Original Research Article

A study on isolation, screening, characterization of indigenous oleaginous bacteria: Evaluation of various carbon and nitrogen sources as substrates for single celled oil producing bacteria

ABSTRACT

Aims: In this study, isolation, screening and characterization of heterotrophic lipid producing bacteria from various oil and fat contaminated sites. In addition influence some carbon and nitrogen sources has been evaluated.

Study design: One way Anova statistical analysis

Place and Duration of Study: Soil samples collected from Hisar, Sirsa, waste water sludge samples collected from Guru Jambheshwar University of Science and Technology, Hisar

Methodology: Isolation and purification of bacterial strains was done by simple plate streak plate method, followed by screening of bacterial strains by Sudan black/Nile Red dye. Genomic DNA was extracted from bacterial strain using Cetyl trimethyl ammonium bromide (CTAB) method. PCR product was sequenced by 16sRNa approach. Effect of various carbon and nitrogen sources on lipid and biomass were evaluated by using gravimetric bligh and dyer method.

Results:

Filamentous bacterial strains were initially isolated using selective culture media further these oleaginous bacterial strains were screened out on the basis of growth rate and lipid content (dcw%) and employed Nile red and Sudan black staining for detection of neutral lipids in cells. The biochemical behavior (biomass production, accumulation of total lipid, substrate uptake by two oleaginous bacteria *Rhodococcus sp.* and *Gordonia sp.* was studied when aforementioned bacteria were cultivated under various carbon and nitrogen sources. Significant differences in the process of lipid accumulation and biomass yield as related to the carbon, nitrogen sources used were observed for both microorganisms. Although glucose containing MSM medium favour production of biomass yield 1.81±0.026 gL^{-1} and 1.63±0.032 gL^{-1} with corresponding high lipid content 16.78%,17.05% in *Rhodococcus opacus* as well as *Gordonia alkanivorans* respectively. Among Various tested ammonium sulphate was found to be best nitrogen source for cultivation of *Rhodococcus opacus* and *Gordonia alkanivorans* as indicated significant (P≤0.05) higher lipid content of 16.55%,17.01 %.

Keywords: Oleaginous bacteria, filamentous bacteria, screening, Sudan black, Nile Red, 16srRNa, Yeast extract, glucose

1. INTRODUCTION

Single-cell microorganisms (SCM) constitute an emergent alternative to source high-value lipids for a series of growing markets demanding low-cost, high-quality alternatives. SCM as a broad class display a series of advantages when compared to plants and animals as lipid sources. In addition to being more genetically accessible, SCM are capable of producing greater bio-diversity and storing higher percentages of lipids [1].Therefore, their productivity per volume and energy input can be up to 5 or 6 times that of plants and even more when compared to animal sources [2,3]. In principle, SCM can achieve greater sustainability to alleviate the increasing problem of sourcing oils for both the fuel and human consumption markets, thus mitigating the continuous increase in commodity oil prices. Recently, utilization of microbial lipid as an alternative feedstock for the production of oleochemicals especially fatty acid methyl esters (FAMEs), which are also known as biodiesel, has drawn interest of scientists to heterotrophic oleaginous microorganisms [4,5]. This encourages scientists to

devote their efforts not only to screen microorganisms which produce high lipid yields by utilization of inexpensive bio-based feedstocks [6-9], but also to produce lipid in a reproducible, high quality and sustainable way [10]. Microbial lipids can also become sources of safe and clean biomaterials at reduced costs and continuous availability [11,12]. Oleaginous microorganisms, such as microalgae, yeasts, fungi and bacteria can produce high levels of lipids and do not need arable lands. As India is agriculture country most of its income comes from agriculture sector through the rural area the population mainly depends on the primary income source both men and women are involved in this sector of agriculture farming [13]. Biodiesel from bacteria is alternative source of income with agriculture sector. This can be done by any individual of any age, sex, qualification with proper guidance, investment and some space along with its primary source of income [14].The fatty acid profiles are dependent on oleaginous microorganism's types and the growth conditions. To fulfill the latter task, several efforts have been conducted by determining the fundamental factors that control the lipid production by oleaginous microorganisms [10] as well as trying to modify and optimize cultivation parameters [15-19]. While heterotrophic bacteria have not been as extensively characterized with respect to their lipid and fatty acid content as other microbes, the available information nonetheless suggests that they can provide an abundant source of neutral lipids as well as specialized lipids [20-22]. Environmental conditions such as temperature, pH, substrate, C/N ratio and oxygen pressure have an effect on the productivity of accumulating lipids [23]. Oleaginous microorganisms that utilize a variety of carbon substrates provide advantages for TAG production from renewable non-food resources such as lignocellulosic biomass [10, 24].Sriwongchai *et al.,* studied that the influence of different nitrogen sources on lipid production using glycerol on *Rhodococcus sp.* for biomass and lipid production. *Rhodococcus erythropolis* was also using concentration of glucose in MSM medium for cultivation of oleaginous cultures, there was significant increase in both biomass yield and lipid content [25]. In this study, isolation, screening and characterization of heterotrophic lipid producing bacteria. In addition of influence some carbon and nitrogen sources on biomass yield and lipid content has been evaluated.

