked by the method described previously with some modifications (Priest, 1977).

Colonies of *Rhodococcus* sp.BGI-11were streaked on the R2A agar supplemented with 1% casein. Plates were then incubated at 15˚C for 05 days and flooded with 1M acetic acid. Clear zone of hydrolysis around the growth demonstrated positive for protease production. For DNase test, isolates were spot inoculated on the surface of DNase agar and plates were incubated at 15˚C for 05 days. Plates were then flooded with 1N hydrochloric acid. Hydrolysis of DNA is indicated by clear zone around the area ofgrowth.

Antimicrobialactivity

Antimicrobial activity of *Rhodococcus* sp.BGI-11 was determined through spot inoculation method. The indicator microorganisms included the multidrug resistance strains of *Candida albicans*, *Klebsiellapneumoniae*, *Acinetobacter* sp., and *Bacillus* sp. while two ATCC strains,*Pseudomonas aeruginosa*(ATCC27853) and *Staphylococcus aureus*(ATCC6538) were also used. Mueller-Hinton agar plates were swabbed with overnight culture of indicator organisms and *Rhodococcus* sp.BGI-11 was spot inoculated on the plates. The plates were incubated at 15˚C for 3 to 5 days and observed for a clear zone around the *Rhodococcus* sp.BGI-11 growth.

Utilization of hexadecane as carbon substrate

Rhodococcus sp.BGI-11 was tested for its ability to degrade hexadecane. Mineral salt medium (MSM) was used as growth medium with hexadecane as the only carbon source. MSM was prepared as described previously (4 g L^{-1} NH_4Cl, 2.5 g L^{-1} K_2HPO_4, 0.5 g L^{-1} NaCl, 0.3 g L^{-1} $MgSO_4.7H_2O$, 0.03 g L^{-1} $FeCl_3.6H_2O$, 0.01 g L^{-1} $CaCl_2$, 0.01 g L^{-1} $MnCl_2.4H_2O$) (Chayabutra and Ju, 2000).Two different concentrations of hexadecane were used, i.e. 0.5% and 1% (v/v). MSMwith hexadecane was inoculated with a suspension of *Rhodococcus* sp.BGI-11 cells, which were pre-grown in R2A broth. The cells were centrifuged and washed 3 times in normal saline to remove any traces of R2A broth. All the flasks were incubated at 15˚C in a shaker incubator at 150 rpm. Growth ($O.D_{600}$) was checked after every 48 hours for 10 days in a microplate reader. Dry weight was also measured at each time point after drying the sample in a lyophilizer (FreeZone 2.5, LABCONO). Two controls were used in the experiment including, MSM with no addition of hexadecane and un-inoculated MSM with hexadecane addition. The second control was added to make sure that degradation is caused by the inoculated strain*Rhodococcus* sp.BGI-11.

Results and discussion

Present study involved isolation of a cold-adapted bacterium from glacier in the Karakoram to explore some of its metabolic potential for industrial and environmental applications. Compared to the other

cold environments, glaciers in the high Karakorum are less explored in terms of their microbial diversity and functionality.

Colony, cell morphologies and biochemical tests

The isolate BGI-11 was isolated from the ice of Batura glacier through sample enrichment technique. Colonies with morphology similar to BGI-11 were present in abundant on the agar plates. The colonies of BGI-11 appeared milky, raised and circular on tryptic soy agar plates (Fig. 1). Gram staining revealed BGI-11 as Gram-positive with coccobacilli cell morphology. Isolate BGI-11 demonstrated positive results for catalase production and citrate utilization while negative for nitrate reduction (Table 1).

Table 1. Physiological and biochemical characteristics of BGI-11.

Temperature Range (˚C)	pH Range	NaCl Range (%)	Biochemical Properties			Cell Morphology (Gram Staining)
			Citrate Utilization	Catalase Test	Nitrate Reduction	
4-35	4-11	0 – 8	+	+	-	+ve (coccobacilli)

Table 2. Growth of*Rhodococcus* sp. BGI-11 in oligotrophic, nutrient rich and selective culture media.

R2A	Luria Bertani Agar	Tryptic Soy Agar	Nutrient Agar	Sabouraud Dextrose Agar	Mannitol Salt Agar	MacConkey Agar	Mueller Hinton Agar
+	+	+	+	+	+	+	+

Molecular identification through 16S rRNAgene based phylogenetic analysis

The 16S rRNAgene sequence of BGI-11 was submitted to EzBioclouddatabase to get the most closely hit strains. BLAST results revealed BGI-11 as closely related to *Rhodococcuserythropolis* species with similarity index >99%. Phylogenetic tree was also constructed and the bootstrap value for BGI-11 and *Rhodococcuserythropolis*NBRC 15567 was 100 (Fig. 2). *Rhodococcus*is widely distributed and cold-adapted species have been routinely reported from cold environments (Rapp *et al*., 2003; Ruberto *et al*., 2005; Maharana and Singh, 2018).

