Results

4.1 Screening of microbial population from natural sources for presence of acyltransferase and related class of enzymes for synthesis of HMGR inhibitors

4.1.1 Sample Collection and Isolation of Microorganisms

A total of 69 bacterial samples isolated from soil, water and human biological fluid, and 12 fungus samples from water and soil were collected during the study period. Serial dilutions of samples were prepared separately with sterile saline. Suitably diluted samples were pour plated to obtain pure cultures using SDA for fungi and LB agar plate for bacteria. Upon incubation for 2-3 days at 28^{o}C, the fungal colonies which had grown were isolated, sub-cultured and preserved in potato dextrose agar (PDA) slants (shown in Figure 4.1) for further experiments.

Discrete bacterial colonies obtained after 24 hours of incubation at 37^{o}C were taken, grown in LB broth and were streaked on agar plates (Figure 4.2) to obtain the pure culture. After obtaining the pure cultures, microbial isolates were preserved in glycerol (10% for fungi, 15% for bacteria) at -80^{0}C.

Figure 4.1: Fungal isolates in potato dextrose agar slants

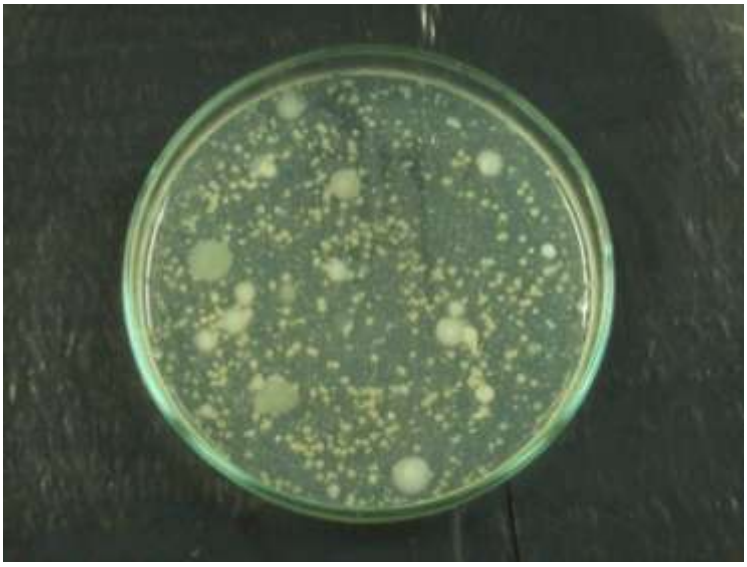


Figure 4.2 (a): Isolation of microbes from samples on LB agar plates

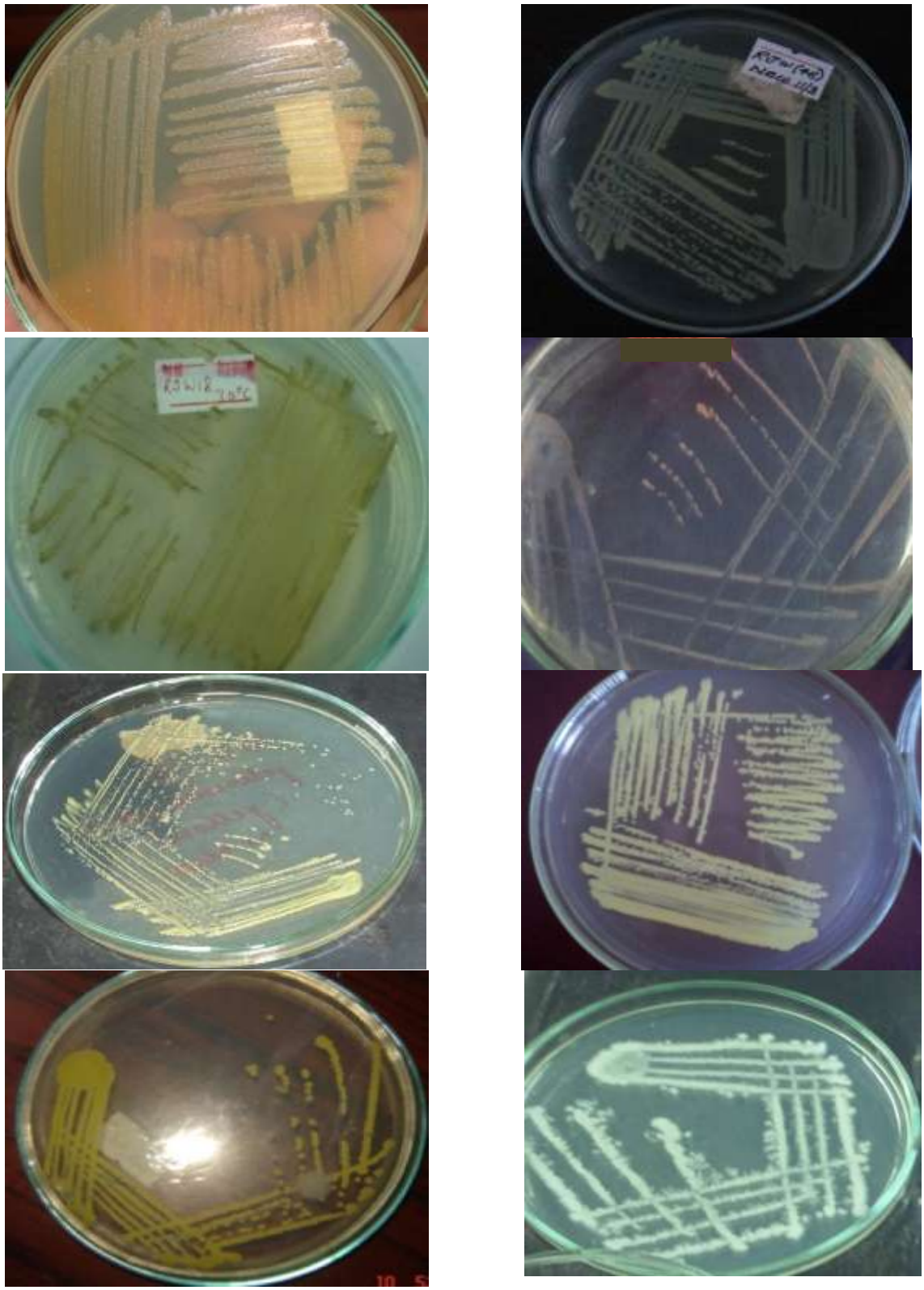


Figure 4.2 (b): Isolation of pure bacteria from mixed bacterial pool on LB agar plates

4.1.2 Primary screening of microbes for production of HMGR inhibitors:

All the isolated microbes (total: 81; fungus: 12; bacteria: 69) were used in biotransformation reaction for bioconversion of precursor molecule, Monacolin J, to HMGR inhibitor, Simvastatin. The product obtained after downstream processing of the biotransformation reaction using the microbial isolates was used for preliminary screening of their HMGR inhibition potential. The primary screening was based on yeast growth inhibition bio-assay. Bioassay guided screening reveals yeast growth inhibition effect for HMGR inhibitors when confronted to *Saccharomyces cerevisiae*. A clear zone of inhibition (ZOI) was found as a result of yeast growth inhibition (Figure 4.3).

Figure 4.3: Plates showing zone of inhibitions of various sizes during screening process.

The size of zone of inhibition was measured to evaluate the yeast growth inhibition capability of the extracted compounds (Table 4.1). Along with the bioassay guided screening, all the extracts were evaluated and estimated for HMGR inhibitor production using thin layer chromatography and UV spectroscopy (200-700nm). The Rf value and λ_{max} was recorded for all the isolated extracts (Table 4.1).

Table 4.1 Primary screening of isolates for production of HMGR inhibitors

Isolate No.	Source	Primary Screening		
		λ_{max}	Rf	Zone size (mm)
Ctrl +ve	***A. terreus***	**238**	**0.72**	**14**
FG 1	Fresh water	254	0.29	0
FG 2	Fresh water	260	0.27	2
FG 3	Soil	231	0.28	8.2
FG 4	Soil	208	0.44	4
FG 5	Soil	267	0.37	3
FG 6	Soil	230	0.11	1
FG 7	**Marine water**	**238**	**0.72**	**14**
FG 8	Soil	254	0.89	6
FG 9	Soil	258	0.43	0
FG 10	Marine water	272	0.39	0
FG 11	Marine water	280	0.41	0
FG 12	Water	250	0.12	4
BG 3	Water	230	0.13	4.8
BG 4	Water	238	0.15	9
BG 5	Water	230	0.23	3
BG 6	Water	230	0.22	1
BG 7	Water	230	0.42	6
BG 8	Soil	232	0.11	0
BG 9	Soil	236	0.27	3
BG 10	Soil	238	0.44	7
BG 11	Soil	238	0.17	1
BG 12	Biological fluid	238	0.43	6
BG 13	Biological fluid	237.5	0.39	6
BG 14	Biological fluid	230	0.21	8
BG 15	Biological fluid	230	0.34	0

Isolate No.	Source	Primary Screening		
		λ_{max}	Rf	Zone size (mm)
BG 16	Biological fluid	238	0.28	9
BG 17	**Biological fluid**	**237.5**	**0.73**	**16**
BG 18	Biological fluid	238	0.48	7.3
BG 19	Biological fluid	238	0.32	6
BG 20	Biological fluid	238	0.71	9
BG 21	Biological fluid	212	0.32	0
BG 22	Biological fluid	228	0.26	4.7
BG 23	Biological fluid	238	0.71	8
BG 24	Biological fluid	228	0.11	0
BG 25	Biological fluid	219	0.13	0
BG 26	Biological fluid	229	0.09	10
BG 27	Biological fluid	230	0.17	0
BG 28	Biological fluid	239	0.11	0
BG 29	Biological fluid	230	0.13	0
BG 30	Biological fluid	238	0.16	8
BG 31	Biological fluid	237.5	0.46	7
BG 32	Biological fluid	230	0.12	0
BG 33	Biological fluid	238	0.11	9
BG 34	Biological fluid	238	0.70	9.4
BG 35	Biological fluid	238	0.19	8.9
BG 36	Biological fluid	230	0.17	4
BG 37	Biological fluid	238	0.35	0
BG 38	Biological fluid	238	0.21	6.5
BG 39	Biological fluid	238	0.36	4
BG 40	Biological fluid	238	0.23	0
BG 41	Biological fluid	238	0.41	9
BG 42	Biological fluid	238	0.29	7.3

Isolate No.	Source	Primary Screening		
		λ_{max}	Rf	Zone size (mm)
BG 43	Biological fluid	238	0.21	6
BG 44	Biological fluid	238	0.31	0
BG 45	Biological fluid	237.5	0.18	3.8
BG 46	Biological fluid	238	0.15	0
BG 47	Biological fluid	238	0.13	0
BG 48	Biological fluid	238	0.26	0
BG 49	Biological fluid	238	0.09	0
BG 50	Biological fluid	230	0.13	0
BG 51	Biological fluid	238	0.15	8
BG 52	Biological fluid	238	0.23	0
BG 53	Biological fluid	238	0.22	0
BG 54	Biological fluid	238	0.42	8
BG 55	Biological fluid	238	0.11	0
BG 56	Biological fluid	238	0.27	0
BG 57	Biological fluid	238	0.17	6.1
BG 58	Biological fluid	238	0.12	0
BG 59	Biological fluid	238	0.28	7.2
BG 60	Biological fluid	238	0.16	0
BG 61	Biological fluid	238	0.23	0
BG 62	Biological fluid	238	0.44	0
BG 63	Biological fluid	237.5	0.36	7
BG 64	Biological fluid	238	0.71	6
BG 65	Biological fluid	238	0.19	5
BG 181	Biological fluid	270	0.23	6
BG 192	Biological fluid	230	0.39	3.9
BG 193	Biological fluid	219	0.11	7.3
BG 188	**Biological fluid**	**238**	**0.72**	**15**

BG 201	Biological fluid	230	0.26	8
BG 212	Biological fluid	254	0.37	6.6

All the total 81 screened samples were evaluated and categorized on the basis of ZOI size, Rf value and λ_{max} (Figure 4.4 and 4.5). The samples which showed larger ZOI (> 10mm, closer to the statins produced by *A. terreus*) and had λ_{max} 238 nm (absorption maxima for natural and semi-synthetic statins), along with their Rf value closer to the Rf value of standard statins produced by *A. terreus* (positive control) were chosen for further characterization and production at pilot scale in the laboratory.

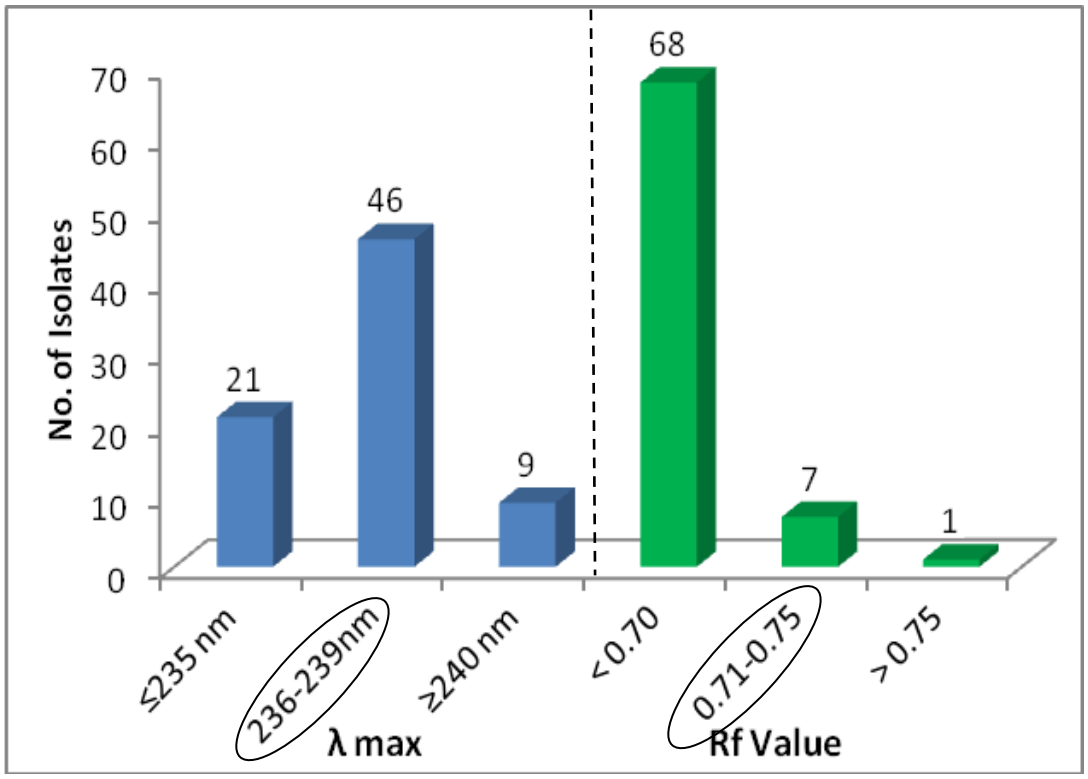


Figure 4.4 Preliminary screening of microbial isolates for HMGR production based on λ_{max} and Rf values (λ_{max} =238 and Rf= 0.72 are values for simvastatin respectively).

