Original Research Article

Isolation and characterization of thermostable lipases from themophilic bacteria isolated from hot water spring

R J Pungle*, S G Sapkal, B D Vyawahare, P R Bhandwalkar, S B Bedwal, R S Pise, S A Rathod

Department of Biotechnology, Shivchhatrapati College, Aurangabad, Maharashtra-431005, INDIA.
Email: patilrohini194@gmail.com

Abstract

Twelve isolates were screened for lipases production from hot water spring, Vajreshwari, Thane, Maharashtra on a Nutrient agar and nutrient broth medium consisting of olive oil. The isolate showed highest lipase activity was VIII which later was identified as *Sphingomonas paucimobilis*. The effect of pH, temperature and substrate concentration was studied on partially purified lipases by ammonium sulphate precipitation from *Sphingomonas paucimobilis*. The partially purified lipase had maximal activity at pH of 7.The optimum temperature for the hydrolysis of olive oil at 60°C.The lipases has ability to hydrolyse used fried oil. The transesterification activity of lipases was used to synthesis biodiesel. The analysis of synthesized biodiesel was performed by FTIR(Fourier transform infrared spectroscopy) and free fatty acid content.

Key Words: Vajreshwari hot water spring, Lipases, Biodiesel, *Sphingomonas paucimobilis*.

***Address for Correspondence:**
Ms. R. J. Pungle, Assistant Professor, Department of Biotechnology, Shivchhatrapati College, Aurangabad, Maharashtra-431005, INDIA.
Email: patilrohini194@gmail.com

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INTRODUCTION

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are serine hydrolases that catalyze the hydrolysis, esterification and transesterification of long-chain triacylglycerols at an oil-water interface. The lipases are able to reverse the reaction in absence of water to form triacylglycerides from fatty acids and glycerols by esterification reaction. The another catalytic activity exhibited by lipases is transesterification utilizing fats, oils and fatty acids to produce alkylesters[2,9,20]. Lipases are carboxylic ester hydrolases attracting an enormous attention due to they are the most versatile and widely used enzyme in biotechnological applications viz. processing of fats and oils, detergents, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture and production of cosmetics and pharmaceuticals, bakery, brewing, biofuels, leather processing and general cleaning. Lipases are produced by microorganisms (bacteria and fungi), plants and animals from that microbial lipases are more useful since they have great variety of catalytic activities and microorganisms are easy to manipulate genetically continuous supply due to absence of seasonal fluctuations and capable of rapid growth on inexpensive media[4,12,16]. Thermostable lipases are in need due to many processes use temperatures around 50°C by having high melting points of the lipids that are used as substrate. The industrial demand for thermostable lipases stimulates the search for microorganisms that produce such thermostable enzymes. The advantage to have elevated temperatures for reaction is favorable changes in most physical properties of fats at high temperature and increased stability of thermostable lipases to organic solvents determine the increased interest in e new thermostable[2,16] The source of thermostable enzyme is thermophilic bacteria which can survive at elevated

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temperature 45°C–80°C[9]. It has been revealed that 99% of bacteria are unexplored in laboratory cultivation from various unexploited regions of the earth[4,12]. Thermophilic lipases show higher thermostability, higher activity at elevated temperature and often show more resistance to chemical denaturation. This makes them ideal tools in industrial and chemical processes where relatively high reaction temperatures and/ or organic solvents are used. The industrial demand search for the thermostable continues to stimulate the microorganism's produces of thermostable enzymes[20]. It has been found out that energy consumption for development made more dependency on fossil fuel which is causing green house effect. The alternative source may the biodiesel (fatty acid alkyl monoester) derived from feedstocks such as vegetable oils and animal fats. Biodiesel is biodegradable, non-toxic, and can be used directly or blended with conventional petrodiesel in unmodified diesel engines. For production of biodiesel alkali treatment used having drawback of saponification in presence of water contamination. To have a adventitious method the lipases are getting interest due to their versatility and stability[5,18]. Thus the present study isolation and screening of thermophilic bacteria for lipases was demonstrated. It was first time *Sphingomonas paucimobilis* was isolated from hot water spring from Vajreshwari, Thane, Maharashtra. Lipases from *Sphingomonas paucimobilis* was studied for production and optimization for physical parameters with exploitation for biodiesel production.

MATERIALS AND METHODS

All the chemicals used in the study were procured from Himedia Pvt. Ltd. And SD Fine India Pvt. Ltd.pH meter, weighing balance (Shimazdu), water bath, Autoclave (Kumar lab product), Incubator (Meta-lab), centrifuge (Remi).