Table 3. Antimicrobial activities demonstrated by *Rhodococcus* sp. BGI-11 against multidrug resistant and ATCC strains using spot inoculation method at 15˚C.

Antimicrobial activity (mm)					
Staphylococcus aureus	*Pseudomonas aeruginosa*	*Bacillus* sp.	*Klebsiella pneumoniae*	*Candida albicans*	*Acinetobacter* sp.
-	-	17.32	-	25.87	-

Table 4. Extracellular enzyme activity demonstrated by *Rhodococcus* sp.BGI-11 using plate assay at 15˚C.

Enzyme activities				
Lipases	Proteases	Cellulases	Amylases	DNases
+	-	-	-	+

Growth characteristics of Rhodococcus sp.BGI-11 at different environmental and nutritional conditions

This strain grewwell at a temperature range of 4-35˚C. Growth pattern was similar at 15, 25 and 35˚C during the first 24 hours, demonstrating the exponential phase of the growth. Growth was slow at 4˚C during the first 24 hours and increased significantly after 48 hours and afterwards. No growth was observed at 45˚C (Fig.3a). Stationary phase was reached during 72 hours for temperatures 15, 25, and 35˚C. Optimum temperature was found above 20˚C. Temperature optimization on agar plates also indicated a wide temperature range for growth, with no growth visible on plates at 45˚C.).

Table 5. Antibiotic susceptibility test of *Rhodococcus* sp.BGI-11 against narrow and broad spectrum antibiotics using disc diffusion method at 15˚C.

Antibiotics (Zone of inhibition in mm)				
Methicillin (10 µg)	Imipenem (10 µg)	Ofloxacin (5 µg)	Vancomycin (5 µg)	Trimethoprim Sulfamethxazole (30 µg)
R	19.80	31.27	27.66	R

According to Morita's (1975) definition, *Rhodococcus* sp. BGI-11 falls in the category of psychrotrophs as it demonstrated a wide growth temperature range (4-35°C). This is also true as unlike deep sea, terrestrial cold environments have more temperature fluctuation, which results in high abundance of the psychrotrophic organisms than true psychrophiles (Russell, 1990).

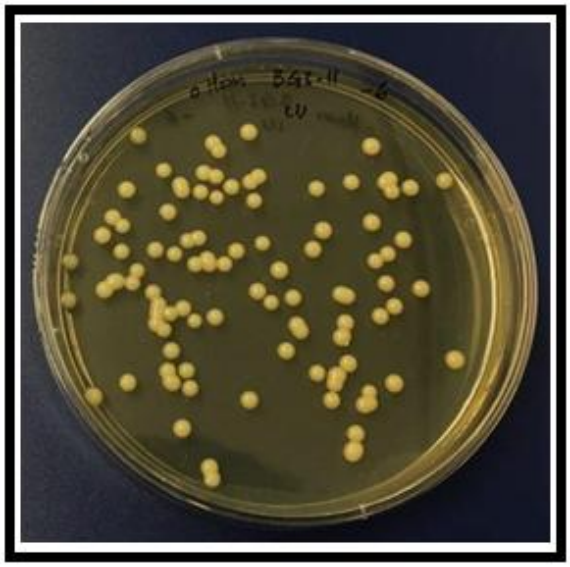

Fig. 1. Pure culture of isolate BGI-11 colonies on tryptic soy agar plate.

Rhodococcus sp.BGI-11 demonstrated good growth at a pH range of 4-11 in broth media. Growth curve pattern was similar at pH 6-11. However,slow growth in the first 24 hours was observed at pH 4 and 5 (Fig. 3b). Stationary phase was reached during 72 hours for pH 6-11. Increase in growth was observed at low pH after 24 hours of incubation.Plate streaking results also demonstrated a wide pH range for *Rhodococcus* sp.BGI-11. On the plates BGI-11 produced visible colonies at the extremes of pH including 3 and 13. *Rhodococcus* sp.BGI-11 demonstrated good growth at a salinity range up to 7% in the broth media. The maximum optical density was achieved at 1-5% NaCl during 72 hours. Growth was also observed at 10% NaCl but could not reach high optical density even after one week (Fig. 4a). *Rhodococcus* sp.BGI-11 produced visible colonies on agar plates supplemented with 8% NaCl, indicating a moderate salinity range. The strain was therefore, considered as moderate halophile or a halotolerant. *Rhodococcus* species have been reported previously from saline environments (Zolfaghar*et al*., 2019).