2. MATERIAL AND METHODS

2.1 Samples collection for bacterial strains isolation

Soil samples collected from Hisar, Sirsa, waste water sludge samples collected from Guru Jambheshwar University of Science and Technology, Hisar in sterile disposable plastic bags and were taken to lab under non-contaminating conditions (**Table 1**)

Table: 1 Samples collection from various sites

S. No	Name of samples	No. samples	Collection sites
Hisar, Sirsa (Haryana)			
1.	Soil	2	Workshop auto market, Sirsa
2.	Soil	2	Workshop auto market, Hisar
3.	Soil	1	Slaughter house, Valmiki Chowk Sirsa,
4.	Soil	1	Petrol pump Sangwan chowk (Sirsa)
5.	Soil	3	Restaurant soil samples (Sirsa)
6.	Soil	2	Vita milk plant, Sirsa
7.	Waste water sludge	1	Guru Jambheshwar University of Science and Technology, Hisar

2.2 Isolation and purification of bacterial strains

Samples were serially diluted to obtain desired dilution so that distinct bacterial colonies appeared in the nutrient agar petriplates. 0.1 ml of 10^{-6} dilution was spread with spreader over agar plated nutrient agar medium in order to get uniform bacterial growth. Inoculated plates were incubated at 30^{o}C for 48hr and heterogeneous bacterial colonies were appeared on plates. Purified strains were obtained by 3-4 times streaking. Pure and isolated colonies maintained on slants containing nutrient agar. Schematic protocol for isolation and purification of bacterial strains is given in **Figure 1.** Composition of nutrient agar is given as under:

Table 2: Chemical composition of Nutrient agar media

Nutrient constituents	Composition gL^{-1}
NaCl	5
Beef extract	3
Peptone	5
Agar	15

pH adjusted 7.5 before autoclaving

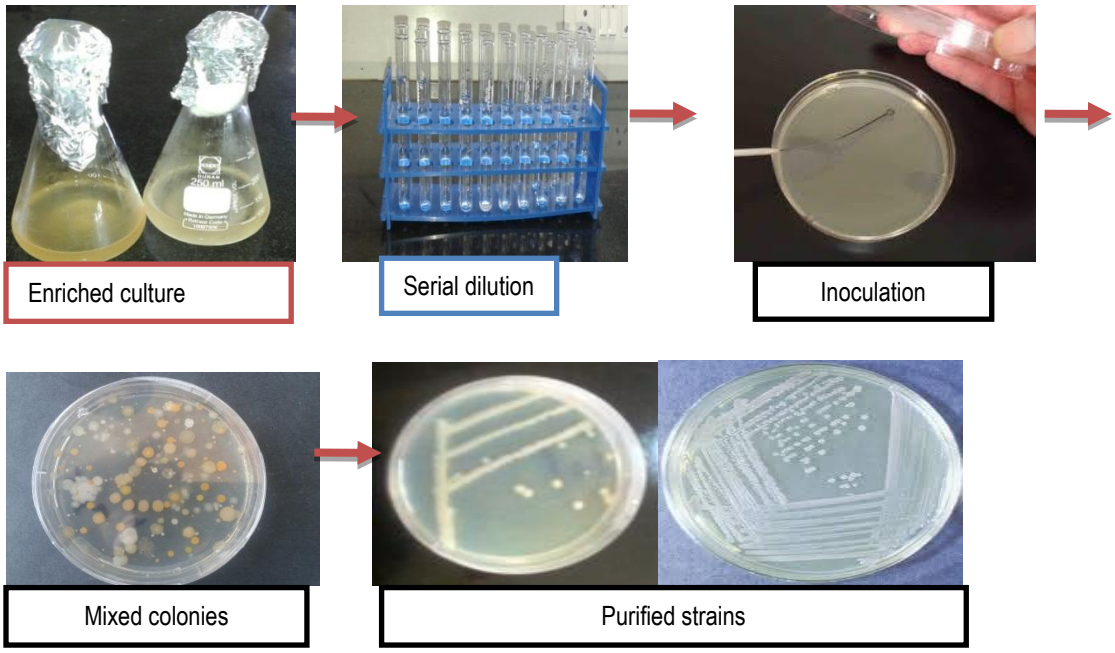


Fig. 1: Schematic protocol for isolation and purification of bacterial strains

2.3 Isolation of filamentous bacterial strains and actinomycetes

For inoculation and isolation of filamentous bacteria two selective media were used named Tryptone glucose yeast extract agar (TGY) and Tryptone yeast extract agar (TYE)[26] Composition of these media are mentioned in **Tables 2, 3** respectively. These two media are growth specific for certain filamentous bacteria only, so a growth in them provisionally confirmed the presence of the respective bacteria and in filamentous bacteria have considerable amount of lipid [26]

Table 2: Composition of Tryptone Glucose yeast extract agar (TGY)

Ingredients	gL^{-1}
Casein-enzymic hydrolysate	10.0
Glucose	5.0
Yeast extract	1.0
Dipotassium phosphate	1.25

pH adjusted 6.8±0.2

Table 3: Chemical composition of Tryptone yeast extract agar (TYE)

Ingredients	gL^{-1}
Tryptone	6.0
Yeast extract	3.0
Agar	15.0

pH adjusted before autoclaving Final pH 7.2 ± 0.2

2.4 Screening of lipid producing bacterial strains (Sudan black & Nile Red staining)

Sudan black staining: Smears of cells were deposited on a glass slide were heat fixed and stained with a 3% (w/v in 70% ethanol) solution of Sudan black B for 10 min, then, immersion of the slide in xylene until it completely was decolorized. The sample was counterstained with safranine (5% w/v in deionized water for 10 sec, washed with water and dried. A few drops of immersion oil were added directly on the completely dry slide, and the cells were examined by phase contrast microscopy [27]

Nile Red staining: Based on preliminary procedure for improved Nile red staining, bacterial cells (0.5 ml) were collected by centrifugation at 5000 rpm (Rotation per minute) for 10 min and washed with distilled water after that washed with physiological saline solution (0.5 ml) several times. Further bacterial samples immersed in Nile red solution (0.5 mg/ml-1 in acetone), mixed with 50 ml glycerol: water mixture (75:25),gently vortex for 1min.After 15 minutes of incubation in darkness, the fluorescence of bacterial samples was measured with fluorescence Olympus Magnus microscope having 420 nm to 580 nm absorption and emission wavelength respectively[42]

2.5 Genomic DNA isolation from bacterial isolates and 16srRNA sequence determination and phylogenetic analysis

Genomic DNA was extracted from bacterial strain using Cetyl trimethyl ammonium bromide (CTAB) method [28]. The PCR product of 16SrDNA was sequenced by Geneombio Technology Pvt. Ltd. Pune (Maharashtra). Nucleotide sequence was analyzed and compared with Gen Bank nucleotide sequence database using the Basic Local Alignment Tool (BLASTn).