Collection of sample: Hot water spring sample was collected in sterile tubes from Vajreshwari, Thane, Maharashtra.

Isolation of Thermophilic Micoorganisms and Screening for Lipase producers: The water samples were streaked over nutrient agar plate for isolation and purification of bacterial culture. The isolates were spot inoculated on lipase medium consisting of nutrient agar with oil and incubated at 60^0C for 24hr, 48hr and 72hr. The Bacterial colonies showing zone of clearance were selected as lipase producer[3,15]. The isolates capable of lipase production were further screened to isolate the best possible lipase producing bacteria based titrimetric assays. The selected isolates were inoculated in Nutrient broth medium consisting olive oil as substrate with Tween 80 as emulsifier and incubated at 60^0C for 24hr, 48hr and 72hr. The enzyme activity of supernatant (crude enzyme) was evaluated. The culture having highest enzyme activity was selected for production of lipases, partial purification of enzyme and optimization[15]. All the experiments were performed in duplicate.

Lipase Production: The composition of lipase production media was: peptone (0.2gm), glucose (0.2gm), $(NH_4)_2SO_4$ (0.1gm), K_2HPO_4(0.2gm), olive oil (1ml), tween80 (1-2 drops) and distilled water (100ml).The 1ml of overnight culture was inoculated in production media and incubated at 60^0C with constant shaking at 120rpm for 48hr. After 48 hours of incubation, the culture was centrifuged at 10,000 rpm for 20 min at 4ºC and the cell free culture supernatant fluid was used as the sources of extracellular enzyme. The lipase activity in the supernatant was determined by the titrimetric method. The bacterial isolate that produced maximum lipase was selected for further work[21].

Enzyme assay: The inoculums was centrifuged at 10,000rpm at 4^0C to pellet out the cells. The titrimetric method was applied for determination of enzyme activity. From collected supernatant 1ml was added in 10% of olive oil substrate with tween 80 as emulsifier. To the reaction mixture 500µl of phosphate buffer was added. The reaction mixture incubated at 60^0C for 1hr with constant shaking at 120rpm. The 500 µl of ethanol was added to stop the reaction. The released fatty acid was titrated with sodium hydroxide (0.05N) in buret with phenolphthalein. Titration was carried out until persistant light pink colour appears. Control experiment was done by mixing phosphate-buffered olive oil with tween 80 emulsion substrate mixture and titrating with sodium hydroxide (0.05 N). The quantity of fatty acid liberated is equivalent to the volume of NaOH used and it was calculated using Eq. (1), where N is the normality of NaOH titrant used (0.05 in this case).

Eq. (1):

µlmole fatty acid per ml sample = (ml NaOH of sample – ml NaOH of control) * N*1000/

Volumn of reaction mixture

One unit (U) of lipase activity is defined as the amount of enzyme that releases from the emulsion substrate 1 µmole of fatty acid per ml per minute under specific assay condition.[9]

Protein Estimation: Protein estimation was performed by Foline lowry method[4].

Partial Purification of Lipase: Lipase purification was carried out at 4°C. The culture medium was centrifuged at 10000 rpm for 20 min to obtain crude enzyme, the supernatant fluid was subjected to precipitation with ammonium sulphate to 30 % saturation and allow to precipitate for 24 hours. The precipitate was collected by centrifugation at 10000rpm at 4^0C for 20min.Lipase activity both in the precipitate. The pellet was dissolved

in TriCl buffer of pH 7.4 and dialyzed for overnight at 4^0C.The dialyzed enzyme was used for characterization of enzyme[21].

Characterization of Lipase: The enzyme was incubated at 60^0C with olive oil emulsified with tween 80 substrate in buffers viz. citrate buffer (for pH 5.0), phosphate buffer (for pH 6.0,7.0,8.0) and Tris-HCl buffer (for pH 9.0,10.0,11.0) under assay conditions and enzyme activity was determined. The optimum temperature for the enzyme activity was determined by incubating the assay mixture at different temperatures (20^0C, 37^0C, 50^0C, 60^0C and 70^0C, 80^0C) and enzyme activity was detected[15]. The effect of substrate concentration for enzyme activity was determined by incubating the assay mixture at different substrate concentration (1%,2%, 4%, 6%, 8%,10% and 12%) and enzyme activity was detected.