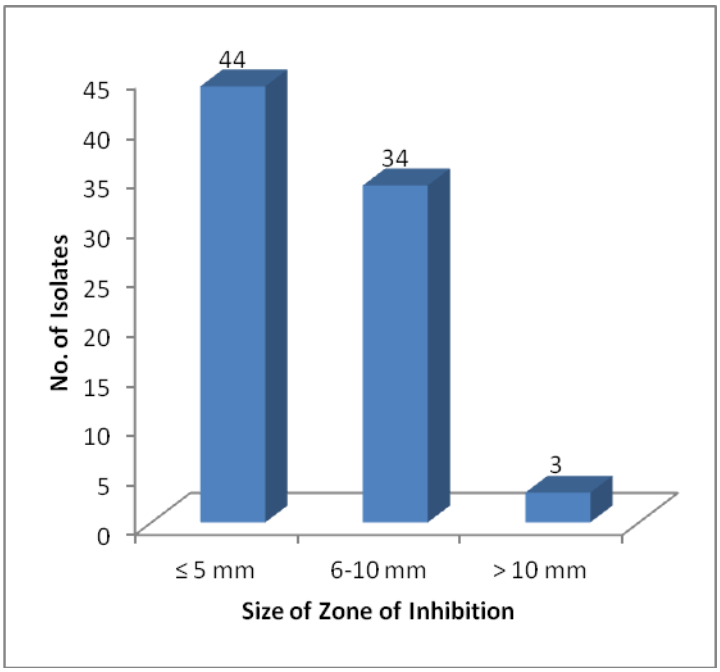


Figure 4.5: Number of microbial isolates vs size of zone of inhibition in yeast growth inhibition bioassay.

4.1.3 Determination of product yield by UV analysis:

Standard stock solution of Simvastatin was prepared by dissolving Simvastatin in methanol (200 µg/ml /ml). The standard solutions were diluted with distilled water to obtain various dilutions (5, 10, 15, 20, 25 and 50 µg/ml). The calibration curve was constructed by plotting drug concentration versus the absorbance values measured on UV spectrophotometer at 238 nm. Further optical densities of samples which showed λ_{max} at 238 nm along with zone of inhibition greater than 7 mm were taken and amount of statin produced was calculated in µg/ml by referring to the standard graph (Figure 4.6 & 4.7).

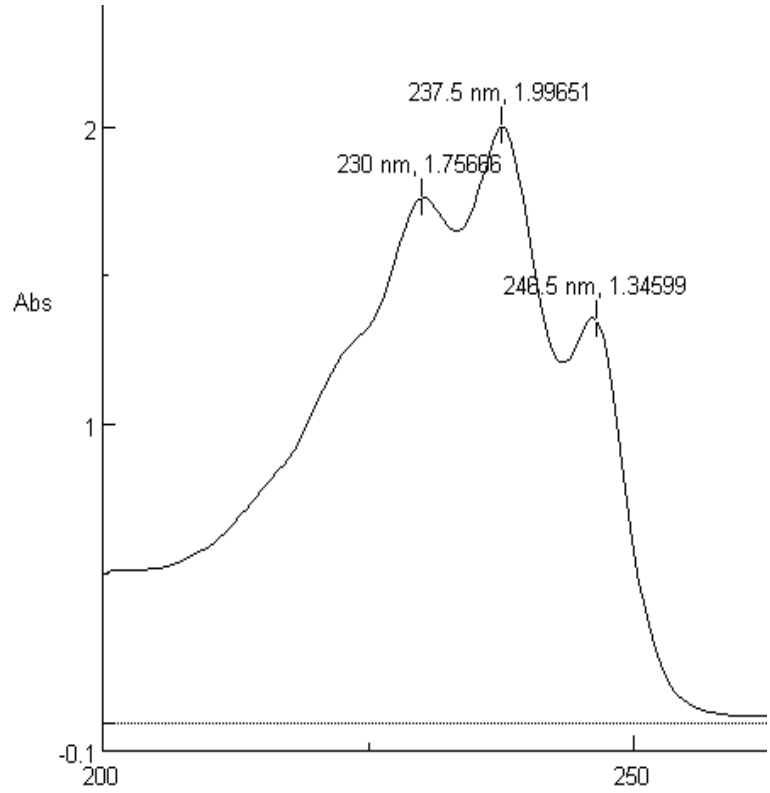


Figure 4.6: UV absorption spectrum of standard Simvastatin

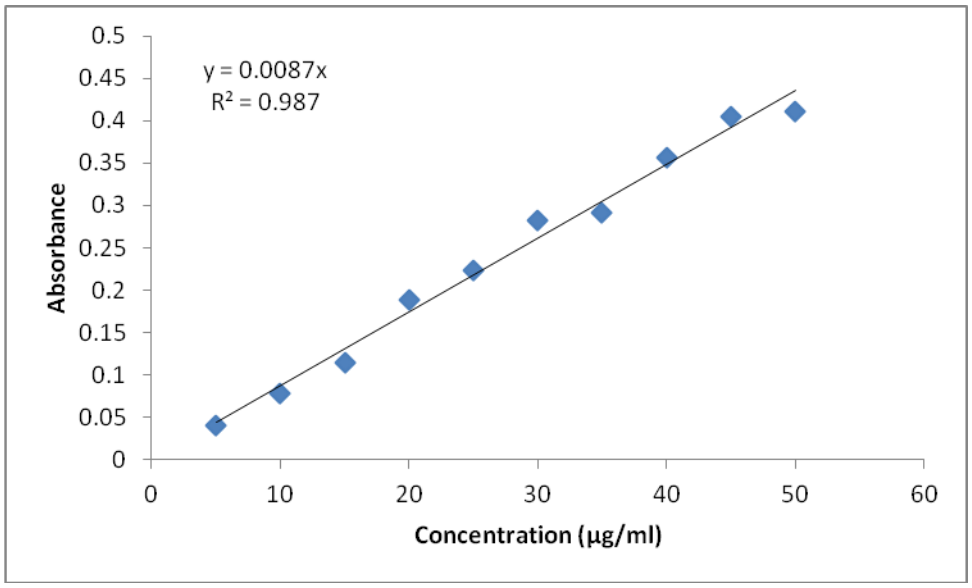


Figure 4.7: Standard calibration graph of Simvastatin

After the primary screening three microbial isolates; one marine derived fungi (FG 7) and two bacterial isolates (BG 17; BG 188) from human biological fluid were found to possess strongest capability for production of HMGR inhibitors (Table 4.2).

Table 4.2: Isolates having high probability to produce HMGR inhibitors

Isolate	Source	λ_{max}	Rf	Zone size (mm)	Yield (µg/ml)
FG7	Marine water	238	0.72	14	121
BG 17	Biological fluid	238	0.73	16	130
BG 188	Biological fluid	238	0.72	12	91

These primary screening results revealed a good correlation between zone of inhibition in bioassay method and yield estimated through UV spectra analysis (Figure 4.8), which confirmed that the isolates showing positive results in primary screening parameters are the strong potential candidates for production of HMGR inhibitors and that the primary screening method was able to identify the HMGR inhibitor producers very efficiently.

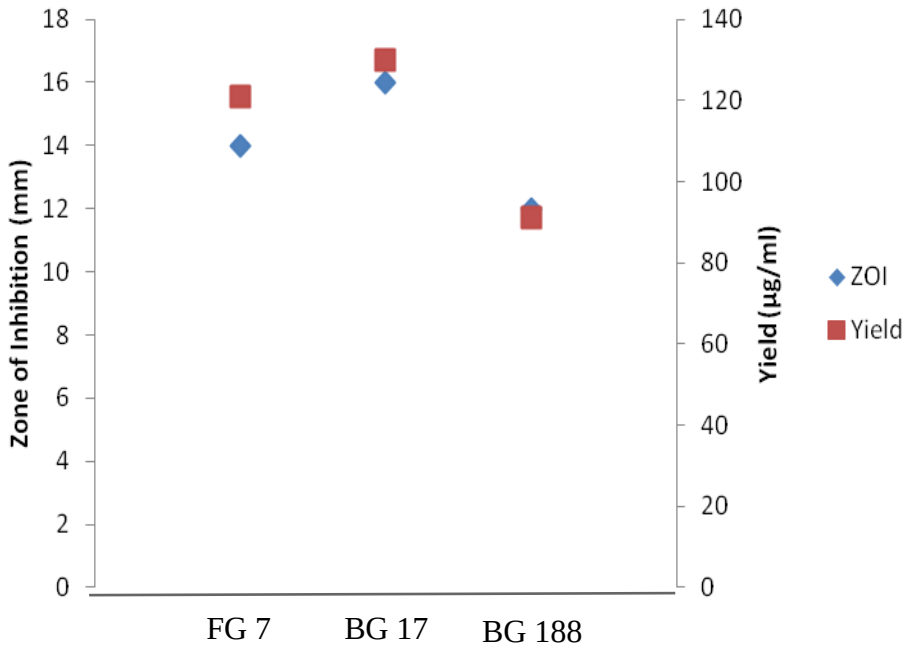


Figure 4.8: Correlation between inhibition zones and HMGR production by isolates.

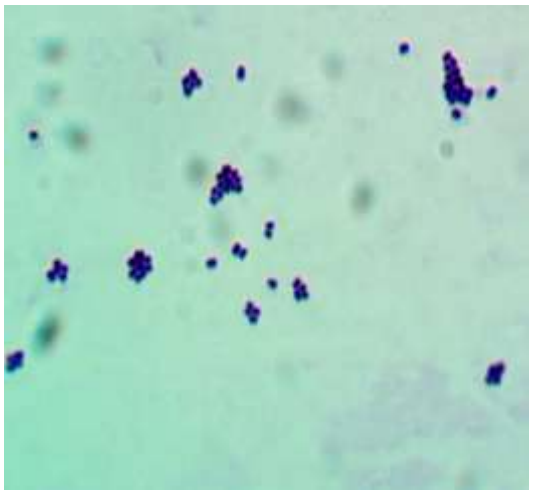
4.1.4 Conclusion of primary screening

In the attempt to screen natural sources for microbes producing HMGR inhibitors, the three isolates which were producing inhibition zone > 10 mm and had Rf value of 0.71 along with λ_{max} at 238nm demonstrated the potential of finding natural sources for microorganisms producing HMGR inhibitors. Further these three prospective microbial isolates were used to accomplish more in-depth investigation on HMGR inhibitor production, strain identification, analytical characterization, molecular biology and in-silico analysis for finding similarities/differences between the fungal and the bacterial genes coding for enzymes involved in synthesis of Simvastatin.

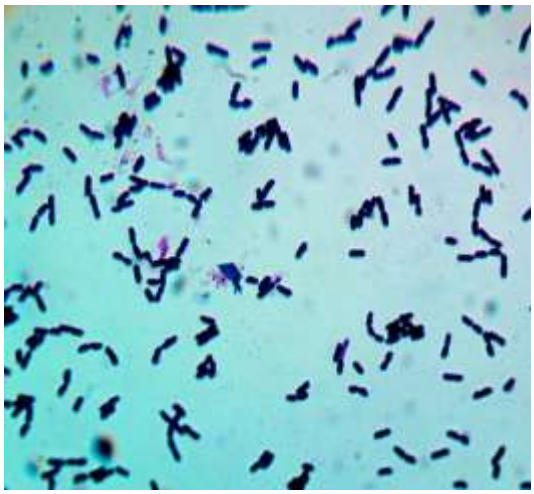
4.2 HMGR inhibitor production by bacterial isolates BG 17 & BG 188

4.2.1 Identification & morphological characterization of bacterial isolates

BG 17 & BG 188, bacteria were isolated from human urine samples. To date numerous studies have been done on various type of fungus for production of HMGR inhibitors, but no bacteria has been found to be able to catalyze the enzyme assisted biosynthesis of statin molecules. In the present study, two bacteria BG 17 & BG 188 were found to possess this property. These bacteria were initially characterized in terms of morphology (color, size, margin, shape, texture) followed by microscopic observations (Gram staining). On LB agar plate, small pin head colonies were observed while greenish colored colonies were obtained on HiChrome agar medium. Morphological characters are described in the Table below and features are shown in Figure 4.9.

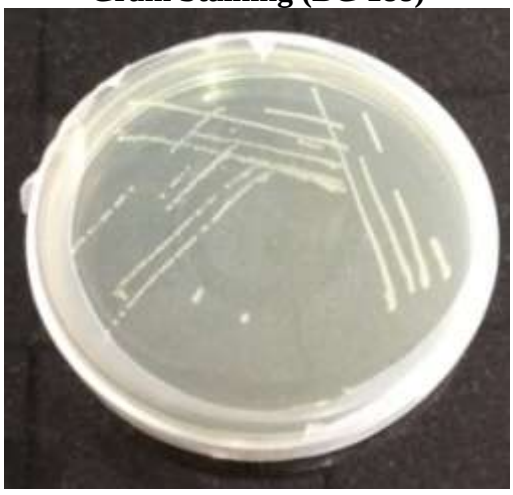


Gram staining (BG 17)

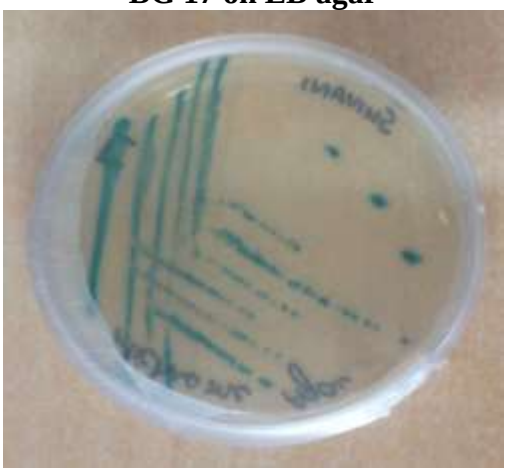


Gram Staining (BG 188)

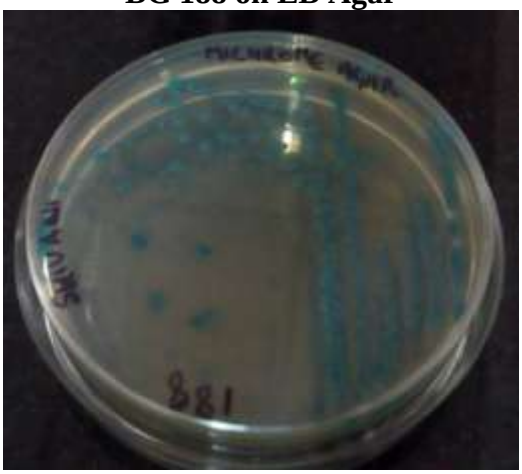
BG 17 on LB agar



BG 188 on LB Agar



BG 17 on Hichrome



BG 188 on Hichrome

Figure 4.9: Morphological features of BG 17 and BG 188

Table 4.3: Morphological characteristics of bacterial strains

Bacterial Strain	Surface	Colour	Shape	Elevation	Opacity	Arrangement	Gram staining
BG 17	Smooth	White	Circular	Convex	Opaque	Pairs	+ve
BG 188	Smooth	Off white	Circular	Flat	Opaque	Chain	+ve

4.2.2 Biochemical Characterization of bacterial strains

Biochemical characterization was performed for both the bacteria based on their ability to enzymatically metabolize specific substrates (Table 4.4)

Table 4.4: Biochemical characteristics of bacterial strains

Tests	BG 17	BG 188
Catalase	-	+
Oxidase	-	+
Citrate utilization	-	+
Indole	-	-
Methyl red	+	-
Voges Proskauer's	+	+
Glucuronidase	-	
Nitrate reduction	-	+
Urease	-	-
H_2S production	-	+
Malonate utilization	-	-
Lysine utilization	-	-
Ornithine utilization	-	-
ONPG	-	-
Phenylalanine Deamination	+	-
Arabinose	-	-
Adonitol	-	-
Esculin Hydrolysis	+	

Cellobiose	+	-
Glucose	+	+
Lactose	+	-
Melibiose	-	-
Raffinose	-	-
Rhamnose	-	-
Saccharose	+	
Sucrose	+	-
Sorbitol	+	-
Trehalose	+	+
Xylose	-	-

In terms of colony morphology (color, shape, size, elevation, margin, consistency, opacity), basic microscopic observations (gram stain, spore stain, cell size) and biochemical tests performed all point towards *Enterococcus sp* for BG 17 and *Bacillus sp* for BG 188.