2.6 Effect of carbon and nitrogen sources on biomass yield and lipid accumulation in screened bacterial strains

In order to test various carbon sources namely fructose, lactose, sucrose, sodium acetate, glucose, glycerol individually added in the production medium. The cultures were inoculated and incubated for 5 days at 30°C. The cultures were then collected and used for total lipid and biomass estimation. To investigate the effects of nitrogen sources, various nitrogen sources *viz.* $(NH4)_2SO_4$, urea, NaNO3, yeast extract and peptone were added 1% in MSM media composition of MSM medium given in (**Table 4**). All the experiments were carried out in triplicates in 250ml flasks containing sterilized Minimal salt medium.

Table 4: Composition of Minimum salt medium

Constituents	gL^{-1}
KH_2PO_4	2
K_2HPO_4	7
$ZnCl_2$	0.01
$MgCl_2$	0.20
$FeCl_3$	0.01
$MnCl_2.4H_2O$	0.01
Na_2SO_4	0.20
NH_4NO_3	1.0
Yeast extract	0.006
$CaCl_2$	0.01

pH adjusted 7.5 before autoclaving.

2.9 Statistical analysis

Statistical comparison between the groups was done by multi factors one-way analysis of variance (ANOVA) and Duncan's multiple-range test, using SPSS version 21.0. The *p*-values that were less than 0.05 were considered significant.

3. RESULTS AND DISCUSSION

3.1 Samples collection and isolation of bacterial strains

A total 12 samples were collected from various fat and oil contaminated sites as shown in **Table 1.** Isolation was carried out by standard streak plate method on nutrient agar medium. Purified strains were maintained on nutrient agar slants. Total 35 bacterial strains were isolated from various contaminated sites.

3.2 Isolation and screening of potent biodiesel producing strains

Two Selective media were used for the inoculation as well as isolation of filamentous bacteria from the sludge and soil samples named Tryptone Glucose Yeast extract (TGY) and Tryptone yeast extract (TYE).These media are specific for the growth of certain filamentous bacteria only, which have substantial amount of lipid. Out of 35 bacterial strains,15 filamentous bacterial strains were isolated by using respective selective culture media as shown in **Fig. 2 (A)**. In preliminary screening by Sudan black B staining and Nile Red staining showed different intensity in color uptake of dye based on their lipids content. Further these strains were screened out on the basis on optical density and lipid content gravimetrically. **Table 5** showing screening of oleaginious bacteria with lipid content and biomass. Isolates S4,S6,S7,S10 and S11 showed maximum lipid production in sudan black blacks staining whereas only two isolates namely S4,S11 showed maximum lipid production in nile red staining. In Sudan black staining, intracellular lipid granules are black in colour and rest are in pink colour **(Fig. 3(B, C)**.

For preliminary screening Sudan black staining have been used by many scientist to screen out oleaginious bacterial strains [12,29,30]. Wheareas neutral lipid or triglycerides appeared as yellow dots, whereas polar lipid were observed in red colour cells by Nile Red staining under fluorescent microscope with excitation wavelength at 420 nm and emission at 580-nm **(Fig.3, D, E)**.Similar results were reported by many workers for lipid staining by using Nile Red dye for intracellular lipid identification [31,32,33].On the basis of high growth rate and lipid content four bacterial strains *viz.* S4,S7,S10,S11 were screened out and characterized by molecular techniques. Furthermore, phylogenetic analysis of 16s rRNA bacterial revealed that these bacterial strains have 99% similarity with *Bravibacillus*, *Bacillus cereus*, *Rhodococcus opacus* and *Gordonia alkanivorans*. as shown in **Fig.4(A-D)**. Additionally after quantitative and qualitative screening, finally two bacterial strains (S4, S11) were selected on the basis of comparatively higher lipid content and biomass for further study. Screened bacterial strains further identified as S4 *Rhodococcus opacus* (KB05) and S11*Gordonia alkanivorans* (KB06) by using molecular tools.