Lipases on used Fried Oil: The ability of lipases to act on high acid value oil was investigated.10% 0f used fried oil was mixed with 0.5ml phosphate buffer and 50µl of enzyme was added and incubated at 60^0C with a control and enzyme activity and free fatty acid content was determined by enzyme assay.

Lipases in biodiesel production: The partially purified lipase was mixed with fried oil and methanol in the ratio of oil, methanol was 1:1 and 0.5ml of lipase was added. The transesterification was operated at 55^0C temperature for 24 hours. The free fatty acid content of the was measured after free fatty acid conversion to biodiesel[10].

The analytical method: Biodiesel from lipase was characterized for free fatty acid content and FTIR analysis. Fatty acid content were determined by titration method[8,10]. FTIR (Bruker) was performed at RUSSA center, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad.[14]

RESULT AND DISCUSSION

Isolation of Thermophilic Microorganisms and Screening for Lipase production: From the hot water spring Vajreshwari, Thane, Maharashtra the bacteria isolated were 12 in number as reported by Pimpliskar, 2014.The isolated microorganisms were plated on lipase medium consisting of nutrient agar with Olive oil and incubated at 60^0C for 24hr and 48hr.The bacterial colonies having clear zone ariond them were considered as lipase producer. Colony no. I,II,III,IV,V,VI,VII,VIII,XIII and XVIII had cleare zone around it. The isolates having clear zone was assayed for maximum enzyme activity by inoculating in Nutrient broth with olive oil and detected for enzyme activity by titrimetric method. From Graph 1 Colony I and VIII has maximum enzyme activity, thus selected for production of lipases. Colony no. I and VIII had maximum enzyme activity in 48hr. The enzyme activity of all isolates was declined in 72hr. Of incubation.

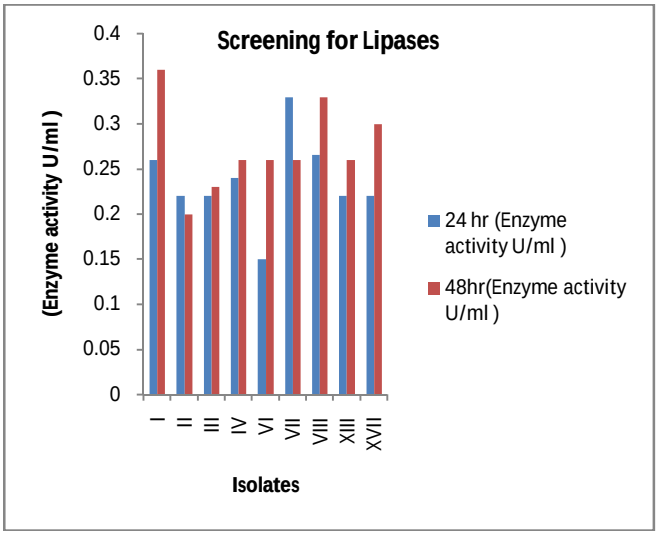

Figure 1: Secondary screening

Identification of Bacteria: Bacteria was identified which had given highest enzyme activity after partial purification of lipases. The bacteria was identified as *Sphingomonas paucimobilis* by VITEK-2 at Dr, Jagatap-Patil Speciality Laboratory, Barashi. The Studies found out that *Sphingomonas paucimobilis* has lipase activity[17].

Table 1: Biochemical Identification by VITEK-2

Well	Test	Mnemonic	Isolate 8	Well	Test	Mnemonic	Isolate 8
2	Ala-Phe-Pro Arylamidase	APPA	–	34	D-Tagatose	dTAG	+
3	Adonitol	ADO	–	35	D-Trehalose	dTRE	+
4	L-Pyrrolydonyl-Arylamidase	PyrA	–	36	Citrate (Sodium)	CIT	–
5	L-Arabitol	IARL	–	37	Malonate	MNT	–
7	D-Cellobiose	dCEL	+	39	5-Keto-D-Gluconate	5KG	–
9	Beta-Galactosidase	BGAL	–	40	L-Lactate alkalinization	ILATk	–
10	H2S Production	H2S	–	41	Alpha-Glucosidase	AGLU	–
11	Beta-N-Acetyl-Glucosaminidase	BNAG	–	42	Succinate alkalinization	SUCT	–
12	Glutamyl Arylamidase pNA	AGLTp	–	43	Beta-N-Acetyl-Galactosaminidase	NAGA	–