The results for temperature, pH and salinity ranges for the strain *Rhodococcus* sp. BGI-11 is very much in accordance with a previous report, working with *Rhodococcus erythropolis* (De Carvalho, 2012). This *Rhodococcus* sp. was able to grow at temperature 4-37˚C, pH 3-11 and salinity up to 7.5% (De Carvalho, 2012).

Rhodococcus sp.BGI-11 demonstrated great versatility in utilization of sugars as growth substrate. The strain was able to use glucose, galactose, lactose, maltose and sucrose as carbon source. The growth pattern was similar in all the sugars supplemented media. Growth with high cell densities were observed for all the sugars (Fig. 4b). A similar result was reported by Munaganti *et al*., (2015) for a *Rhodococcus* species.

Their results also revealed utilization of glucose, galactose, lactose, maltose and sucrose as carbon source by the *Rhodococcus* species used in their study. Likewise BGI-11, their *Rhodococcus* strain also exhibited positive activity for catalase and negative for nitrate reduction.

Glycerol and sugar waste molasses were used as substrate for the growth of *Rhodococcus* sp.BGI-11. The strain demonstrated good growth in glycerol and molasses (Fig. 5).

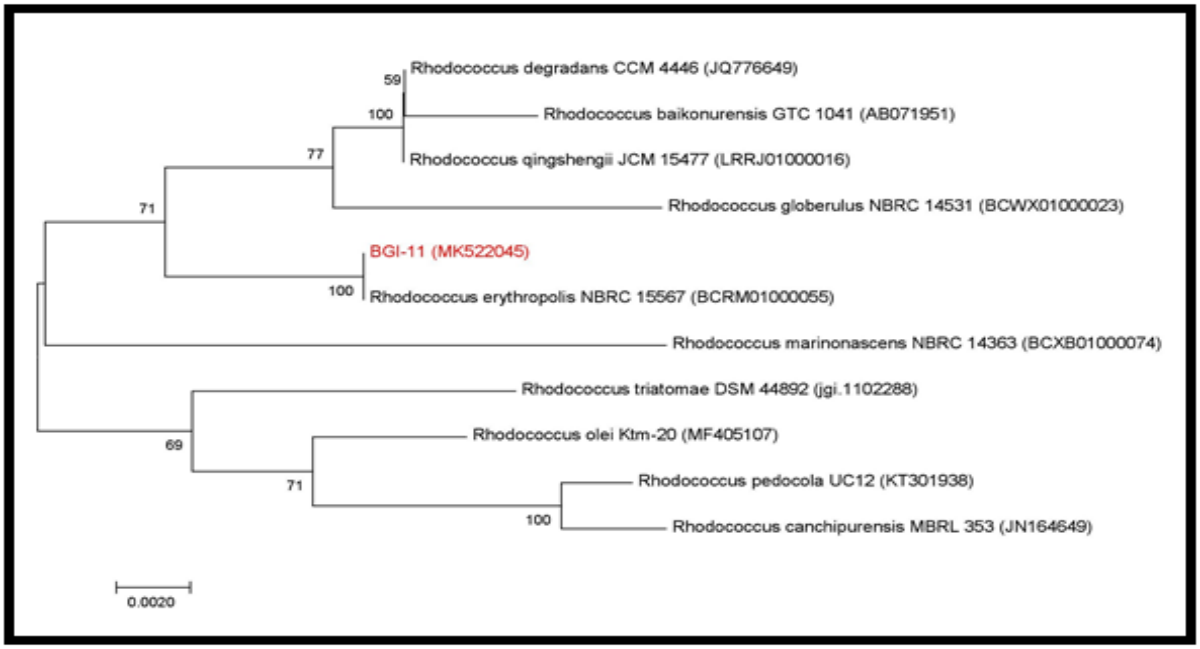

Fig. 2. Phylogenetic tree constructed for the glacial isolate BGI-11 (red color) and closely hit strains, using neighbor-joining method with a bootstrap value (%) greater than 50 from 1000 replicates. Numbers in the brackets are GenBank accession numbers for the 16S rRNA sequences.