Fig. 2 (A) Filamentous bacterial strains growing on selective media

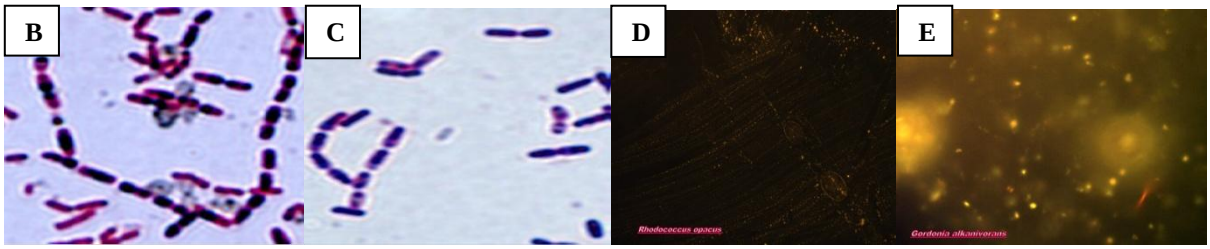


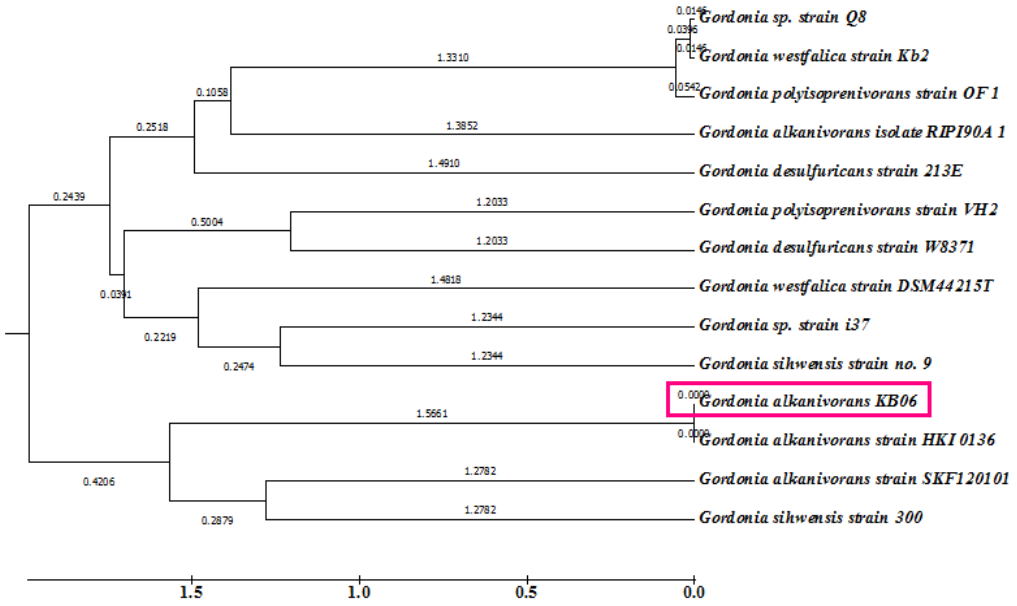
Fig. 3: Images of Sudan black and Nile red staining of *Rhodococcus sp.* (B, D);*Gordonia sp.*(C,E) under phase contrast microscope (1000 x)

Table : 5 Screening of oleaginous bacterial strains

Oleaginous Bacterial isolates	Sudan black staining	Nile Red staining	OD, 600 nm	Lipid content (DCW) g/l
S1	++	-	2.101	1.97 ± 0.023^{h}
S2	++	+	2.136	2.07 ± 0.034^{g}
S3	+	--	2.052	1.48 ± 0.011^{i}
S4	+++	+++	2.136	3.11 ± 0.025^{a}
S5	++	+	1.921	2.31 ± 0.032^{f}
S6	+++	++	2.301	2.64 ± 0.013^{e}
S7	+++	+	2.489	2.78 ± 0.020^{d}

S8	++	+	2.135	2.33±0.011[f]
S9	+	-	1.844	1.95±0.022[h]
S10	+++	++	2.520	2.87±0.030[c]
S11	+++	+++	1.816	3.08±0.015[b]

156 + : good lipid visibility, ++ : Moderate lipid visibility, +++ : Maximum lipid visibility, - : No growth