13	D-Glucose	dGLU	+	44	Alpha-Galactosidase	AGAL	–
14	Gamma-Glutamyl-Transferase	GGT	–	45	Phosphatase	PHOS	–
15	Fermentation Glucose	OFF	–	46	Glycine Arylamidase	GlyA	–
17	Beta-Glucosidase	BGLU	–	47	Ornithine Decarboxilase	ODC	–
18	D-Maltose	dMAL	+	48	Lysine Decarboxylase	LDC	–
19	D-Mannitol	dMAN	+	52	Decarboxylase Base	0DEC	–
20	D-Mannose	dMNE	+	53	L-Histidine assimilation	IHISa	–
21	Beta-Xylosidase	BXYL	–	56	Courmarate	CMT	+
22	Beta-Alanine arylamidase pNA	BAlap	–	57	Beta-Glucuronidase	BGUR	–
23	L-Proline Arylamidase	ProA	–	58	O/129 Resistance (comp. vibrio.)	O129R	–
27	Palatinose	PLE	–	59	Glu-Gly-Arg-Arylamidase	GGAA	–
29	Tyrosine Arylamidase	TyrA	–	61	L-Malate assimilation	IMLTa	–
31	Urease	URE	–	62	Ellman	ELLM	–
32	D-Sorbitol	dSOR	–	64	L-Lactate assimilation	ILATa	–
33	Saccharose / Sucrose	SAC	–		Organisms identified by VITEK-2		Sphingomonas pausimobilis

Partial Purification of Lipase: Partial purification of lipases was carried out by 30% ammonium sulphate precipitation. It was noted down that enzyme was precipitated at 30% saturation only as precipitate has enzyme activity. The specific enzyme activity was increased after precipitation of lipase. The specific enzyme activity in crud lipases from colony VIII was more than colony I. Thus for further studies colony VIII was taken.

Table 2: Partial purification of lipases

No.	Name	Protein concentration (mg/ml)	Enzyme activity (U/ml)	Specific activity (U/ml/mg)
	Crude extract			
1	I	46.77	0.11	0.002
	VIII	3.5	0.27	0.077
	Ammonium sulphate precipitation			
2	(30%)	1.58	8.1	5.12
	VIII			

Characterization of Lipases: Optimization for pH: The enzymes have effect over activity due to hydrogen ion concentration. Thus evaluation of effect of pH at various pH buffers from pH 5 to pH 11 was performed by using olive oil as substrate and incubating for 1 hr. at 60^0C. It was found that The enzyme activity of Lipases was maximum at pH 7.0 (Graph 2). The enzyme is active at pH 5-pH 6.

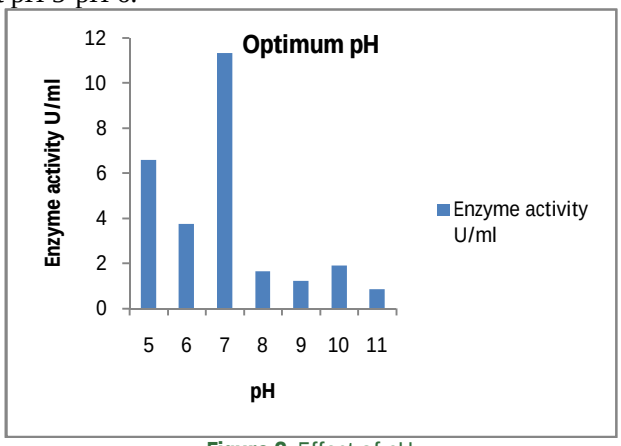

Figure 2: Effect of pH

Optimization for Temperature: Temperature is the critical factor for enzyme activity. The enzyme activity was determined by incubating reaction mixture at various temperatures ranging from 20^0C to 70^0C. It is evident from Graph 3 that gradual increase in enzyme activity of Lipases upto 60^0 C and then decrease. The enzyme activity was maximum at 60^0C.

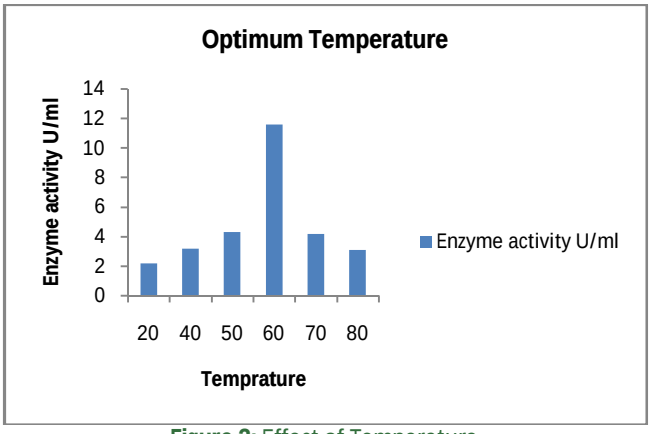

Figure 3: Effect of Temperature

Effect of Substrate concentration: The effect of substrate concentration for enzyme activity was determined by incubating the assay mixture at different substrate concentration (1%, 2%,4%,6%,10% and 12%) and enzyme activity was detected. Optimum Enzyme activity was detected 14% at substrate concentration.The substrate concentrations has to be determined to obtain the saturation point of enzyme.