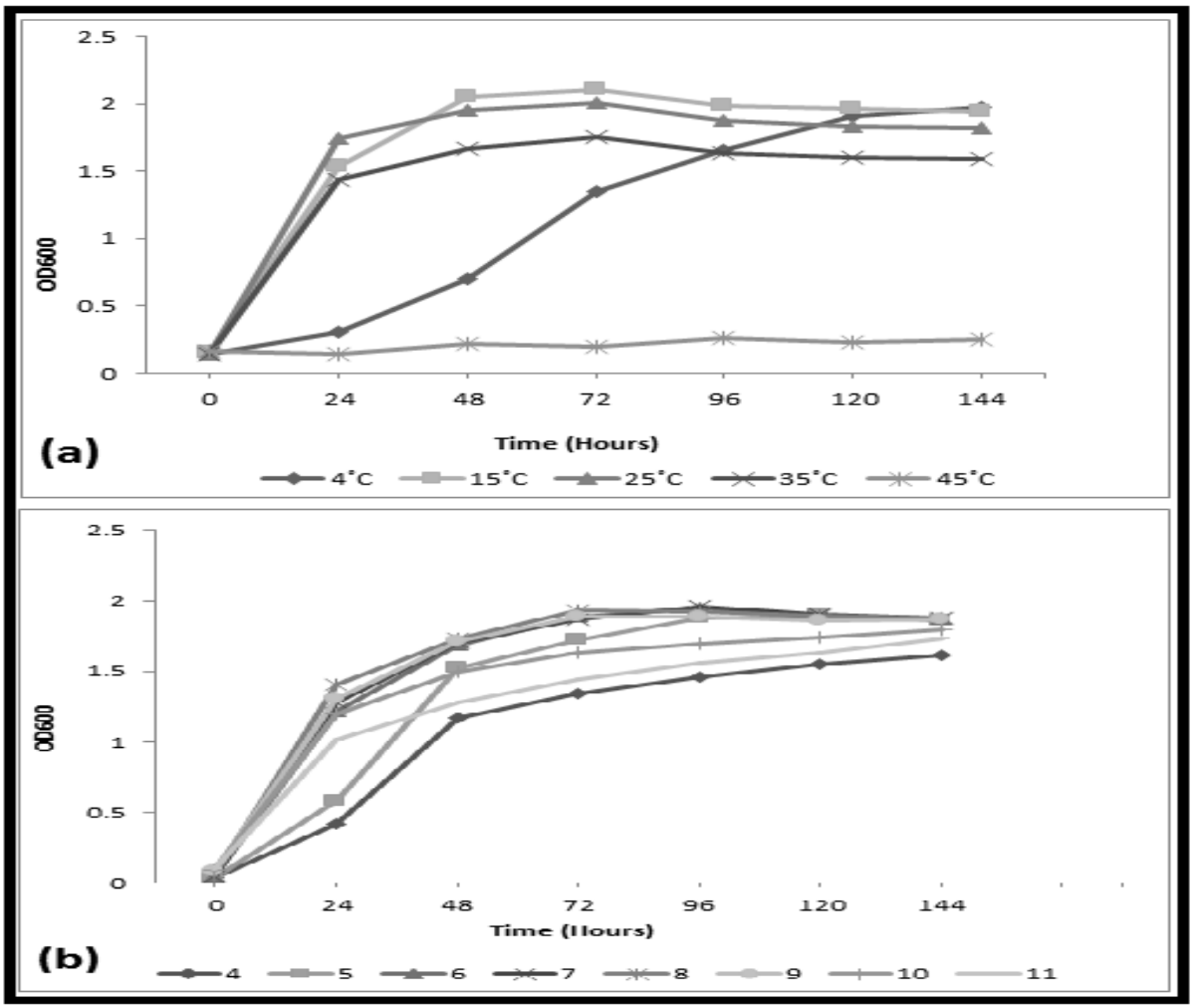

Fig. 3. Growth curve (OD_{600}) of *Rhodococcus* sp.BGI-11 at different (**a**) temperatures (4-45˚C) and (**b**) pH (4-11).

The maximum growth was observed in presence of 2% molasses after 144 hours, sustaining growth for a longer time. Also tryptic soy broth, a nutrient rich medium was used as a control. The maximum growth was observed in MSM with glycerol and molasses than tryptic soy broth. Fast growth and higher productivity by *Rhodococcus* species using glycerol as growth substrate has been previously reported (Herrero *et al.*, 2016). In a recent study, higher biomass and lipid production in a *Rhodococcus* species were observed when molasses was used as a carbon substrate (Saisriyoot *et al.*, 2019).