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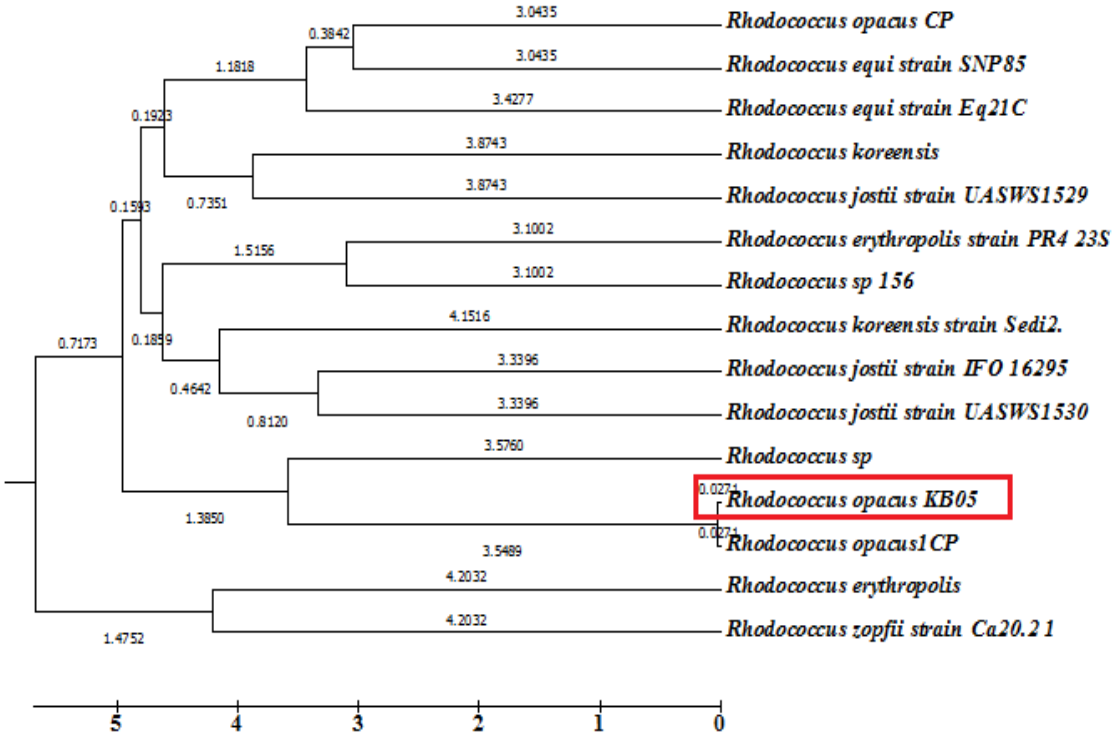
Rhodococcus opacus CP
Rhodococcus equi strain SNP85
Rhodococcus equi strain Eq21C
Rhodococcus koreensis
Rhodococcus jostii strain UASWS1529
Rhodococcus erythropolis strain PR4 23S
Rhodococcus sp 156
Rhodococcus koreensis strain Sedi2.
Rhodococcus jostii strain IFO 16295
Rhodococcus jostii strain UASWS1530
Rhodococcus sp
Rhodococcus opacus KB05
Rhodococcus opacus1CP
Rhodococcus erythropolis
Rhodococcus zopfii strain Ca20.2 1
3.0435
0.3842
3.0435
1.1818
3.4277
0.1923
3.8743
3.8743
0.1593
0.7351
3.1002
1.5156
3.1002
4.1516
0.7173
0.1859
3.3396
0.4642
3.3396
0.8120
3.5760
0.0274
1.3850
0.0274
3.5489
4.2032
4.2032
1.4752
5
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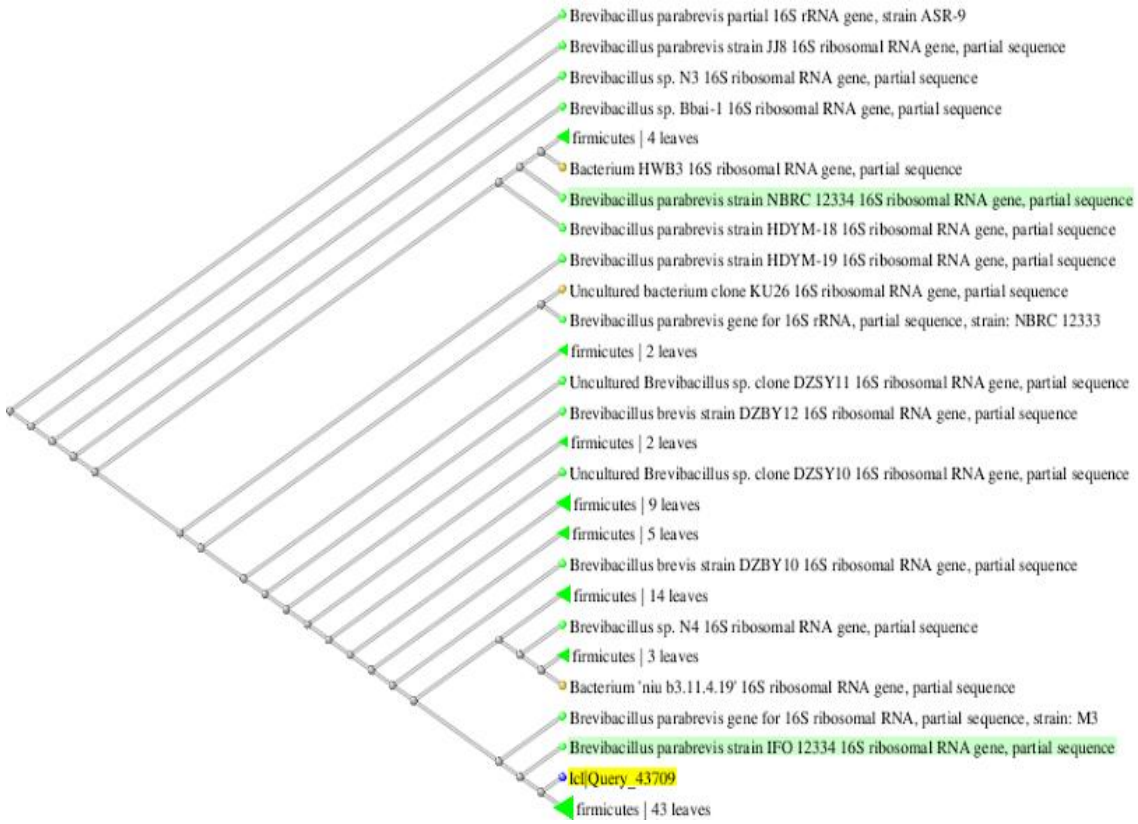
Brevibacillus parabrevis partial 16S rRNA gene, strain ASR-9
Brevibacillus parabrevis strain JJ8 16S ribosomal RNA gene, partial sequence
Brevibacillus sp. N3 16S ribosomal RNA gene, partial sequence
Brevibacillus sp. Bbai-1 16S ribosomal RNA gene, partial sequence
firmicutes | 4 leaves
Bacterium HWB3 16S ribosomal RNA gene, partial sequence
Brevibacillus parabrevis strain NBRC 12334 16S ribosomal RNA gene, partial sequence
Brevibacillus parabrevis strain HDYM-18 16S ribosomal RNA gene, partial sequence
Brevibacillus parabrevis strain HDYM-19 16S ribosomal RNA gene, partial sequence
Uncultured bacterium clone KU26 16S ribosomal RNA gene, partial sequence
Brevibacillus parabrevis gene for 16S rRNA, partial sequence, strain: NBRC 12333
firmicutes | 2 leaves
Uncultured Brevibacillus sp. clone DZSY11 16S ribosomal RNA gene, partial sequence
Brevibacillus brevis strain DZBY12 16S ribosomal RNA gene, partial sequence
firmicutes | 2 leaves
Uncultured Brevibacillus sp. clone DZSY10 16S ribosomal RNA gene, partial sequence
firmicutes | 9 leaves
firmicutes | 5 leaves
Brevibacillus brevis strain DZBY10 16S ribosomal RNA gene, partial sequence
firmicutes | 14 leaves
Brevibacillus sp. N4 16S ribosomal RNA gene, partial sequence
firmicutes | 3 leaves
Bacterium 'niu b3.11.4.19' 16S ribosomal RNA gene, partial sequence
Brevibacillus parabrevis gene for 16S ribosomal RNA, partial sequence, strain: M3
Brevibacillus parabrevis strain IFO 12334 16S ribosomal RNA gene, partial sequence
lcl|Query_43709
firmicutes | 43 leaves

162

163

Fig. 4. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strain (A) S4, *Rhodococcus opacus* (B) S11, *Gordonia alkanivorans* (C) S7, *Brevibacillus parabrevis* (D) S10, *Bacillus cereus* with other universal identified species.

3.3 Effect of carbon sources on biomass and lipid yield in screened bacterial strains

As depicted in (**Fig. 5 A, B)** carbon sources have significant (P≤0.05) effects on biomass yield and lipid content in oleaginous microbes viz. *Rhodococcus sp.* and *Gordonia alkanivorans*. Significant (P≤0.05) high cell density as well as cell dry weight was obtained with glucose and fructose as the carbon source. In addition, cells cultivated in a medium containing glucose yielded significant (P≤0.05) high lipid content 16.78%,17.05% with corresponding significant (P≤0.05) biomass yield 1.81±0.026 gL^{-1} and 1.63±0.032 gL^{-1} in *Rhodococcus opacus* as well as *Gordonia* alkanivorans respectively. Quite poor biomass and lipid content were observed from sodium acetate in both screened bacteria, while sucrose was also a favorable carbon source for biomass yield 1.59±0.023gL^{-1} in *Gordonia alkanivorans*. Glycerol also found be suitable carbon source for biomass yield in *Rhodococcus opacus*. Hence all carbons sources including control somewhat supported significant (P≤0.05) higher biomass and lipid content except sodium acetate in *Gordonia alkanivorans* and *Rhodococcus opacus*.