4.2.3 Identification by 16SrRNA gene sequencing approach

The 16S ribosomal gene was amplified using primers designed for conserved regions of the 16S rRNA gene and polymerase chain reaction. The amplified product was sequenced and the obtained DNA sequence was analyzed for identification of the bacterial strains. Based on a blast analysis BG 17 was found to be *Enterococcus faecalis.*

The sequence obtained was:

CAATGGNCGAAAGTCTGACCGAGCACGCCGCGTGAGTGANGAAGGTTTTCGGATCGT
AAAACTCTGTTGTTAGAGAAGAACAAGGACGTTAGTAACTGAACGTCCCCTGACGGT
ATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTG
GCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCT

GATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAG

TGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGA

GGAACACCAGTGGCGAAGGCGNCTCTCTGGTCTGTAACTGACGCTGATGCTCGAAAG

CGTGGGGANCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTG

CTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCNAACGCATTAAGCACTCCG

CCTGGGGAGTACGACCGCAAGGTTGAAACTCAA

Strain Designation	Closest phylogenetic affiliation	Max ident
17	*Enterococcus faecalis* ATCC 19433(T)16S ribosomal RNA gene partial sequence (ASDA01000001)	99.63%

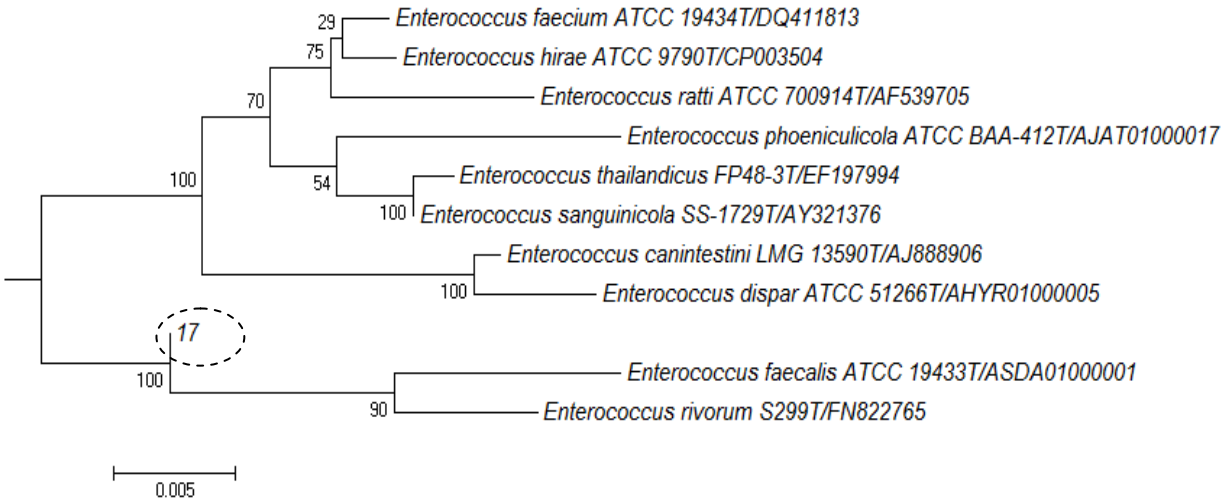


Figure 4.10: Phylogenetic identification of BG 17 based on 16S rRNA gene sequencing

Similar analysis was performed for the PCR product obtained from PCR amplification of DNA isolated from BG 188 and the strain was identified as *Bacillus anthracis*. The sequence obtained was:

GAAGGCGACTTTTTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAA

CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGG

GTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCNTGGGGAGTACGG

CCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG

TGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAAC

CCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGT

CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTT

AGTTGCCATCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAA

GGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTAC

AATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCG

TTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGAAT

CGCGGATCAGCATGCCGCGGGAANGCCCNC

Strain Designation	Closest phylogenetic affiliation	Max ident
188	*Bacillus anthracis* ATCC 14578 (T)16S ribosomal RNA gene partial sequence (AB190217)	99.53%

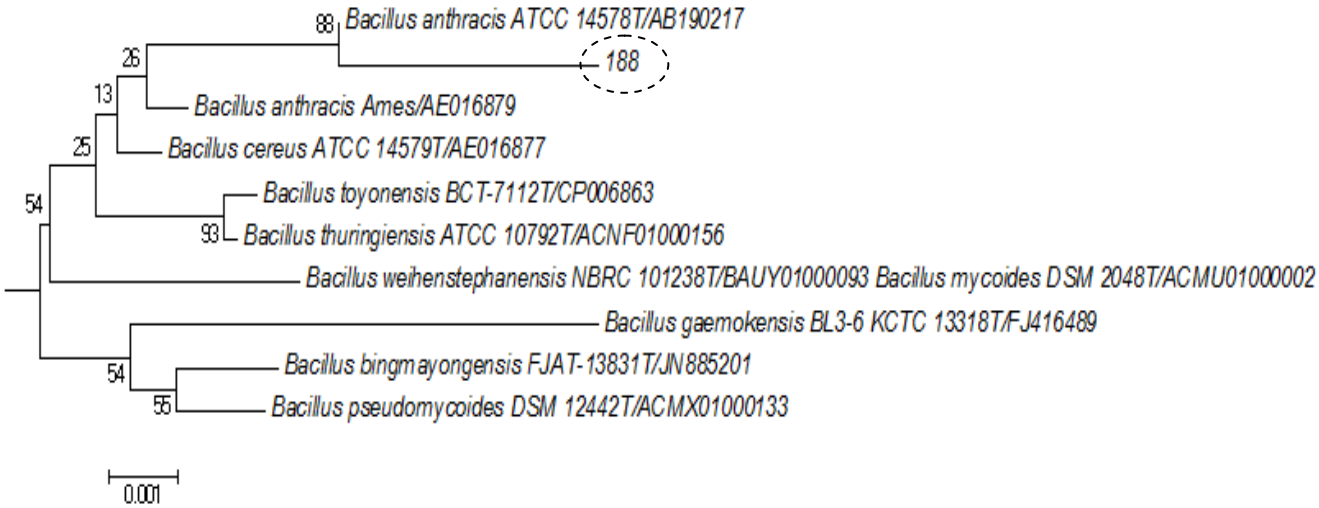


Figure 4.11: Phylogenetic identification of BG 188 based on 16rRNA gene sequencing

These bacteria were used in bio-fermentation reaction as described in materials and methods. After downstream processing the product SK-03 and SK-04 obtained respectively from BG 17 and BG 188 were used for various analytical characterization and biological evaluation for their HMGR inhibition property.

4.2.4 Optimum culture conditions and maximum yield of the product

Temperature is one of the most important factors influencing the activity of bacterial enzymes. The effect of temperature on growth of both the bacteria was studied for forty eight hours (Fig 4.13). The result showed that both of them are mesophilic bacteria. The strains BG 17 and BG 188 showed optimum growth at 37°C and 30°C respectively. Hence these temperatures were chosen for detailed study further.

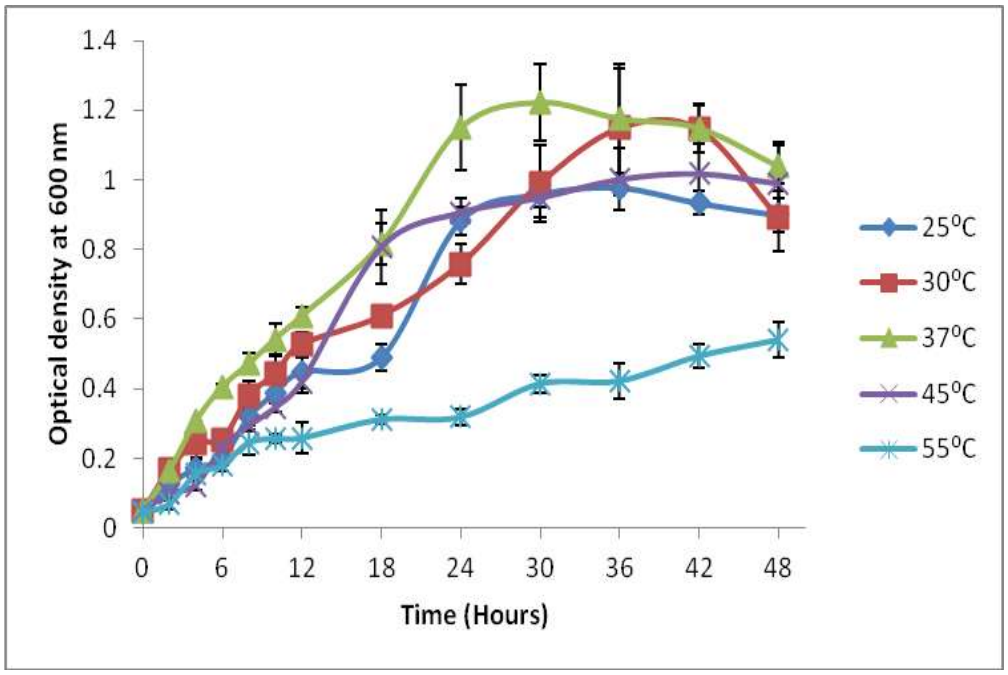


Figure 4.12: Growth of BG 17 in Luria broth at corresponding temperatures. Data are expressed as mean± standard deviation (n=3).

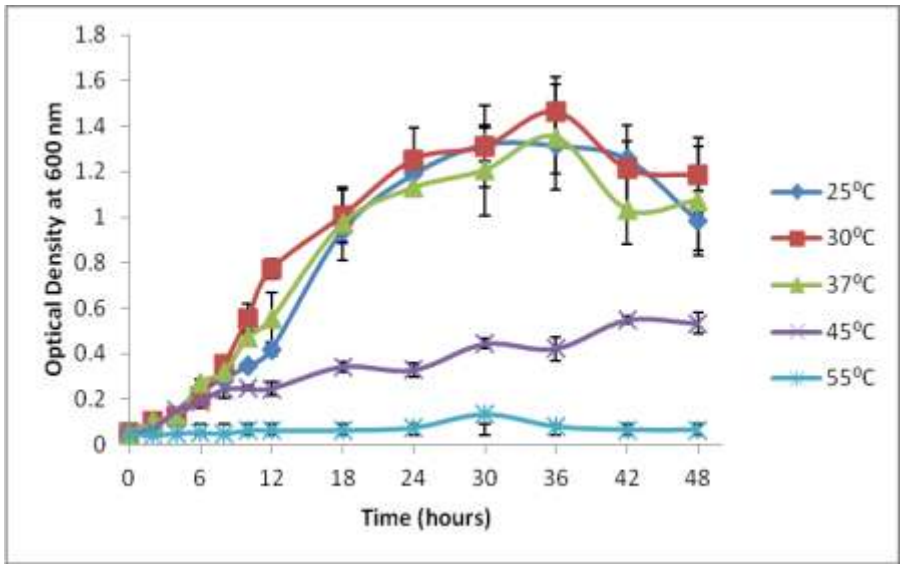


Figure 4.13: Growth of BG 188 in Luria broth at corresponding temperatures. Data are expressed as mean± standard deviation (n=3).

pH is one of the several important factors that influence the growth of bacteria. The effect of hydrogen ion concentration (Fig 4.15) showed that the bacteria grew optimally in the pH range from 6.5 to 8.0 with optimum growth at pH 7-7.5.

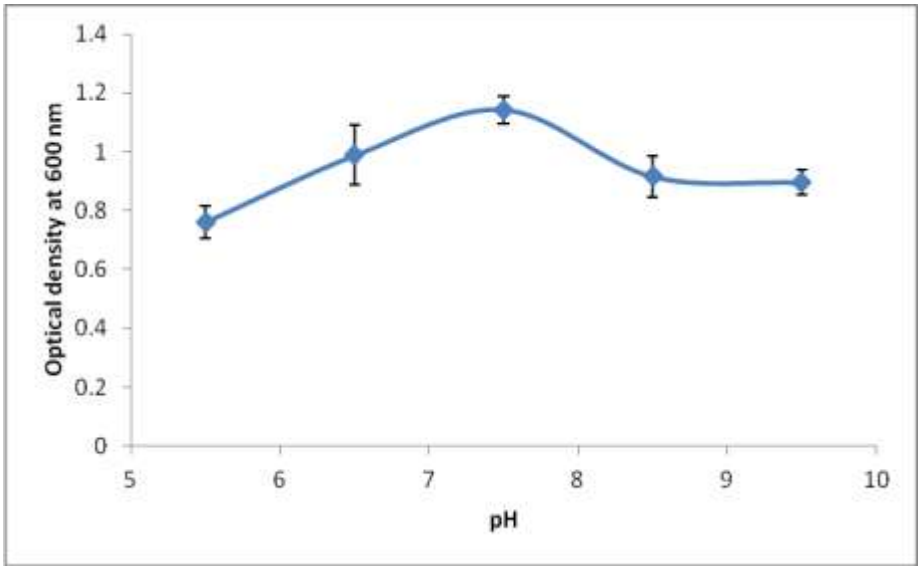


Figure 4.14: Growth of bacteria, BG 17 in Luria broth after 48 hours of incubation at 37°C and corresponding pH. Data are expressed as mean± standard deviation (n=3).