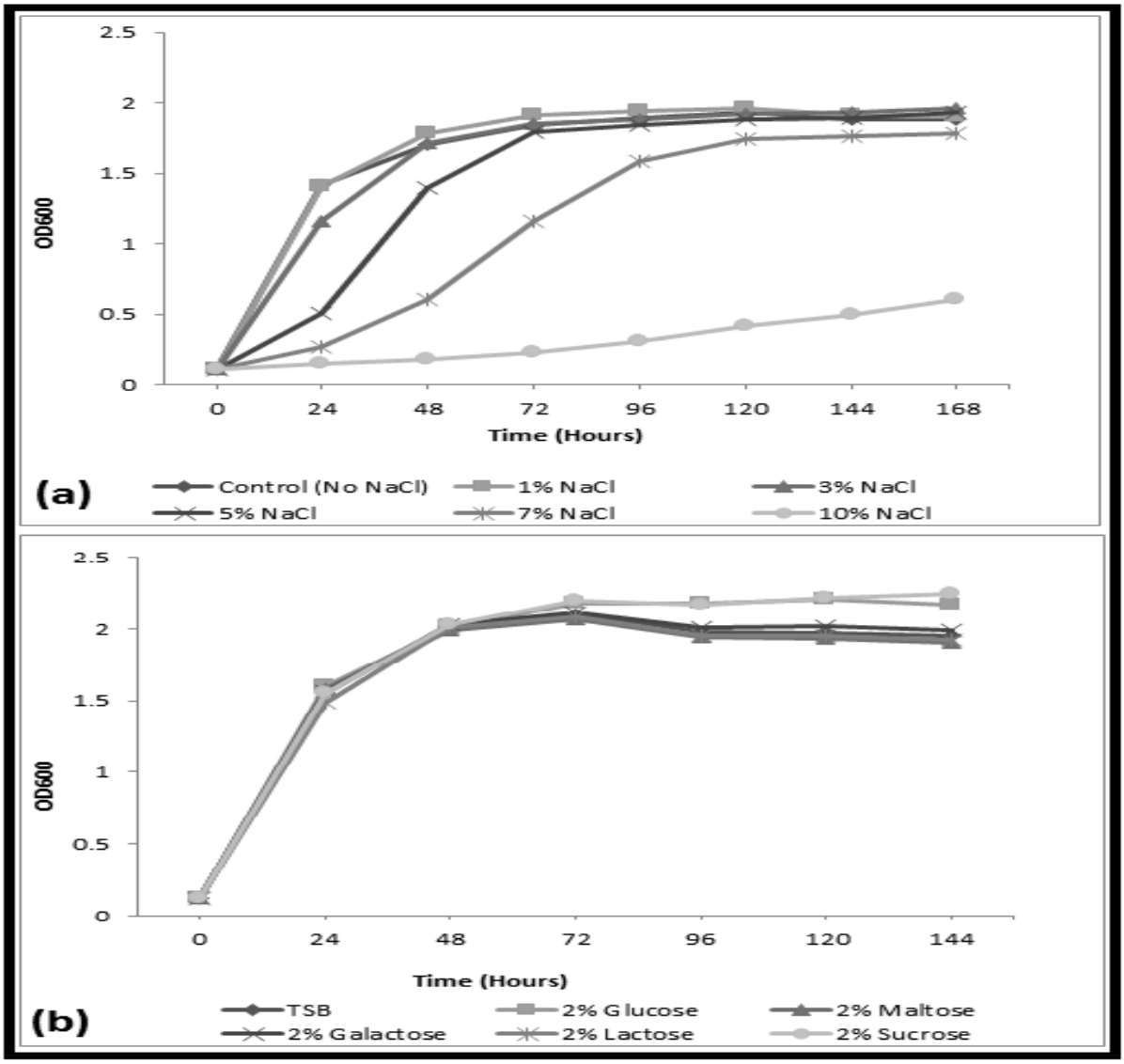

Fig. 4. Growth curve using optical density (OD_{600}) of *Rhodococcus* sp.BGI-11 at different (**a**) salinity (0-10%) and (**b**) carbon sources (glucose, galactose, lactose, maltose, sucrose).

Rhodococcus sp.BGI-11 exhibited great versatility to withstand and grow in multiple stressful conditions. These physicochemical conditions included low temperature, extremes of pH and high salt concentration. Organisms which grow in more than one extreme conditions are called polyextremophile (Pikuta *et al.*, 2007). *Rhodococcus* sp.BGI-11 is therefore, considered as a polyextremophile.