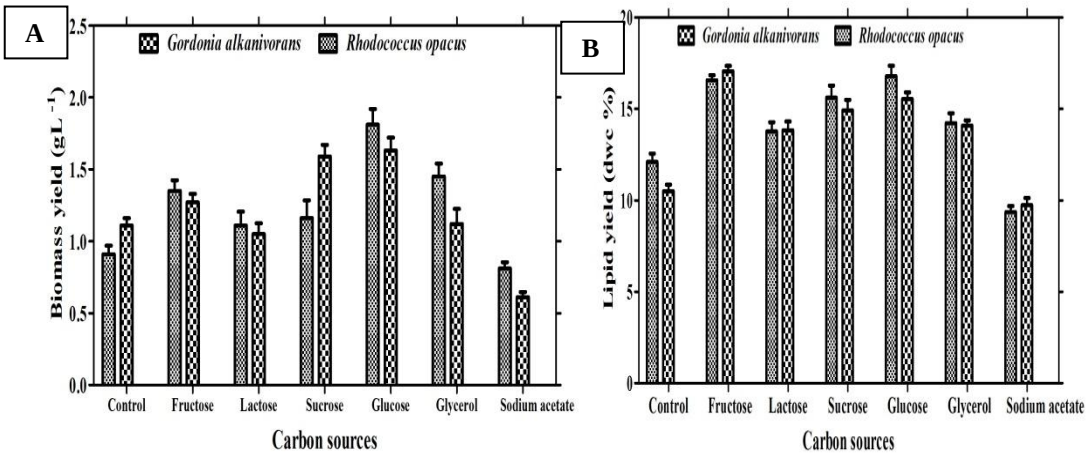

Fig. 5. Effect of carbon sources on biomass yield (A) lipid accumulation (B) in *Gordonia alkanivorans* and *Rhodococcus opacus*

Sriwongchai *et al.*, explored that glucose as a sole carbon source reached highest dry biomass and lipid yield in *R. erythropolis* [34] Vipra *& his co-workers* found that in *Y. lipolytica,* maximum biomass was obtained using glucose in the medium [35]. These results strongly supported our work. While glucose and fructose is easily taken up by microbial cells, disaccharides like sucrose or lactose must be first hydrolyzed to monosaccharides or must have specific transport system before entering microbial cells as advocated by Perez-Garcia *et al.,*[36]. *Aeromonas* sp. KMITL-R4.4 had maximum biomass and lipid contents when cultured with glucose and fructose [37] as we found in our present study. Some report found *mycophenolate* strain could produce gamma linoleic acid, with using glucose as carbon source and the concentration of oil was up to 66% (w/w), with using starch as carbon source, the fat content was 41.2% [37-38]

3.4 Effect of nitrogen sources on lipid accumulation and biomass yield in screened bacterial strains

Statistical comparison suggested that various nitrogen sources have significant (P≤0.05) effects on biomass yield and lipid content as shown in **(Fig. 6 A, B)**. Ammonium sulphate was the best nitrogen source for cultivation of *Rhodococcus opacus* and *Gordonia alkanivorans* as indicated significant(P≤0.05) higher lipid content of 16.55%,17.01 % respectively, followed by yeast extract, beef extract. In yeast extract *Rhodococcus sp.* and *Gordonia alkanivorans* showed significant (P≤0.05) higher biomass yield 1.61±0.030 and 1.47±0.025gL^{-1} respectively. Among various nitrogen sources, inorganic nitrogen salts viz. NaNO3, urea exhibited quite poor biomass and lipid content in oleaginous microbes. These finding suggested that screened bacterial strains had the ability to utilize inorganic nitrogen sources, particularly in the ammonium form for maximum cell lipid production.

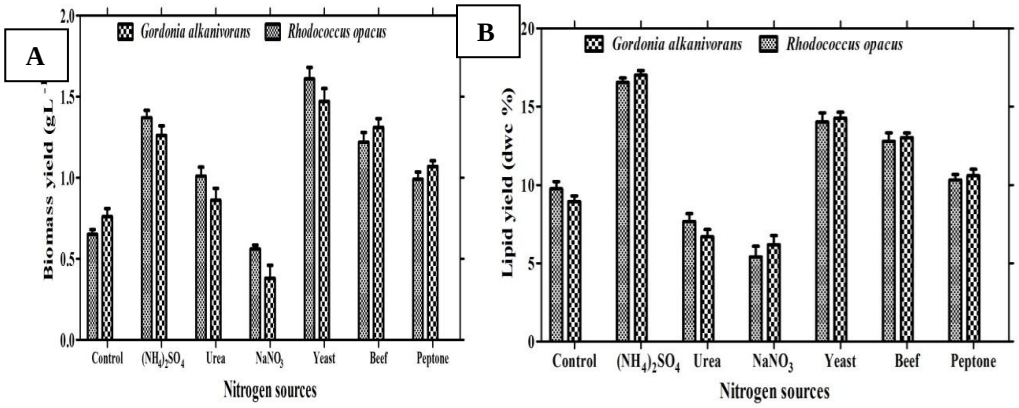

Fig. 6 Effect of nitrogen sources on (A) Biomass yield, (B) lipid content DCW%

Huang et al., [39] studied the effect of diverse kinds of nitrogen sources affected microbial lipid synthesis and reported that NH4NO3 and urea as nitrogen source was ideal for the growth of cells, but using the above two kinds of nitrogen source, the amount of oil synthesis is very low; peptone, beef extract were the best nitrogen source for oil production, but the cell growth was severely affected by peptone, beef extract medium. Liang et al.,[40] Zhao et al., [41] stated that concentration of nitrogen has important effect on the synthesis of microbial oil. The research showed that potassium nitrate and urea were used as a nitrogen source for fermentation of *Mortierella*, which could accelerate oil production and dry cell weight. In addition, the utilization of urea as a nitrogen source required urease activity in cells in order to hydrolyze urea to ammonium which subsequently incorporated into cellular components [37]