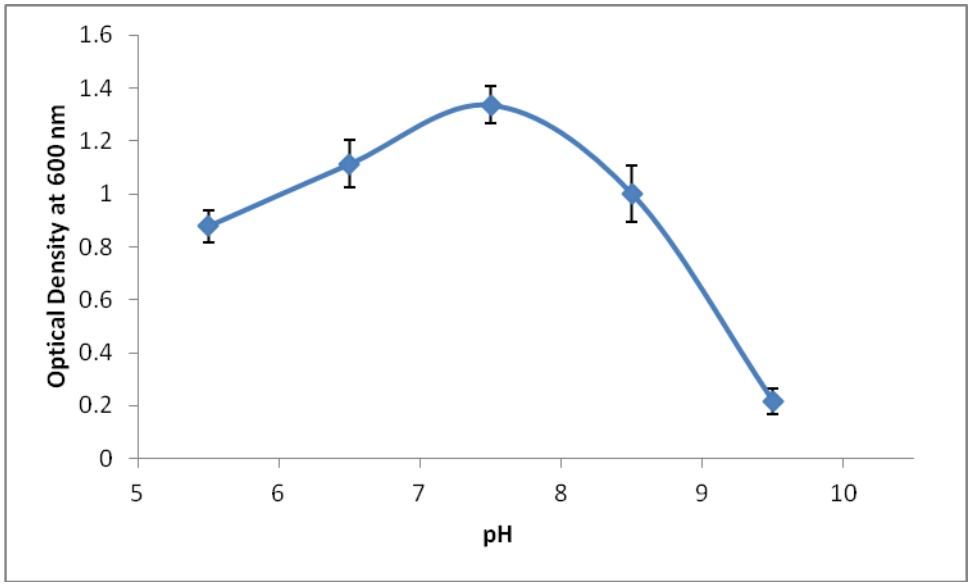


Figure 4.15: Growth of bacteria, BG 188 in Luria broth after 48 hours of incubation at 30°C and corresponding pH. Data are expressed as mean± standard deviation (n=3).

Temperature and pH are the most important factors influencing the activity of bacterial enzymes. The effect of temperature and pH on fermentation yield of Simvastatin was studied for twenty four hours. As both of them are mesophilic bacteria, both the strains showed optimum production at 30 and 37°C. An altered pH can lead to loss or enhanced enzymatic activity. The effect of hydrogen ion concentration showed that both bacteria showed maximum yield at pH 7.5. Similarly, both the isolates showed maximum yield of Simvastatin after 24 hours when tested for varying time period from 16-72 hrs at their pH and temperature optima (Table 4.5)

Table 4.5: Optimum conditions for maximum product yield

	BG 17			BG 188		
Temp (^{0}C)	Absorbance	Dilution	Conc. (µg/ml)	Absorbance	Dilution	Conc. (µg/ml)
25	0.4012	-	46.11	0.273	2	62.75862
30	0.33	3	113.79	0.259	2	**89.42**
37	0.283	4	**130.11**	0.389	2	78.62069
45	0.402	2	92.41	0.342	2	59.54023
55	0.272	2	62.52	0.113	2	25.97701
pH						
5.5	0.326		37.471264	0.231	2	53.10345
6.5	0.388	2	89.195402	0.332	2	76.32184
7.5	0.376	3	**129.65**	0.394	2	**90.57**
8.5	0.274	2	62.988506	0.329	2	75.63218
9.5	0.152		17.471264	0.254	2	58.3908
Time (hrs)						
12	0.28	2	64.367816	0.314	2	72.18391
24	0.375	3	**129.31**	0.391	2	**89.88**
36	0.31	3	106.89655	0.382	2	87.81609
48	0.257	2	59.08046	0.334	2	76.78161
60	0.405		46.551724	0.287	2	65.97701
72	0.346		39.770115	0.119	2	27.35632

4.2.5 Biological evaluation of the product SK-03 and SK-04

4.2.5.1 Yeast growth inhibition bio-assay- effect on yeast HMGR

Since the HMG-CoA reductase activity is essential for yeast growth, the growth kinetics of appropriate yeast strain (*S. Cerevisiae*) in the presence of commercial statin and novel inhibitor was measured. Based on OD_{600} readings growth curves were plotted for each culture. When the culture media was supplemented with SK-03 (product from BG 17) and SK-04 (product from BG 188), 73% and 65% yeast growth inhibition was observed as compared to the buffer supplemented culture (Figure 4.16). Similar effect on yeast growth inhibition was observed in a plate assay. A clear zone of inhibition was observed around SK-03 and SK-04 loaded wells on the yeast inoculated YEPD plate (Figure 4.17).

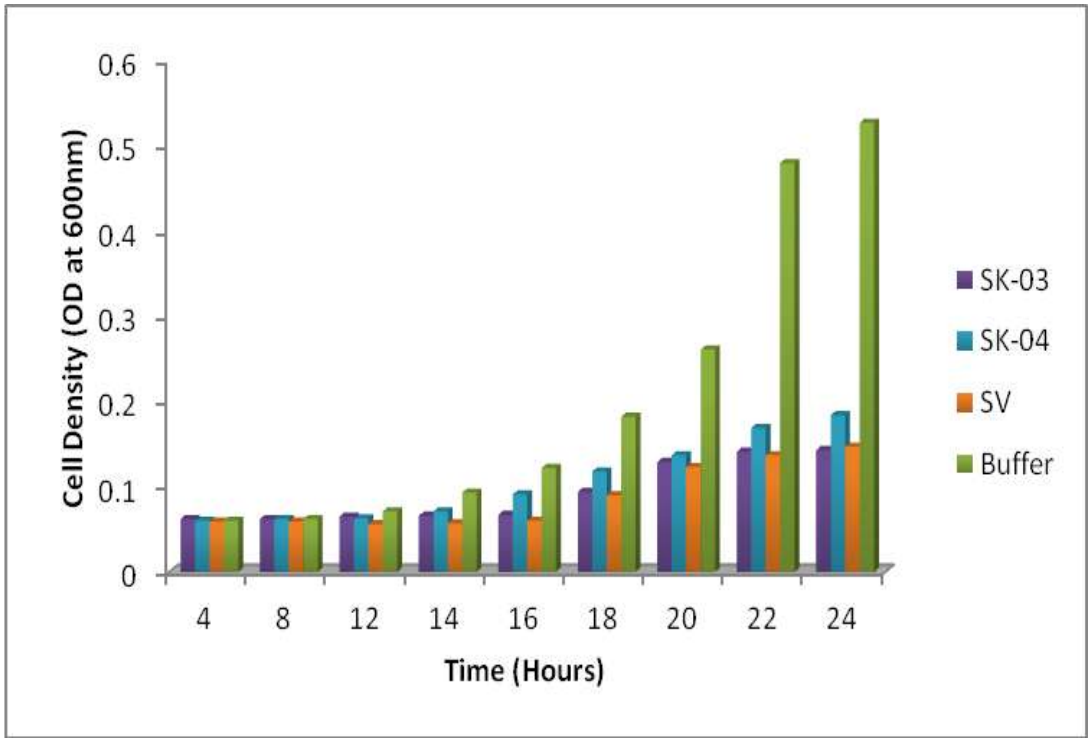


Figure 4.16: Yeast growth inhibition bioassay-broth assay

Figure 4.17: Yeast growth inhibition bioassay-plate assay

4.2.5.2 Effect on recombinant yeast strains expressing human HMGR enzyme

The effects of SK-03 and SK-04 on the level of expression of HMGR gene encoding enzyme involved in mevalonate biosynthesis were investigated in the recombinant yeast strain MB10-1D carrying human *HMGR* gene, whose expression was induced to 1.36 and 1.29 folds after SK-03 and SK-04 treatment respectively as compared to buffer treated cells. SK-02 is the fungal extract described in section 4.3.2 (Fig. 4.18). Data normalization was carried out in relation to the transcript of the housekeeping 35S rRNA

gene. Statistical significance was calculated using student t-test. P-value less than 0.05 was considered to be significant. P-value was found to be 0.0174, 0.0044, and 0.0030 for SK-02, SK-03 and SK-04 respectively.

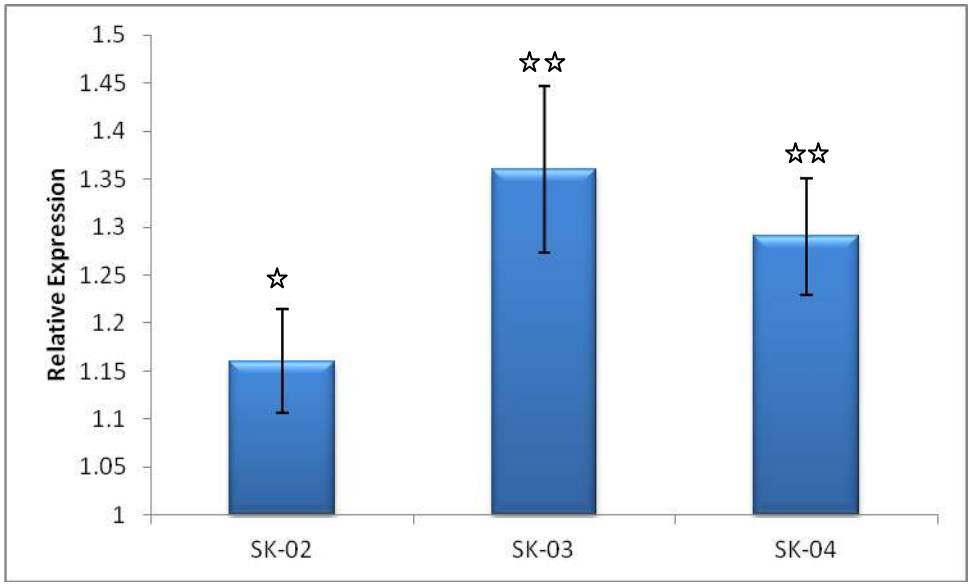


Figure 4.17: Quantitative real-time RT-PCR analysis of HMGR gene encoding enzymes of mevalonate biosynthesis pathway after extract treatment compared to buffer-treated cells. Data are expressed as mean± standard deviation (n=3). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

4.2.5.3 Triglyceride Release Assay

To directly test the relationship between statin sensitivity and triglyceride levels, we measured the triglyceride content of the cell lines (MDA-MB 231) before and after treatment. Treatment with SK-03 and SK-04 reduced the levels of triglycerides released in to the medium by 32% and 22.7 % when compared to that of the control treatment as shown in Table 4.6.

Table 4.6: Triglyceride release assay

	TG conc. (mg/dl)	% inhibition
Methanol	74.33	Nil
MJ	74.33	Nil
LV	45.98	38.1
SV	43.68	41.2
SK-03	50.57	32.0
SK-04	57.47	22.7

4.2.5.4 Cytotoxicity assay- MTT (methylthioazole tetrazolium) assay

It has been proved that statins are not only capable of reducing cardiac disease related mortality, but cancer incidence is also reduced by 28-33%. Statins are shown to be cytotoxic agents that partially explain theri antitumor activity and support for their therapeutic use as a cancer chemopreventive agent. Both SK-03 and SK-04 showed cytototoxicity against breast cancer cell line, MDA MB-231 with IC_{50} of 1mg/ml and 0.5 mg/ml respectively, which proved their anti-proliferative activity.

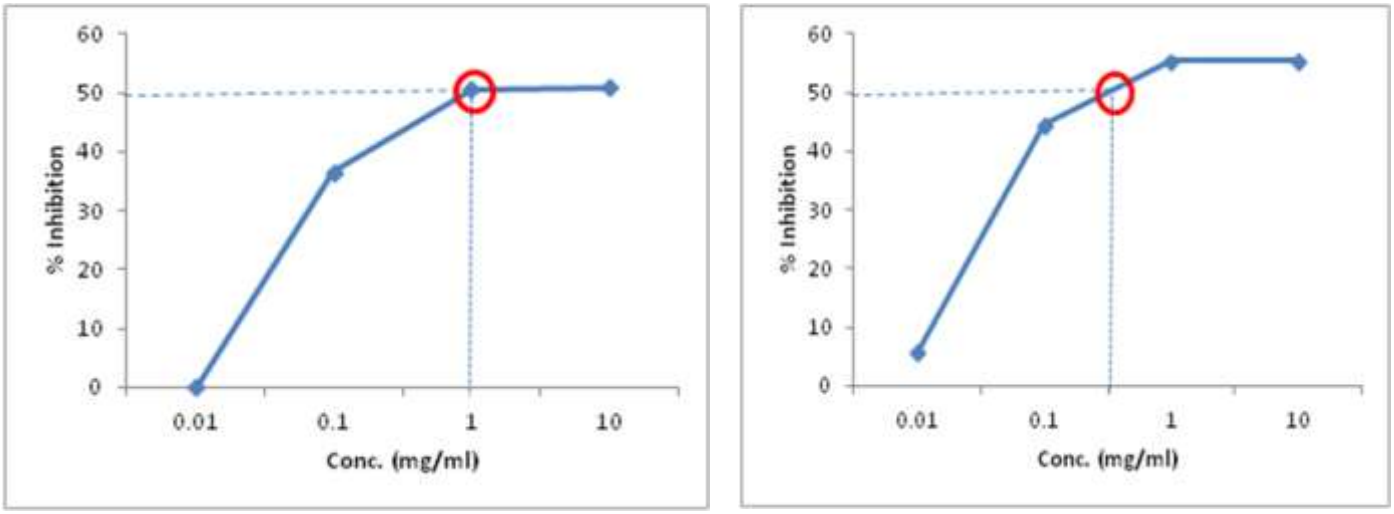


Figure 4.18: MTT assay with SK-03 (left) and SK-04 (right) in MDA MB-231 cells

4.2.6 Analytical Characterization of the product SK-03 and SK-04

4.2.6.1 Thin Layer Chromatography

A TLC separation method was developed for primary screening which effectively separated the major fermentation co-metabolites. The Rf values of the fungal extract SK-03 and SK-04 obtained in TLC (using Dichloromethane: Ethyl acetate in ratio of 7: 3 solvent systems) was found to be similar to that of the standard statin molecule with Rf value 0.72 (see Figure below). These results clearly indicated that the metabolite produced by the novel strains is a statin molecule (Figure 4.19)