Growth on different culture media

Rhodococcus sp.BGI-11 was able to grow at all the culture media used, including R2A, Luria Bertani agar, tryptic soy agar, nutrient agar, Sabouraud dextrose agar, mannitol salt agar, MacConkey agar and Müeller Hinton agar (Table 2). Surprisingly, the strain was also able to grow inSabouraud dextrose agar (SDA) which is designed for growth of yeast and

fungi. The low pH of this medium favors growth of fungi and inhibits bacteria. *Rhodococcus* sp.BGI-11 was able to grow well in SDA as this strain can tolerate very low pH.Similarly, BGI-11 also exhibited good growth on mannitol salt agar (MSA). The MSA medium is designed for particular group of bacteria which can tolerate high concentration of salt (7.5%) such as the *Staphylococcus aureus*. BGI-11 also demonstrated good growth at the higher concentration of salt used (8%). The strain also demonstrated growth on MacConkey agar, a selective medium which inhibits Gram-positives and favors Gram-negative bacteria. Growth on R2A was observed within 2 days of streaking, R2A is a medium of choice for isolation of bacteria from oligotrophic environments such as the glacier (Hong Zhang *et al.*, 2010).

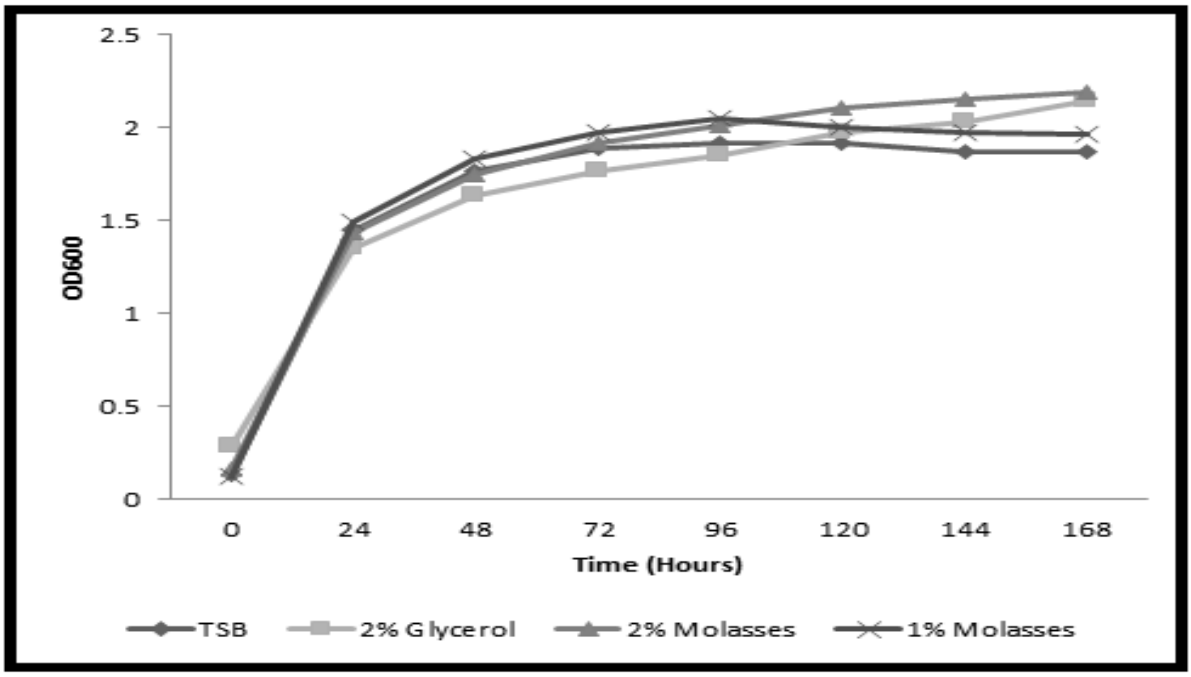

Fig. 5. Growth curve using optical density (OD_{600}) of *Rhodococcus* sp.BGI-11 at different concentration of molasses and glycerol.