4. CONCLUSION

Biodiesel is a cost-effective and renewable fuel that can potentially be produced in microbes. Fatty acid methyl esters (FAMEs) are common components of biodiesel and can be synthesized either from triacylglycerol or free fatty acids (FFAs). Microorganism *viz.* microalgae and bacteria are ideal candidates, which can completely replace petroleum based fuel with biofuel, which is due to higher productivity, higher lipid content, rapid growth and no competition with food crops for agricultural land. Filamentous bacterial strains were initially isolated using selective culture media. These fifteen oleaginous bacterial strains were screened out on the basis of growth rate and lipid content (dcw%) and employed Nile red and Sudan black staining for detection of neutral lipids in cells. Based on quantitative and qualitative screening, four potent oleaginous bacterial strains were screened out. In addition, screened strains can utilize both inorganic and organic nitrogen sources for growth and lipid accumulation but organic nitrogen sources has much more significant effects for lipid production in comparison with inorganic nitrogen source. For heterotrophic cultivation, among carbon sources glucose was found to be most suitable carbon source for both algal and bacterial strains. Among various tested nitrogen sources $(NH_4)_2SO_4$ is the best nitrogen source for cultivation of bacteria namely *Rhodococcus opacus* and *Gordonia alkanivorans* in the form of high lipid content.

REFERENCES

1. Palmer, J. D., & Brigham, C. J. Feasibility of triacylglycerol production for biodiesel, utilizing Rhodococcus opacus as a biocatalyst and fishery waste as feedstock. *Renewable and Sustainable Energy Reviews*, 2016;56, 922-928.
2. Singh, A., Nigam, P. S., & Murphy, J. D. Mechanism and challenges in commercialisation of algal biofuels. *Bioresource technology*,2011; 102(1), 26-34.
3. Merchant, S. S., Kropat, J., Liu, B., Shaw, J., & Warakanont, J. TAG, You're it! *Chlamydomonas* as a reference organism for understanding algal triacylglycerol accumulation. Current opinion in biotechnology,2012;23(3), 352-363.

4. Li, Q., Du, W., & Liu, D. Perspectives of microbial oils for biodiesel production. Applied microbiology and biotechnology, 2008; 80(5), 749-756.
5. Meng X, Yang J, Xu X, Zhang L, Nie Q, Xian M. Biodiesel production from oleaginous microorganisms. Renewable energy. 2009; 31;34(1):1-5.
6. Voss I, Steinbüchel A. High cell density cultivation of *Rhodococcus opacus* for lipid production at a pilot-plant scale. Applied microbiology and biotechnology. 2001;1;55(5):547-55.
7. Gouda MK, Omar SH, Aouad LM. Single cell oil production by *Gordonia sp.* DG using agro-industrial wastes. World Journal of Microbiology and Biotechnology. 2008;1;24(9):1703.
8. Li M, Liu GL, Chi Z, Chi ZM. Single cell oil production from hydrolysate of cassava starch by marine-derived yeast *Rhodotorula mucilaginosa* TJY15a. Biomass and bioenergy. 2010;31;34(1):101-7.
9. Queiroz MI, Hornes MO, da Silva-Manetti AG, Jacob-Lopes E. Single-cell oil production by cyanobacterium Aphanothece microscopica Nägeli cultivated heterotrophically in fish processing wastewater. Applied energy. 2011;88(10):3438-43.
10. Kosa M, Ragauskas AJ. Lipids from heterotrophic microbes: advances in metabolism research. Trends in biotechnology. 2011;29(2):53-61.
11. Garay LA, Boundy-Mills KL, German JB. Accumulation of high-value lipids in single-cell microorganisms: a mechanistic approach and future perspectives. Journal of agricultural and food chemistry.2014; 62(13):2709-27.
12. Bajwa, K., & Bishnoi, N. R. Single cell oil of bacterial strains as a new source of high-value biodiesel: isolation and screening for storage lipids in cytoplasm. Annals of Biology. 2016;32(1), 1-6.
13. Seraphim P, Michael K, George A Single cell oil (SCO) production by *Mortierellaisabellina* grown on high-sugar content media. BioresourTechnol. 2004; 95:287–291.
14. Certik M, Balteszova L, Sajbidor J. Lipid formation and clinolenic acid production by *Mucorales* fungi grown on sunflower oil. Appl Microbiol Biotechnol. 1997; 25:101–105.
15. Li Y, Zhao ZK, Bai F. High-density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fed-batch culture. Enzyme and microbial technology. 2007; 2;41(3):312-7.
16. Beopoulos A, Cescut J, Haddouche R, Uribelarrea JL, Molina-Jouve C, Nicaud JM. *Yarrowia lipolytica* as a model for bio-oil production. Progress in lipid research. 2009;30;48(6):375-87.
17. Kurosawa K, Boccazzi P, de Almeida NM, Sinskey AJ. High-cell-density batch fermentation of Rhodococcus opacus PD630 using a high glucose concentration for triacylglycerol production. Journal of Biotechnology. 2010;30;147(3):212-8.
18. Subramaniam R, Dufreche S, Zappi M, Bajpai R. Microbial lipids from renewable resources: production and characterization. Journal of industrial microbiology & biotechnology. 2010;1;37(12):1271-87.
19. Wu H, Li Y, Chen L, Zong M. Production of microbial oil with high oleic acid content by *Trichosporon capitatum*. Applied energy. 2011;88(1):138-42.
20. Alvarez HM, Kalscheuer R, Steinbüchel A. Accumulation and mobilization of storage lipids by *Rhodococcus opacus* PD630 and *Rhodococcus ruber* NCIMB 40126. Applied microbiology and biotechnology. 2000;16;54(2):218-23.
21. Alvarez, H., & Steinbüchel, A. Triacylglycerols in prokaryotic microorganisms. *Applied microbiology and biotechnology*, 2002;60(4), 367-376.
22. Patnayak, S., & Sree, A. Screening of bacterial associates of marine sponges for single cell oil and PUFA. Letters in applied microbiology, 2005;40(5), 358-363.
23. Nisha, A., & Venkateswaran, G.Effect of culture variables on mycelial arachidonic acid production by *Mortierella alpina*. Food and Bioprocess Technology,2011; 4(2), 232-240.
24. Nasr, M. M., Nahvi, I., Keyhanfar, M., & Mirbagheri, M. (2017). The effect of carbon and nitrogen sources on the fatty acids profile of *Mortierella vinacea*. Biological Journal of Microorganism,2017;5(20):1-8
25. Sriwongchai S, Pokethitiyook P, Pugkaew W, Kruatrachue M, Lee H. Optimization of lipid production in the oleaginous bacterium *Rhodococcus erythropolis* growing on glycerol as the sole carbon source. African Journal of Biotechnology. 2012;11(79):14440-7.
26. Gangadhara R, Mohammad Munawar T, Diwakar Reddy K, Prasad NB, Jayasimha Rayalu D. A study on the probabilities of the production of biodiesel from naturally isolated bacterial sources. International Journal of Pharmacy & Life Sciences. 2013;1;4(2).