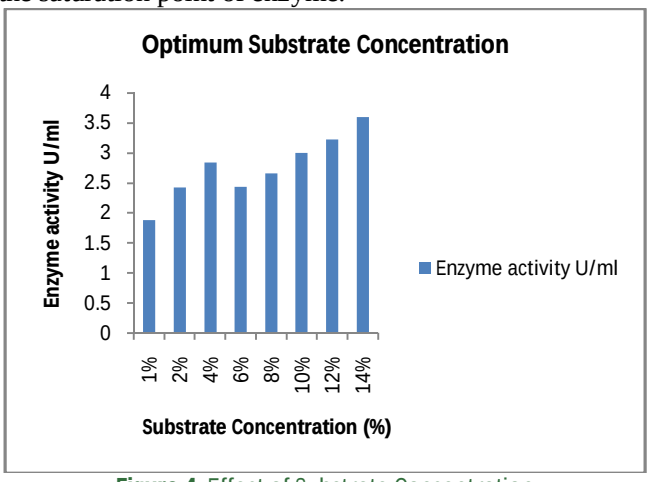

Figure 4: Effect of Substrate Concentration

Lipases on used Fried Oil: The lipases were applied on fried oil obtained from the sweet shop. It was observed from Graph 5 the content of fatty acid was increased after treating lipases with fried oil suggesting lipases effective over fried oil.

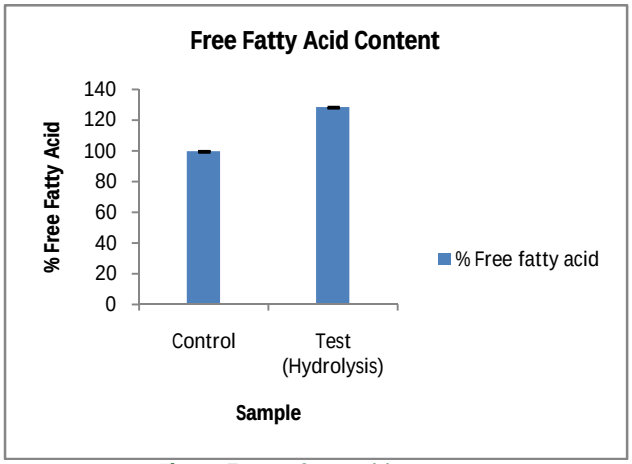

Figure 5: Free fatty acid content

Lipases in biodiesel production: Analysis of Free Fatty acid: The free fatty analysis after transesterification reaction carried out in presence of lipases with used fried oil and methanol was reported according to Graph 6 as fatty acid content was reduced from 99.5% to 4.23% suggesting the conversion of free fatty acid to FAME.