Antimicrobial activities

Rhodococcus sp.BGI-11 demonstrated antimicrobial activity against 2 of the 5 indicator microorganisms including a *Bacillus* sp. and *Candida albicans*. No activity was observed for the other three bacteria (Table 3). Activity against *Candida albicans*and *Bacillus sp*. was surprising as these strains were multidrug resistant acquired from a tertiary care hospital. Representatives from the genus *Rhodococcus* have been reported for antibiotic production previously (Borisova, 2011; Claverías *et al.*, 2015). Similarly, Munaganti *et al.*, (2015) reported antimicrobial activity exhibited by *Rhodococcus erythropolis* species against a number of microorganisms including *Bacillus* species and *Candida albicans*. However, their report did not mention whether these indicator strains were multidrug resistant or from a culture collection.

Extracellular enzyme activities

Rhodococcus sp.BGI-11 demonstrated activity for 2 enzymes including, lipases and DNases (Table 4). No activity was observed for proteases, cellulases and amylases. Cold active enzymes maintain high catalytic activity at low temperatures and have relevance to many industrial processes. The high activity at low temperature negates the heating steps, saving energy. *Rhodococcus* sp.BGI-11 demonstrated lipase activity suggests its use in oil (fats) removal.Production of cold-active lipase enzymes by *Rhodococcus* species have been previously reported (Yu and Margesin, 2014; Maharana and Singh, 2018).

Antibiotic susceptibility test

Antibiotic susceptibility test revealed *Rhodococcus* sp.BGI-11 resistant to 2 antibiotics including, methicillin and trimethoprim/sulfamethxazole. The strain was found sensitive to imipenem, ofloxacin and vancomycin (Table 5). The broad-spectrum oflaxacin and narrow spectrum vancomycin had the maximum zone of inhibition against *Rhodococcus* sp. BGI-11.Antibiotic resistance is common in oligotrophic environments as the scarcity of nutrients creates a competition among the microbes, resulting in production of antimicrobial compounds by some and development of resistance by the others.