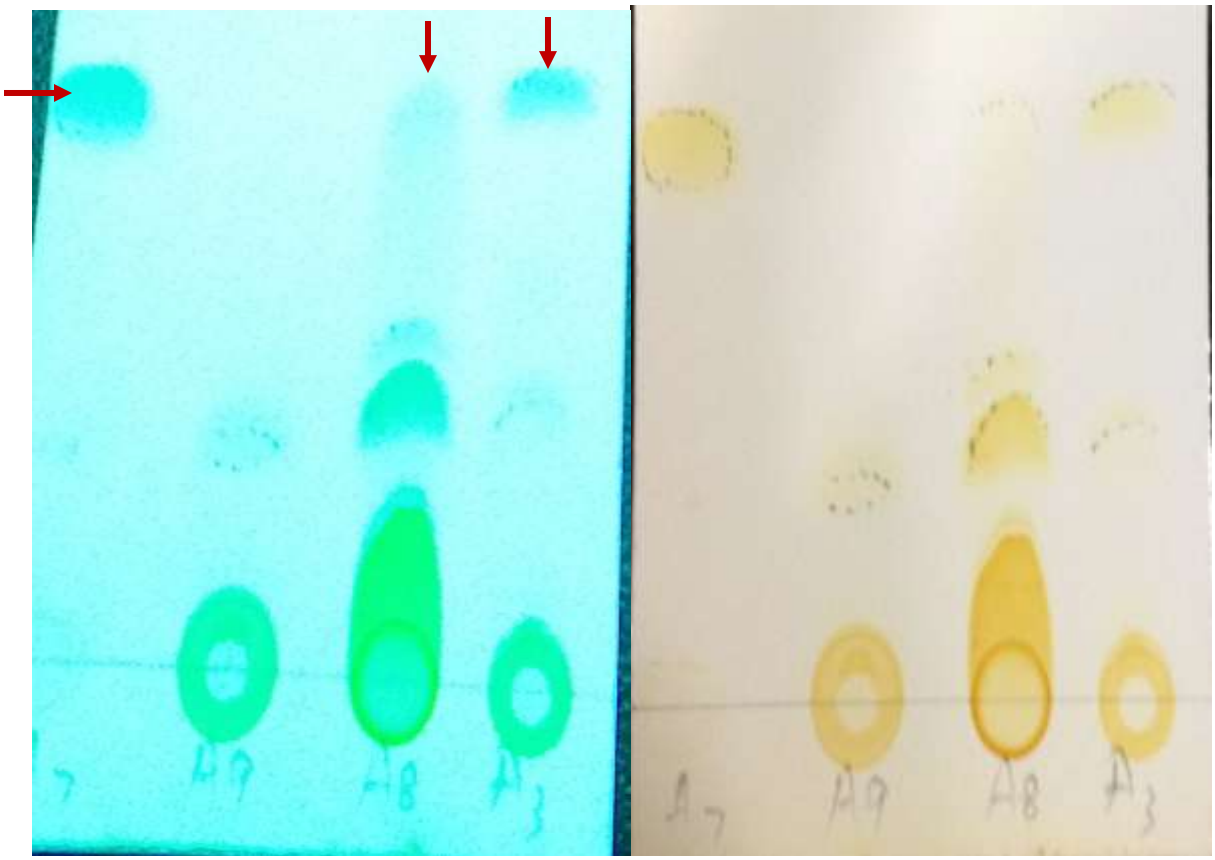


Figure 4.19: Thin layer chromatography, under UV light (L) and iodine stained plate (R) (A7: SV; A9: MJ; A8: SK-04; A3 SK-03)

4.2.6.2 High Performance Liquid Chromatography

The results of HPLC chromatogram of product isolated from the bacterial fermentation medium of BG 17 and BG 188 strain is shown in Fig.4.21.The chromatogram reveals that the compound has a retention time of 7.7 min. No peak was found in the fermentation media where reactants were not provided (Figure 4.21 c)

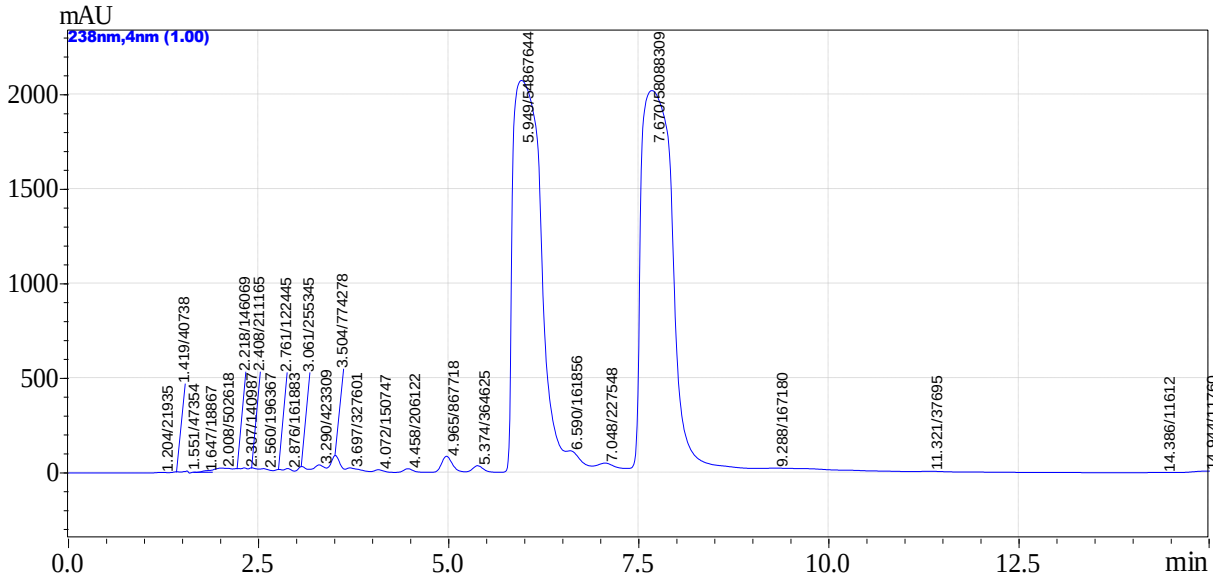


Figure 4.20 (a): Standard Monacolin J & Simvastatin (MJ at 5.9 min and SV at 7.69 min)

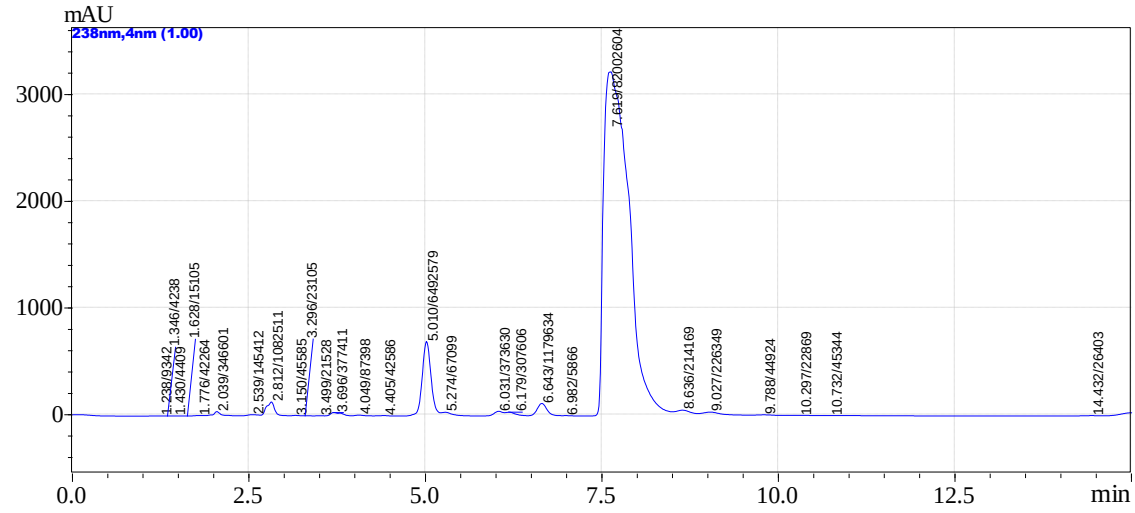


Figure 4.20 (b): HPLC chromatogram for the extracted fermentation product (7.6 min)

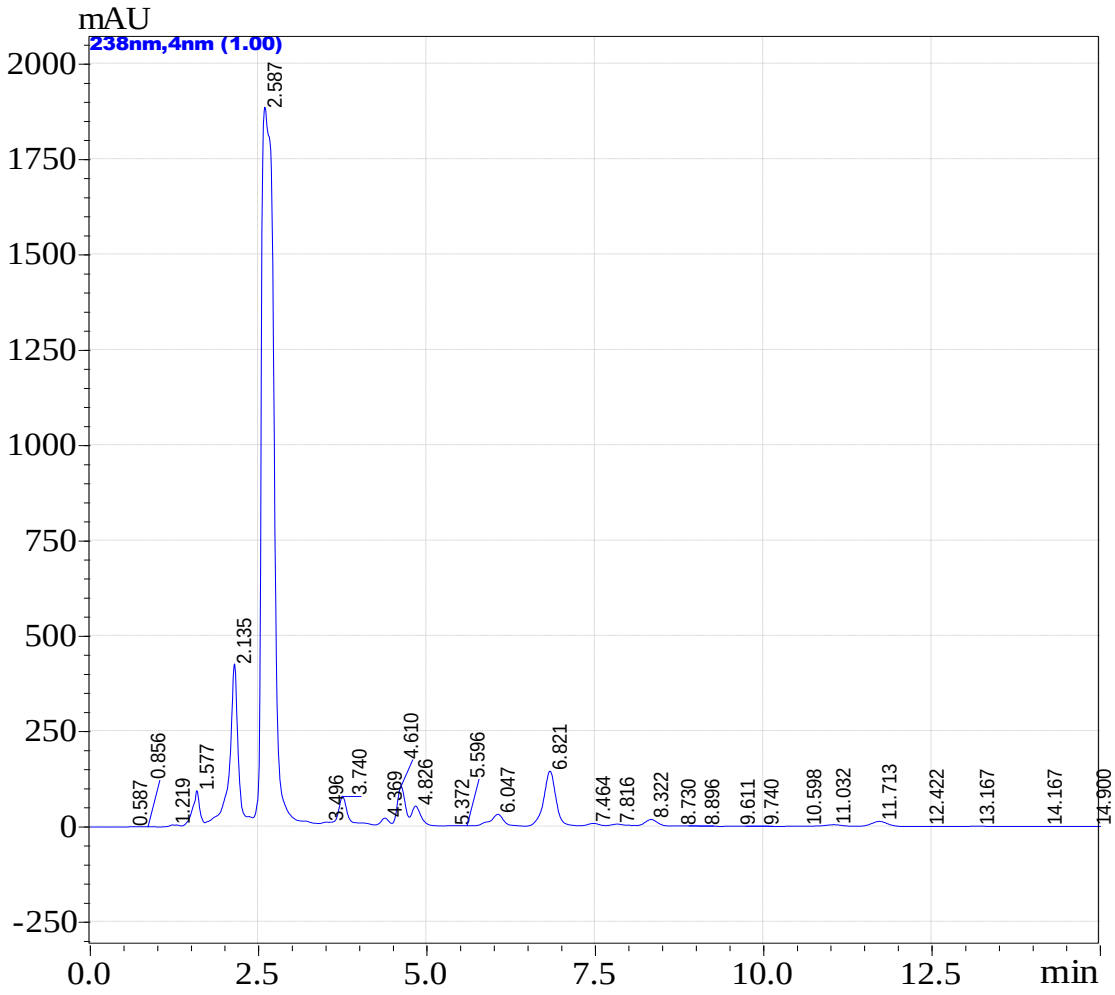


Figure 4.21: HPLC chromatogram for blank (fermentation media without reactants)

4.2.6.3 Mass Spectroscopy

The mass spectrum of SK-03 and SK-04 in positive ionization mode (Fig. below) exhibits an M+ peak at m/z 441atomic mass unit (amu) which corresponds to the exact calculated molar mass of sodium adduct of Simvastatin (418.5231+23). The mass spectrum was compared to the mass spectrum of Simvastatin to confirm that the isolated compound is similar to Simvastatin.