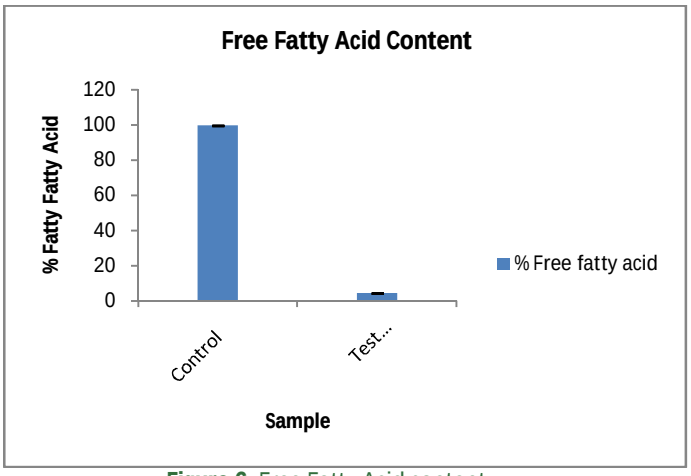

Figure 6: Free Fatty Acid content

FTIR Analysis: For the confirmation of trasesterification reaction and identification of Fatty acid methyl ester the FTIR analysis was carried out. The FTIR analysis of aqueous phase (Graph 7) was with bands at 3328.84 cm^{-1} corresponding to –OH group. According to the peaks 2945.20cm^{-1} and 2833.70cm^{-1} fall between characteristic peaks of lipids at 3100cm^{-1}-2800cm^{-116}. The amid III and amid II bands were represented by 1652.86cm^{-1} and 1410.15cm^{-1} respecrively. Carbohydrate specific peaks were 1111.90cm^{-1} and 1017.64cm^{-1}. FTIR analysis confers the presence of protein which was congugated with carbohydrate group suggesting enzyme may be glycoprotein[11]. The lipid moity observed was may be due to emulsion of lipids or bound lipid to lipases.

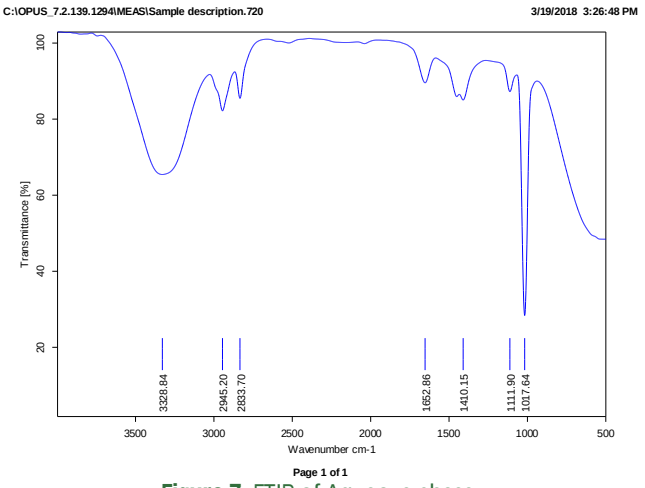

Figure 7: FTIR of Aqueous phase

The FTIR results of oil-methanol phase (Graph 8) obtained demonstrates that peak at 2922.04cm^{-1} and 2854.10 cm^{-1} were typical of lipids of C-H stretching. peak at 1457.62 cm^{-1} was due to asymmetric stretching of $–CH_3$ in FAME and 1158.60 cm^{-1} was characteristic of O-CH_3 typical of biodiesel. In the region from 1800-1700

cm^{-1}, it can be observed peaks that can be attributed to the stretching of C=O, typical of esters. The peak 1370.13 cm^{-1} can be attributed to the glycerol group O-CH_2 (mono-, di- and triglycerides) 1075–1100 cm^{-1}, covering the asymmetric axial stretching of O-CH_2-Ce-CH_2-OH[16].The reaction was in progress as it was observed for peaks 1370.13 cm^{-1} and 1075–1100 cm^{-1}

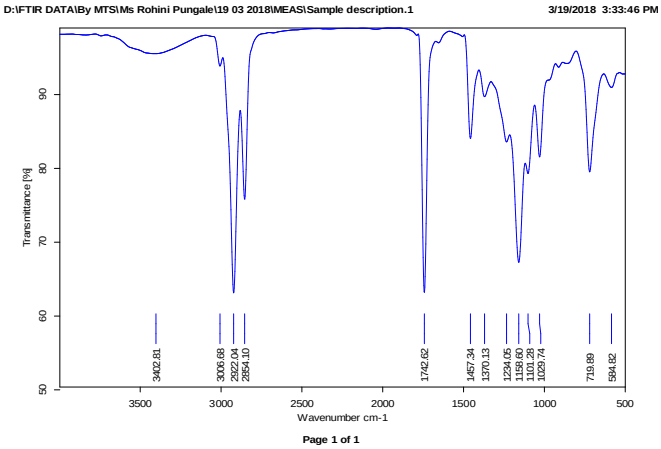

Figure 8: FTIR of Oil and Methanol mixture treated with Lipases

CONCLUSION

From Vajreshwari Hot water spring 12 bacteria were isolated. From that Sphingomonas paucimobilis was identified a culture having maximum lipase activity. *Sphingomonas paucimobilis* was first time evaluated for production, partial purification of of lipase as well as characterization for optimum pH, temperature and substrate concentration as per cited references. Partially purified lipase can hydrolyse used fried oil and its trasesterification reaction exploited for biodiesel synthesis by methanol and used fried oil as substrate. Isolated lipases will be beneficial to utilize waste in the form of used fried oil for a eco-friendly energy generation source i.e. biodiesel.

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