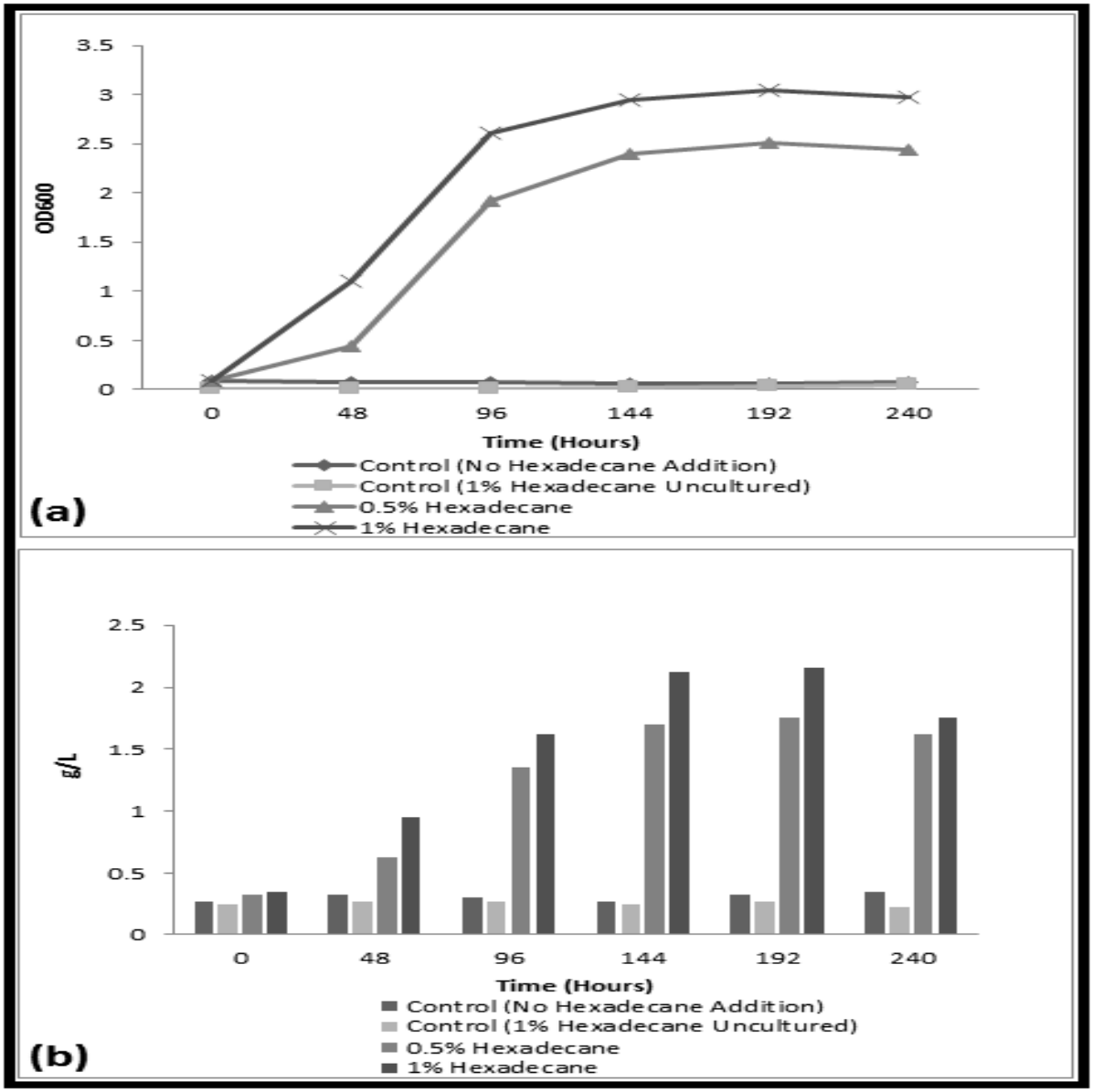

Fig. 6. Growth of *Rhodococcus* sp.BGI-11 in 0.5% and 1% hexadecane, controls include no addition of hexadecane and 1% uncultured hexadecane supplemented medium (**a**) Growth curve (OD_{600}) (**b**) Dry weight biomass (g/L).

Hexadecane utilization

Hexadecane was used as representative petroleum product to check the ability of *Rhodococcus* sp.BGI-11 for the utilization of long chain hydrocarbons. The strain demonstrated maximum growth in the test flask with 1% hexadecane, reaching to an optical density of ~3 in 144 hours. The strain also grew well in the MSM with 0.5% hexadecane (Fig. 6a). No

growth was observed in the two controls throughout the growth period. These controls included MSM with no hexadecane and MSM with 1% hexadecane lacking the *Rhodococcus* sp.BGI-11 inoculum (Fig. 6a).The second control was added to make sure turbidity is solely caused by the cell growth.Likewise, maximum biomass dry weight was observed in MSM with 1% hexadecane, reaching to 2.13 g/L after 144 hours. Maximum dry weight in 0.5% hexadecane supplemented MSM was 1.75 g/L after 192 hours. No biomass production was observed in the controls (Fig. 6b). *Rhodococcus* species are one of the well-studied bacteria for biodegradation due to their ability to use a variety of hydrocarbons. Cold-adapted *Rhodococcus* species have been reported for hydrocarbon degradation in cold environments (Whyte *et al.*, 1999; Bej *et al.*, 2000; Rapp *et al.*, 2003; Ruberto *et al.*, 2005). A psychrotrophic *Rhodococcus* species isolated from the Arctic soil utilized a variety of hydrocarbons including, hexadecane at 5°C (Whyte *et al.*, 1998). In another study, two strains identified as *Rhodococcus fascians* utilized a number of aliphatic and aromatic hydrocarbons (including hexadecane) as principal carbon source at a range of 4-35°C (Yakimov *et al.*, 1999). Petroleum hydrocarbons are one of the most common contaminant of soil and water. Many environments contaminated with hydrocarbon may have other stressful conditions including low temperature, low or high pH, increased pressure and salinity (Margesin and Schinner, 2001). Bioremediation of the hydrocarbons in such environment needs microorganisms which have the capacity to deal with more than one stressful conditions. *Rhodococcus* sp. BGI-11 demonstrated this ability as it grew well at low temperature, extremes of pH and moderate salinity.

Conclusion

Rhodococcus sp. BGI-11 is a psychrotroph with wide temperature and pH range for growth. The strain exhibited activities for extracellular enzymes and antimicrobial compounds. This strain can utilize sugar waste molasses and also glycerol as cheap growth substrates.The most exciting characteristic of this stain is its ability to utilize hexadecane as carbon substrate. The strain BGI-11 is able to grow well at low temperature and can tolerate salt concentration upto 8% (w/v). These characteristics makes BGI-11 a potential candidate for bioremediation of petroleum hydrocarbons in deep ocean, an environment also characterized by low temperature and moderate salinity. Molasses can be used as cheap substrate for large scale production of enzymes and metabolites. Molasses can also be added to hydrocarbon contaminated sites to speed up (biostimulation) the biodegradation process. Environmental pollution with organic compound is a global threat particularly, hydrocarbon contamination in the marine environments is increasing. Cold-adapted bacteria with the ability to cope the harsh environmental conditions becomes crucial for the cleanup process.

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