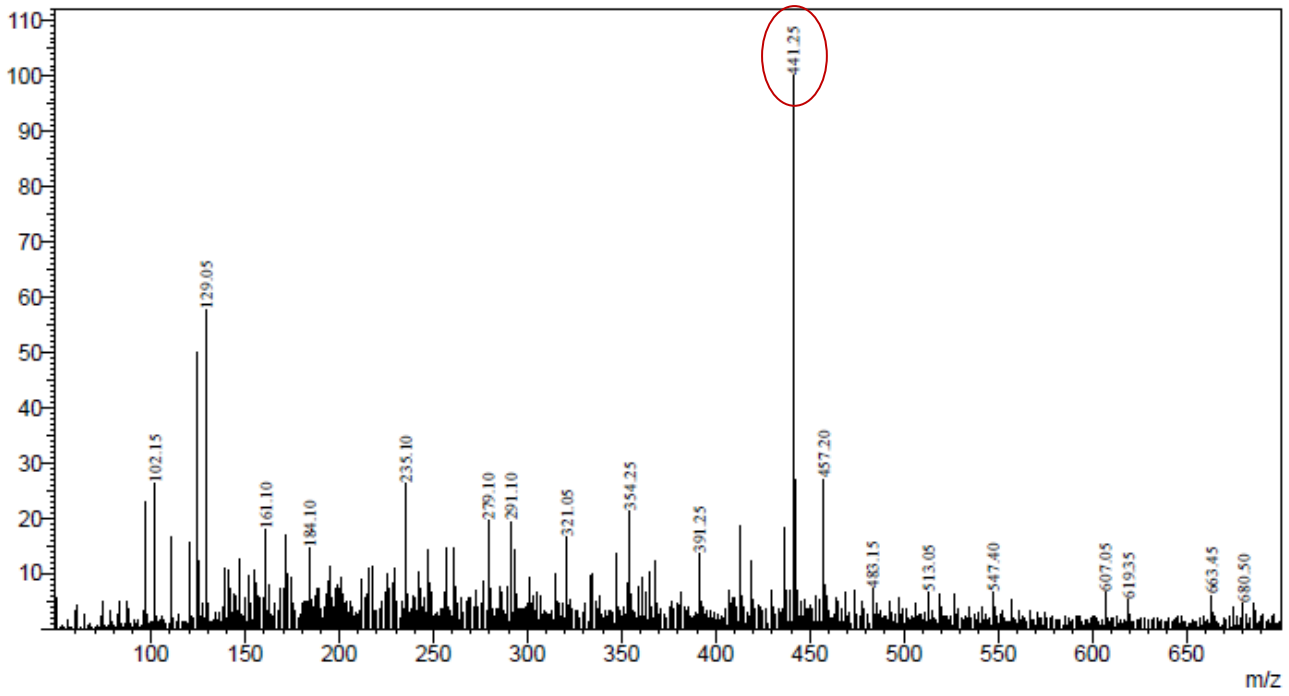


Figure 4.22: Mass spectrum of SK-03

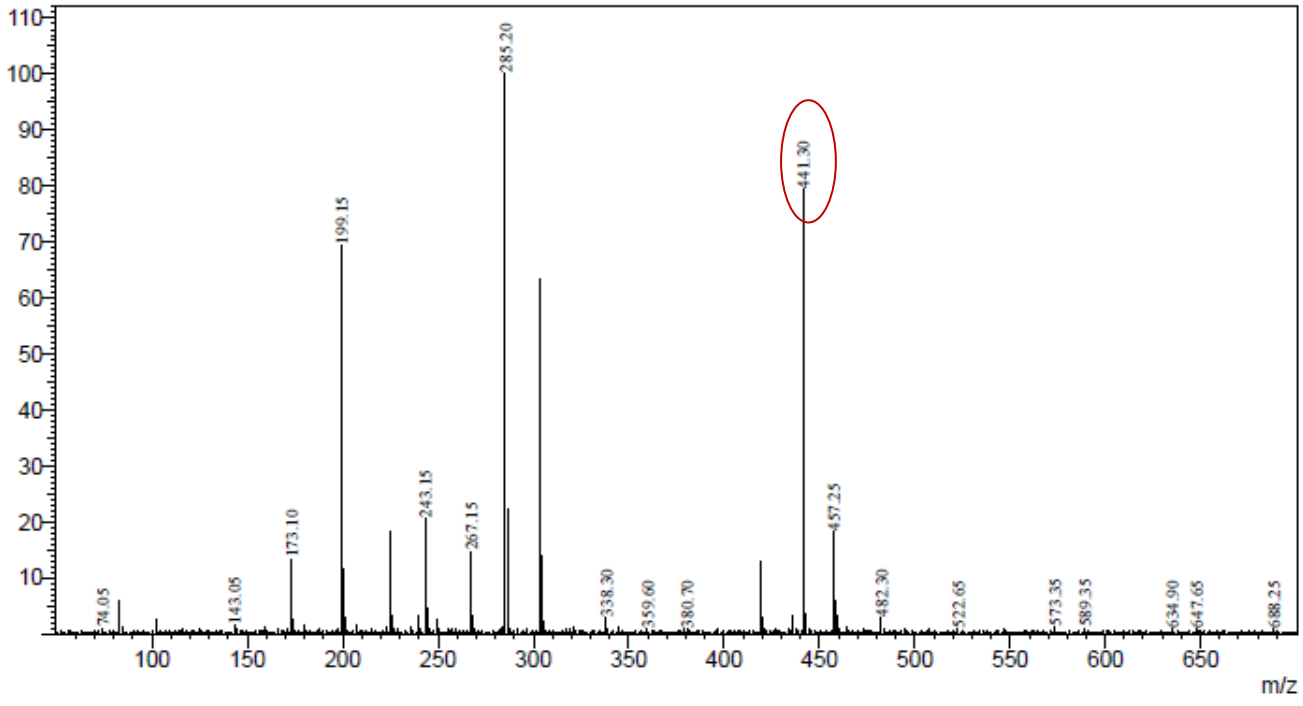


Figure 4.23: Mass spectrum of SK-04

4.2.6.4 Infra Red Spectroscopy

The FTIR spectra of SK-03 and SK-04 are shown in Figure 4.24 and 4.25a &b. The spectrum presented characteristic peaks at 3541 cm-1 (alcohol O-H stretching vibration), 3003.27 cm-1 (olefinic C-H stretching vibration), 2335.87 cm-1 (methyl and methylene C-H asymmetric stretching), 1629.90 cm-1 (lactone and ester carbonyl stretch), 1440.87 cm-1, 1375 cm-1 (Methyl and Methylene bending vibration), 1033.88 cm-1 (ester C-O-C symmetric bend), 918.15 cm-1 (alcohol C-OH stretch), 819.77 cm-1 (tri-substituted olefinic C-H) and 750.33 cm-1 (meta disturbed benzene-strong) confirming the presence of Lovastatin in the samples. Lovastatin containing the lactone ring gives characteristic peak at 1629.90 cm-1.

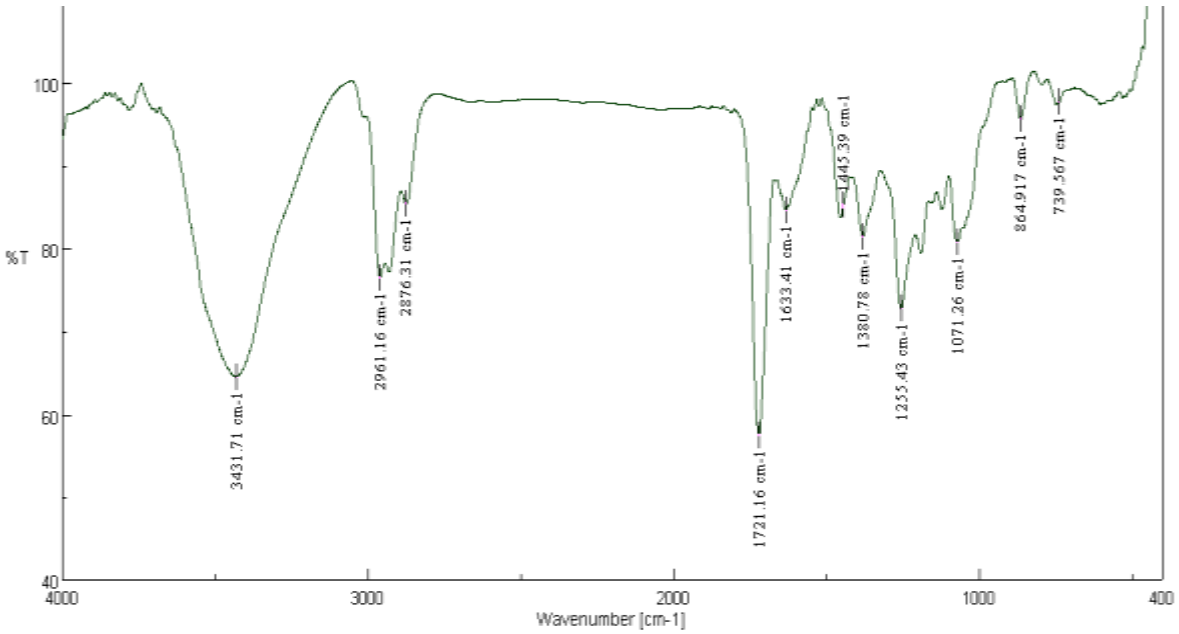


Figure 4.24: Infra Red spectrum for Simvastatin standard

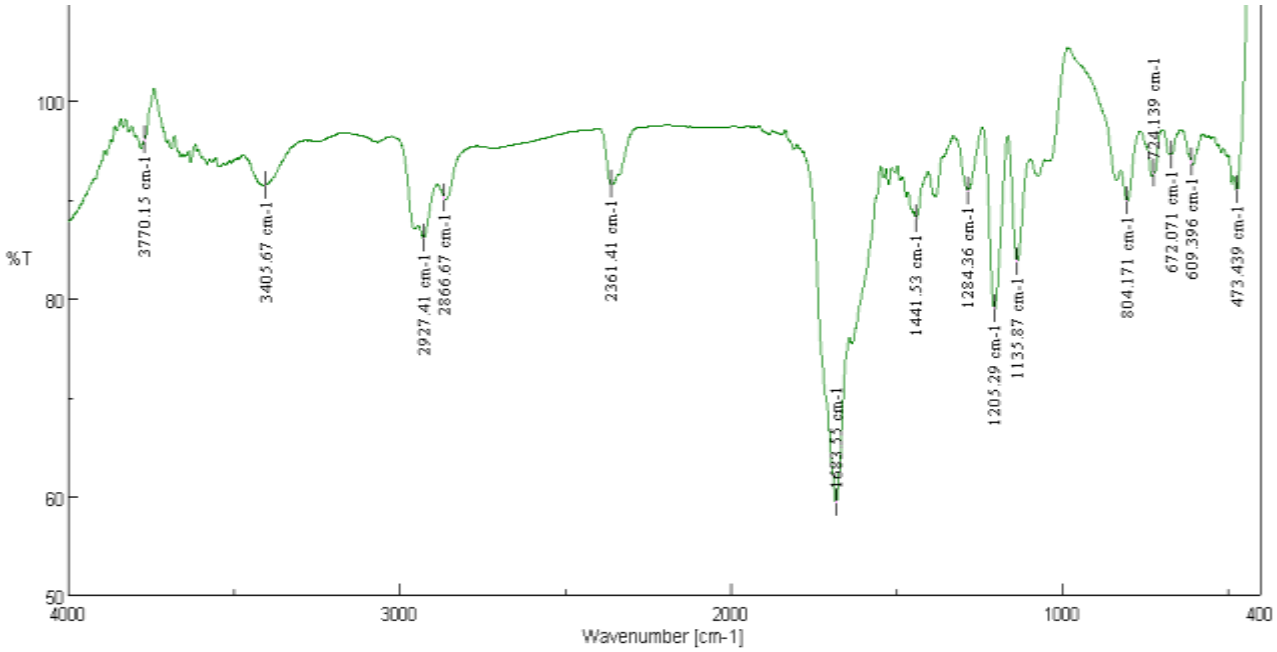


Figure 4.25 (a): Infra Red spectrum for SK-03

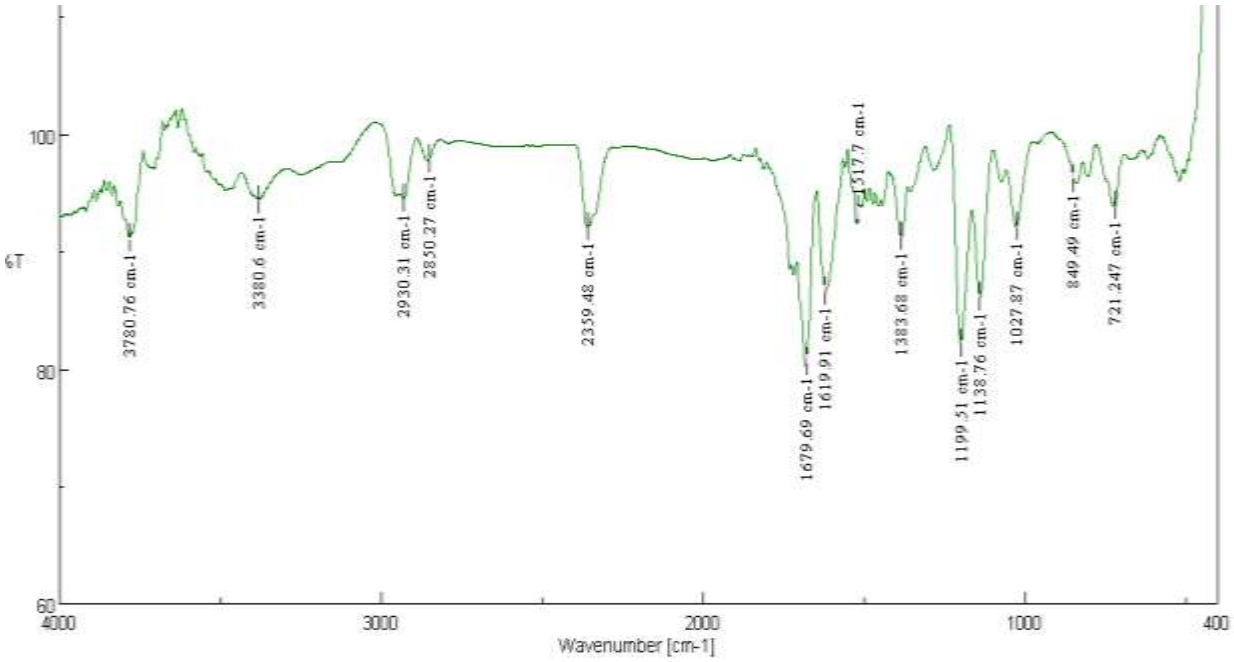


Figure 4.25 (b): Infra Red spectrum for SK-04

Table 4.7: Summary of Infra red spectroscopy results

SK-03	SK-04	SIMVASTATIN	Functional group	Range
3405.67 cm^{-1}	3461.27 cm^{-1}	3548cm^{-1} (alcohol/phenol O-H stretching vibration)	Alcohol/Phenol O-H Stretch	3550 - 3200
2927.44 cm^{-1} 2866.67 cm^{-1}	2928.38 cm^{-1} 2850.27 cm^{-1}	2944cm^{-1} 2820cm^{-1}	Methyl Methylene	1260 cm^{-1} - 2960 cm^{-1} 1470 cm^{-1} - 2925 cm^{-1}
1683.55 cm^{-1}	1677.77 cm^{-1}	1709cm^{-1} (lactone and ester carbonyl stretch C=O)	Ester C=O Stretch	1670-1820 cm^{-1}
1441.53 cm^{-1}	1516.74 cm^{-1}	1455cm^{-1} (C-C stretch in ring aromatic)	aromatic C═C	1400-1600cm^{-1}
1205.29 cm^{-1}	1198.51cm^{-1}	1266ccm^{-1}, 1060cm^{-1} (lactone and ester C-O-C bending vibration)	Ester C-O-C bending vibration)	1000-1300 cm^{-1} two bands or more

4.3 HMGR inhibitor production by marine fungal isolate FG 7

4.3.1 Identification and morphological characterization of the fungal isolate

FG 7 is a fungi isolated from a marine source (from Manipal, Malpe and Kapu region, in Karnataka located on the west coast of India (13° 20' 59" N latitude and 74° 43' 11" E longitude). To date numerous studies have been done on terrestrial fungus for production

of HMGR inhibitors, but no marine derived fungus has been explored for this property. This marine derived fungus, a potential producer of HMGR inhibitor, was initially characterized in terms of morphology (color, size, margin, shape, texture) followed by microscopic observations (lactose cotton blue staining).

On the potato dextrose agar plate, the colonies were brown centered with cream margins while in the flask in broth it made small balls. The lactose cotton blue staining showed biseriated spherical conidiophores with long hyphae (Figure 4.27).

The strain was identified by Agarkar Research Institute, Pune as *Aspergillus cervinus.*

The fungus was used in bio-fermentation reaction as described in materials and methods. After downstream processing the product (SK-02) was used for various analytical characterization and biological evaluation for its HMGR inhibition property.

Figure 4.26: Morphological characterization of fungal isolate FG 7

[FG 7 grown in broth (above), FG 7 on potato dextrose agar plate (left below) and lactose blue staining (right below)]

4.3.2 Biological evaluation of the product SK-02

4.3.2.1 Yeast growth inhibition bio-assay- effect on yeast HMGR

Since the HMG-CoA reductase activity is essential for yeast growth, the growth kinetics of appropriate yeast strain (*S. cerevisiae*) in the presence of commercial statin and novel inhibitor SK-02 was measured. Based on OD_{600} readings growth curves were plotted for each culture. The inhibitory effect on yeast growth was observed in cultures supplemented with novel inhibitor SK-02 while no growth inhibition was observed in culture supplemented with buffer alone (Figure 4.27 a). When the culture media was supplemented with SK-02, 62.8% yeast growth inhibition was found as compared to the buffer supplemented culture. Similar effect on yeast growth inhibition was observed in the plate assay. A clear zone of inhibition was observed around SK-02 loaded well on the yeast inoculated YEPD plate (Figure 4.27 b).