27. Legat A, Gruber C, Zangger K, Wanner G, Stan-Lotter H. Identification of polyhydroxyalkanoates in Halococcus and other haloarchaeal species. Applied microbiology and biotechnology.2010 ;1;87(3):1119-27.
28. Ausubel, F.M., Brent, R.., Kingston, R..E., Moore, D.D., Seidman, J.G., Smith J.A., & Stuhl, K. Current protocol in molecular biology. (1987) Wiley, New York.
29. Jape, A., Harsulkar, A., & Sapre, V. R. Modified Sudan Black B staining method for rapid screening of oleaginous marine yeasts. International journal of current microbiology and applied sciences. 2014; 3(9), 41-46.
30. Shruthi, P., Rajeshwari, T., Mrunalini, B. R., Girish, V., & Girisha, S. T. Evaluation of Oleaginous Bacteria for Potential Biofuel. Int. J. Curr. Microbiol. App. Sci, 2014; 3(9), 47-57.
31. Cooksey, K. E., Guckert, J. B., Williams, S. A., & Callis, P. R. Fluorometric determination of the neutral lipid content of microalgal cells using Nile Red. Journal of microbiological methods, 1987; 6(6), 333-345.
32. Matsunaga, T., Matsumoto, M., Maeda, Y., Sugiyama, H., Sato, R., & Tanaka, T. Characterization of marine microalga, *Scenedesmus sp.* strain JPCC GA0024 toward biofuel production. Biotechnology Letters, 2009; 31(9), 1367-1372.
33. Abdo, S. M., Ahmed, E., El-Enin, S. A., El Din, R. S., & Ali, G. E. D. G. Qualitative and quantitative determination of lipid content in microalgae for biofuel production. J. Algal Biomass Utln., 2014; 5(3), 23-28.
34. Sriwongchai, S., Pokethitiyook, P., Kruatrachue, M., Bajwa, P. K., & Lee, H. Screening of selected oleaginous yeasts for lipid production from glycerol and some factors which affect lipid production by *Yarrowia lipolytica* strains. The Journal of Microbiology, Biotechnology and Food Sciences, 2013; 2(5), 2344.
35. Vipra, A. A., Desai, S. N., Roy, P., Patil, R., Raj, J. M., Narasimhaswamy, N., & Sriram, B. Antistaphylococcal activity of bacteriophage derived chimeric protein P128. BMC microbiology, 2012;12(1), 1-10.
36. Perez-Garcia, O., Escalante, F. M., de-Bashan, L. E., & Bashan, Y. Heterotrophic cultures of microalgae: metabolism and potential products. Water research, 2011; 45(1), 11-36.
37. Ongmali, R., Phunpruch, S., & Thawornchaisit, U. Cellular lipid production of a heterotrophic bacterium isolated from poultry processing wastewater. Songklanakar in Journal of Science & Technology, 2014; 1 (3), 1-20.
38. Papanikolaou, S., Chevalot, I., Komaitis, M., Marc, I., & Aggelis, G. Single cell oil production by *Yarrowia lipolytica* growing on an industrial derivative of animal fat in batch cultures. Applied Microbiology and Biotechnology,2002;58(3), 308-312.
39. Huang, J., Shi, Q., Zhou, X., Lin, Y., Xie, B., & Wu, S. Studies on the breeding of *Mortierella isabellina* mutant high producing lipid and its fermentation conditions. Wei sheng wu xue tong bao, 1997; 25(4), 187-191.
40. Liang, Y., Beardall, J., & Heraud, P. Changes in growth, chlorophyll fluorescence and fatty acid composition with culture age in batch cultures of *Phaeodactylum tricornutum* and *Chaetoceros muelleri* (Bacillariophyceae). Botanica Marina, 2006; 49(2), 165-173.
41. Zhao.C., Zhi Gang Liu., Qingli.Z.,Guorong S., Tang. X, Wenshan Shi & Xinli Lu. Effects of culture conditions on single cell oil accumulation. Journal of Chemical and Pharmaceutical Research 2015; 7(3): 2189-2191.
42. Mohammady, N.G. E., Rieken, C.W., Lindell, S.R., Reddy, C. M.,Taha, H.M., PuiLing, L., & Carmichael, C. A. Age of nitrogen deficient microalgal cells is a key factor for maximizing lipid content. Research Journal of Phytochemistry,2012;6(2), 42-53.