4.3.2.2 Effect of SK-02 on recombinant yeast strains expressing human HMGR enzyme

The effects of SK-02 on the level of expression of HMGR gene encoding enzyme involved in mevalonate biosynthesis were investigated in the recombinant yeast strain MB10-1D carrying human *HMGR* gene, whose expression was induced to 1.16 fold after SK-02 treatment as compared to buffer treated cells. The data is shown in Figure 4.18

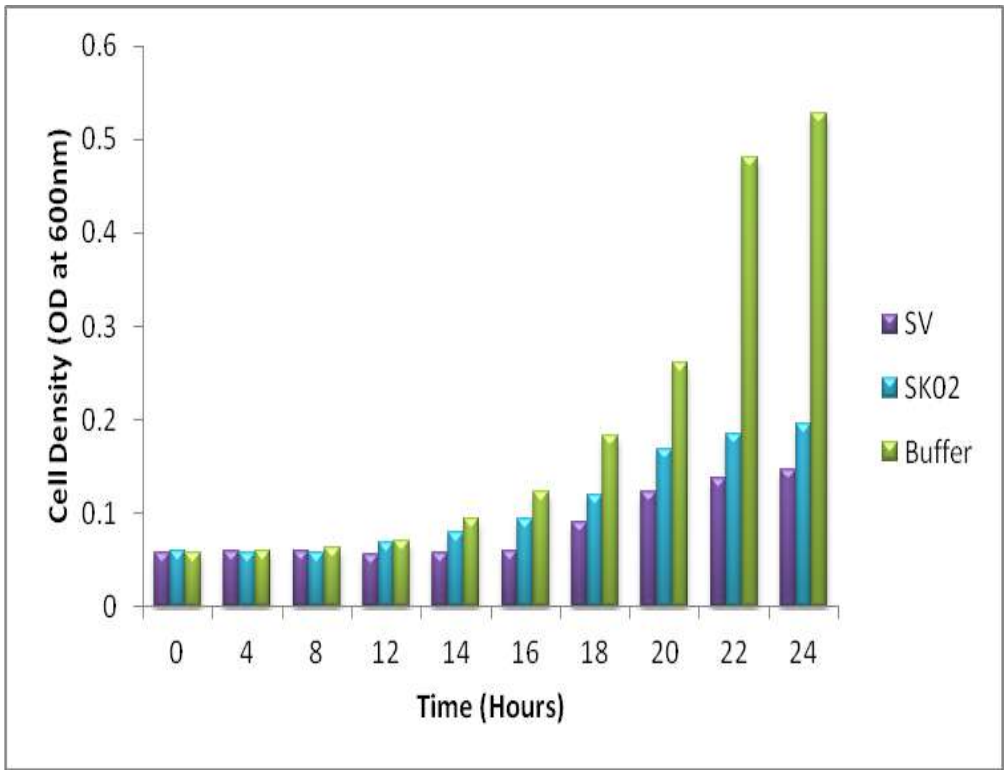


Figure 4.27 a: Yeast growth inhibition bioassay-broth assay

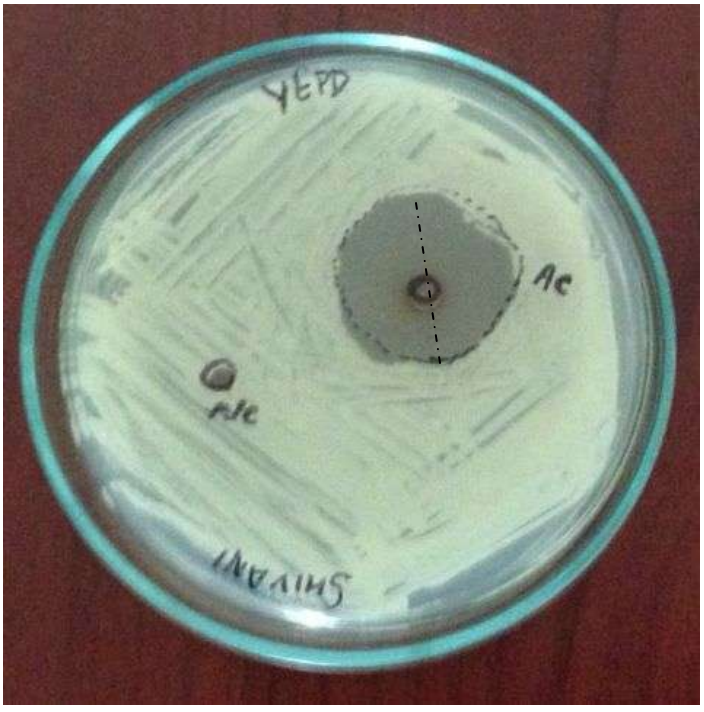


Figure 4.27 b: Yeast growth inhibition bioassay-plate assay

4.3.2.3 Triglyceride (TG) Release Assay

To directly test the relationship between statin sensitivity and triglyceride levels, we measured the triglyceride content of the cell lines MDA-MB 231 before and after treatment. Treatment with SK-02 reduced the levels of triglycerides released in to the medium by 33 % when compared to that of the control treatment, Table 4.7.

Table 4.7: Triglyceride Release Assay (SK-02)

	TG conc. (mg/dl)	% inhibition
Methanol	74.33	Nil
MJ	74.33	Nil
LV	45.98	38.1
SV	43.68	41.2
SK-02	49.81	33.0

4.3.3 Analytical Characterization of the product SK-02

4.3.3.1 Thin Layer Chromatography

A TLC was done as described earlier. The Rf values of the fungal extract SK-02 obtained in TLC (using Dichloromethane: Ethyl acetate in ratio of 7: 3 solvent systems) was found to be similar to that of the standard statin molecule with Rf value 0.72 (see Figure below). These results clearly indicated that the metabolite produced is Simvastatin.

Figure 4.28 Thin layer chromatography (SV on left and SK-02 spotted on right side)

4.3.3.2 High Performance Liquid Chromatography (HPLC)

The result of HPLC chromatogram of SK-02 is shown in Figure 4.29. The chromatogram reveals that the compound has retention time of 7.7 min, confirming the presence of Simvastatin.

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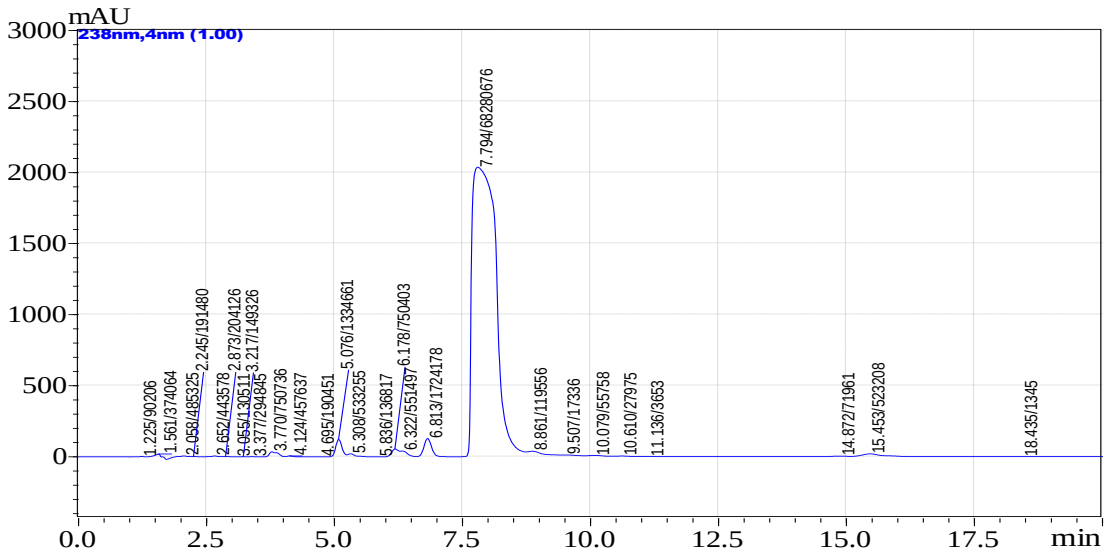

Figure 4.29: HPLC chromatogram of SK-02

4.3.3.3 Mass Spectroscopy

Mass spectrum of SK-02 in positive ionization mode (Fig. 4.30) exhibits an M+ peak at m/z 441 amu which corresponds to the exact calculated molar mass of Simvastatin + sodium (418.5231 +23), other fragment ions are at m/z 320.10, 307.05, 285.13, 274.2, 199.11, 171.05, 145.1. The fragmentation pattern of SK-02 is same as the fragmentation pattern of Simvastatin.

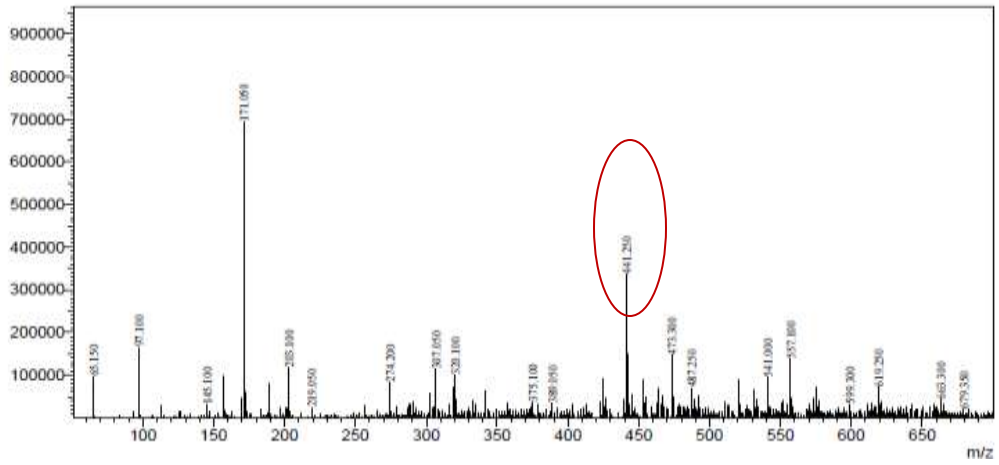

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Figure 4.30: Mass spectrum of SK-02

4.3.3.4 Infra red spectroscopy

The IR spectrum of SK-02 is shown in Figure 4.31. The spectrum presented characteristic peaks at 3541 cm-1 (alcohol O-H stretching vibration), 3003.27 cm-1 (olefinic C-H stretching vibration), 2335.87 cm-1 (methyl and methylene C-H asymmetric stretching), 1629.90 cm-1 (lactone and ester carbonyl stretch), 1440.87 cm-1, 1375 cm-1 (Methyl and Methylene bending vibration), 1033.88 cm-1 (ester C-O-C symmetric bend), 918.15 cm-1 (alcohol C-OH stretch), 819.77 cm-1 (trisubstituted olefinic C-H) and 750.33 cm-1 (meta disturbed benzene-strong) confirming the presence of statin in the sample.

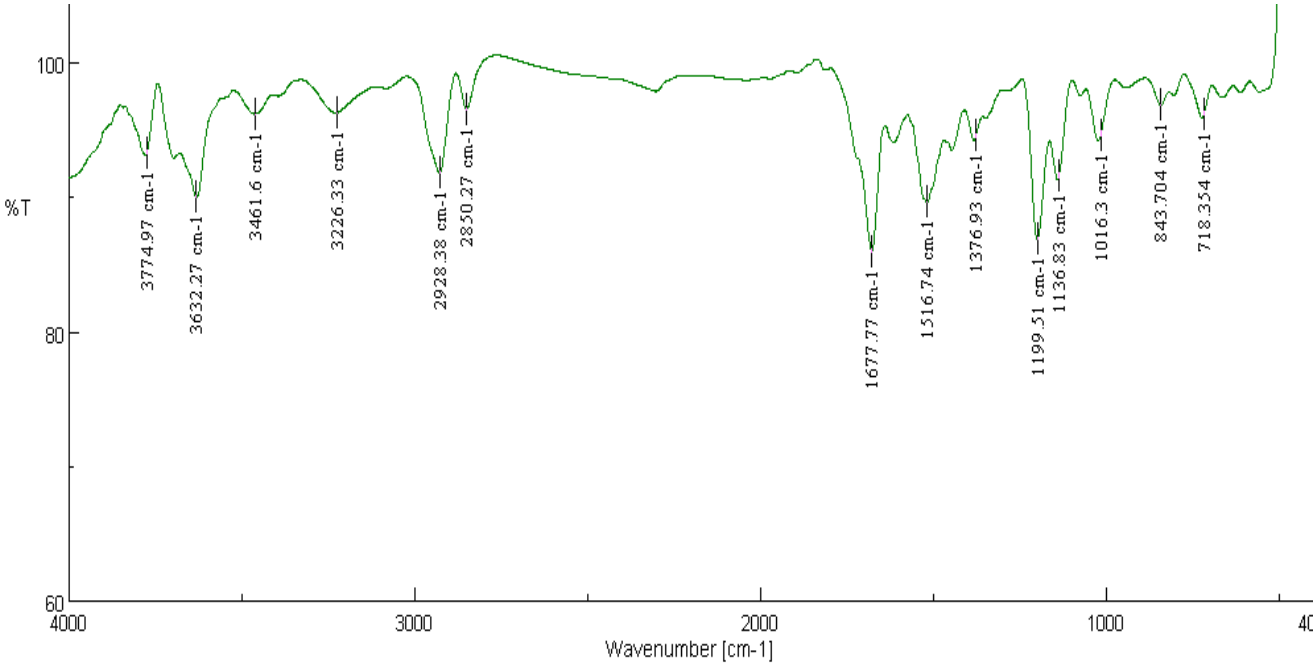

Figure 4.31: IR spectrum for SK-02

4.4 Molecular biology and bioinformatics analysis

4.4.1 Amplification of acyltransferase coding gene and sequencing of amplicons for DNA isolated from fungus FG 7:

Genomic DNA was extracted from the fungal isolate FG 7 and quantitated on nano drop. 100 ng of extracted DNA was used as template and amplified using the primers LV-F and LV-1R to generate a PCR product of 399 bp and LV-2F and LV-R to generate a PCR product of 998 bp. The PCR conditions optimized in this study gave a specific PCR product in sufficient quantity for further analysis. A readable sequence and single band was obtained by PCR amplification (Figure 4.32).

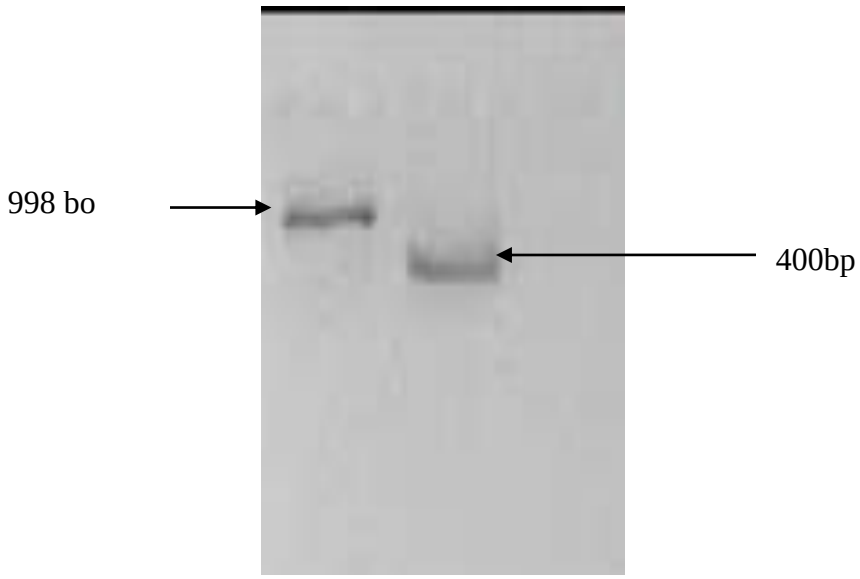

Figure 4.32: The PCR product on agarose gel (marker lane not shown)

The fragments were sequenced using reserve primers. The sequences obtained were 378 bp and 978 bp , which were then analyzed further.

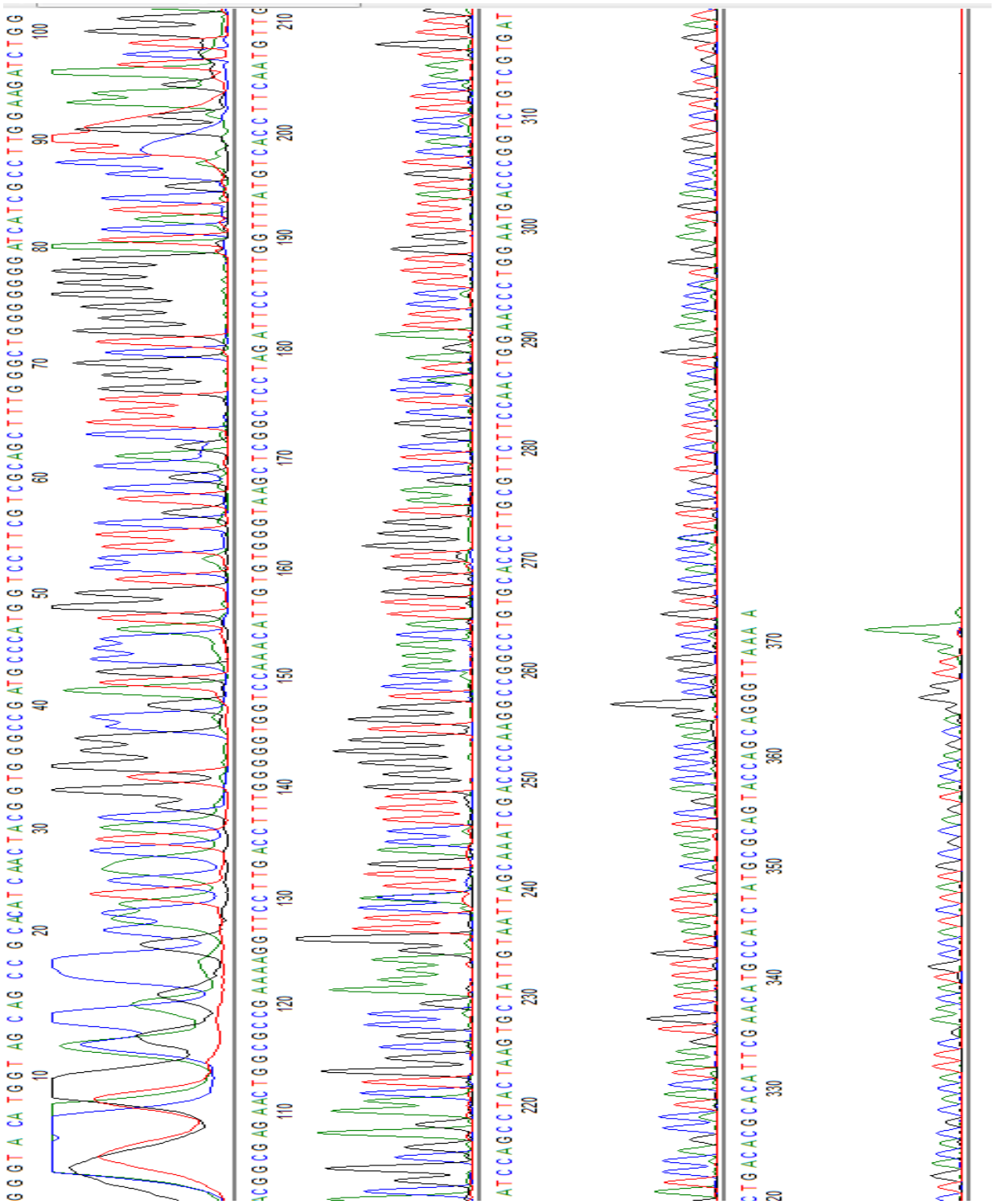

Figure 4.33: Chromatogram of nucleotide sequence of Lov D (400 bp fragment)

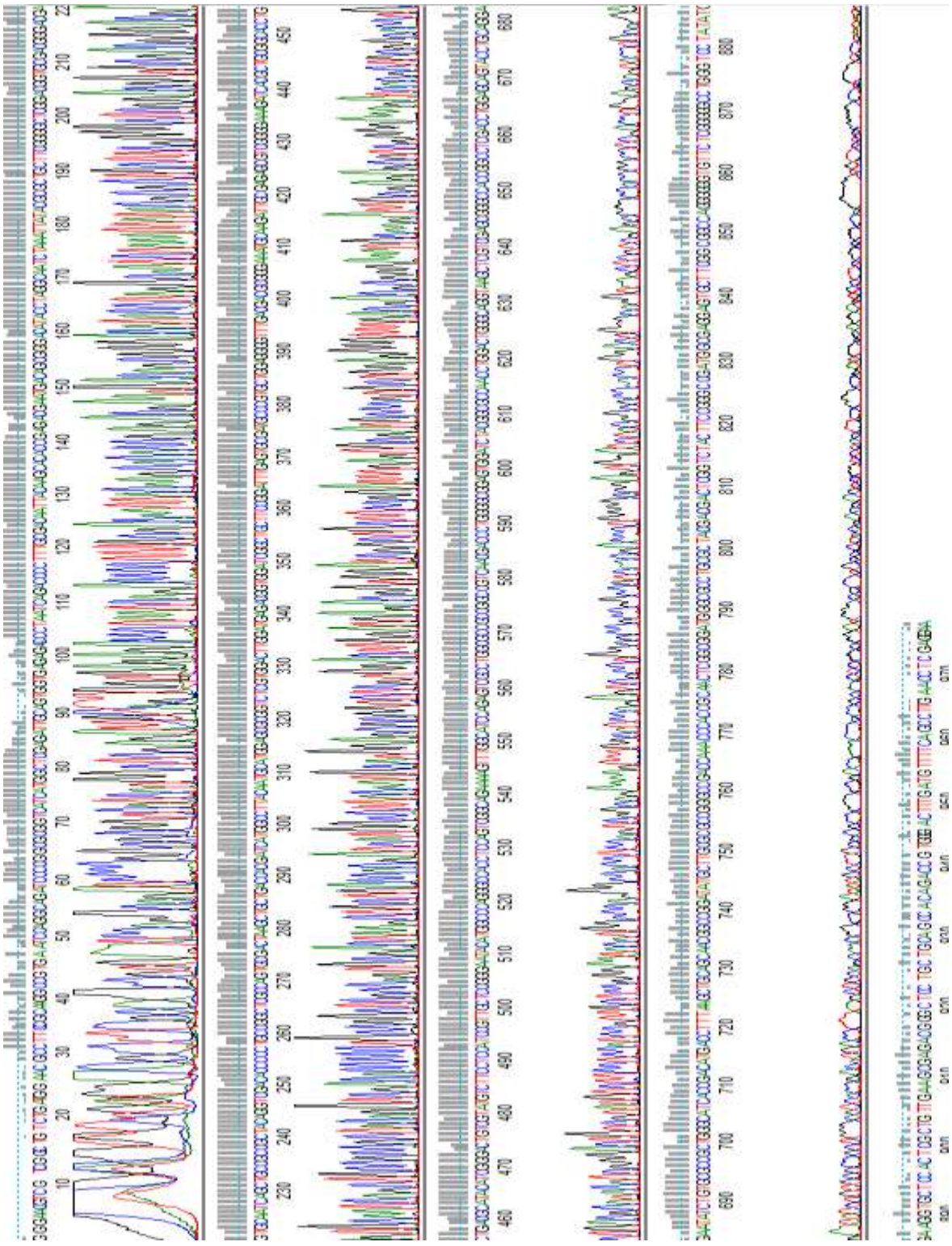

Figure 4.34: Chromatogram of nucleotide sequence of Lov D (1000 bp fragment)

>G05_11070-AC-400-LV-1R sequence exported from chromatogram file

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GCGAGAACTGGCGCCGAAAAGGTTCCTTGACCTTTGGGGGTGGTCCAAACAT
TGTGTGGGTAAGCTCGGCTCCTAGATTCCTTTGGTTTATGTCACCTTCAATGTT
GATCCAGCCTACTAAGTGCTATTGTAATTAGCAAATCGACCCCAAGGCCGGC
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TAAAA

>F05_11070-AC-1000-LV sequence exported from chromatogram file

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AGCAACGGCCGGATATGCTTGCGCGCCGGGCCGACCAAACCCACCGCAACT

CGGCGGATGGGCGCCTGCGCTACGACGACTCGGTCTACTTCCGGGCCGATGG

CGAGGAGTGCTTCGGCGGCCAGGGGGGTGTTCTCGGGGGCCTGGGTCCTATA

TGAAGGTGCTCCACTCGCTGTTGAAGCGAGACGGGCTCCTGCTGCAGCCACA

GACCGTGGGACTTTGATGTTTTCAGCCTTGAACCTCGAGGAA

4.4.2 Homology identity

The lov D (acyltransferase) reference sequences used for the sequence alignment was retrieved from Genbank. Based on the scoring index the most similar sequences were aligned with the other representative sequences. The BLASTn search was performed for both the sequences obtained during the course of this study. The subject and query sequences showed 97% identity, showing the presence of acyltransferase in the marine fungus *A. Cervinus* responsible for bio-catalytic activity leading to production of semi synthetic Simvastatin.

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Sbjct  684   CAAGCTGCTGACCACGATCATGGCCCTACAATGCATGGAGCGCGGTCTCGTGGACTTGGATGAGACGGTGGATAGGCTGCTTCCGGATTT  595

Query  368   GAGTGCGATGCCCGTGCTGGAGGGGTTTGACGACGCGGGAAATGCAAGATTGCGAGAGCGTCGGGGAAAGATCACGCTGCGGCACCTGCT  457
             ||| |||||||||||||||||||||||||||||||||||| |||||||| ||||||||||||||| ||||||||||||||||||||||||
Sbjct  594   GAGCGCGATGCCCGTGCTGGAGGGGTTTGACGACGCGGGAAACGCAAGAT-GCGAGAGCGTCGGGGGAAGATCACGCTGCGGCACCTGCT  506

Query  458   GACGCATACATCGGGACTGTCGTATGTCTTCCTCCATCCGTTGCTCCGGGAATACATGGCCCAGGGCCACCTCCAGTCGGCAGAAAAGTT  547
             ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  505   GACGCATACATCGGGACTGTCGTACGTCTTCCTCCATCCGTTGCTCCGGGAATACATGGCCCAGGGCCACCTCCAGTCGGCAGAAAAGTT  416

Query  548   TGGCATCCAGAGTCGCCTGGCGCCGCCGGCCGTCAACGACCCTGGGGCGGAGTGGATCTACGGCGCCAACCTGGACTGGGCAGGTAAGCT  637
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbjct  415   TGGCATCCAGAGTCGCCTGGCGCCGCCGGCCGTCAACGACCCTGGGGCGGAGTGGATCTACGGCGCCAACCTGGACTGGGCGGGTAAGCT  326

Query  638   CGTCGAGCGGGCCACCGGCCTCGACCTGGAGCAGTACCTGCAGGAGAATATCTGTGCGCCGCTGGGCATCACCGACATGACCTTTAAGCT  727
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  325   CGTCGAGCGGGCCACCGGCCTCGACCTGGAGCAGTACCTGCAGGAGAATATCTGTGCGCCGCTGGGCATCACCGACATGACCTTTAAGCT  236

Query  728   GCAGCAACGGCCGGATATGCTTGCGCGCCGGGCCGACCAAACCCACCGCAACTCGGCGGATGGGCGCCTGCGCTACGACGACTCGGTCTA  817
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  235   GCAGCAACGGCCGGATATGCTTGCGCGCCGGGCCGACCAAACGCACCGCAACTCGGCGGATGGGCGCCTGCGCTACGACGACTCGGTCTA  146

Query  818   CTTCCGGGCCGATGGCGAGGAGTGCTTCGGCGGCCAGGGGGGTGTTCTCGGGGGCCTGGGTCCTATATGAAGGTGCTCCACTCGCTGTTG  907
             ||||||||||||||| |||||||||||||||||||||| |||||||||||  |||||||||||||||||||||| ||||||||||||
Sbjct  145   CTTCCGGGCCGATGGAGAGGAGTGCTTCGGCGGCCAGGGGG-TGTTCTCGGGC-CCTGGGTCCTATATGAAGGTGCTTCACTCGCTGTTG  58

Query  908   AAGCGAGACGGGCTCCTGCTGCAGCCACAGACCGTGGGACTTTGATGTTTTCAGCCT  964
             ||||||||||||||||||||||||||||||||||||| |||| |||||||| ||||||
Sbjct  57    AAGCGAGACGGGCTCCTGCTGCAGCCACAGACCGTGG-ACTT-GATGTTT-CAGCCT  4
```

Figure 4.35: BLAST analysis of FG 7 gene sequence with subject

4.4.3 Amplification of acyltransferase coding gene for bacteria:

The genomic DNA extracted from bacterial isolates BG 17 and BG 188 and quantitated on nano drop. 100 ng of extracted DNA was used as template and amplified using the primers LV-F and LV-1R to generate a PCR product (expected product size, 399 bp) and LV-2F and LV-R to generate a PCR product (expected product size, 998 bp). Sequences similar to fungal Lov D genes were not detectable in any of the bacterial DNA isolates.

4.4.4 In silico analysis to identify bacterial homologous gene and its putative biological function

4.4.4.1 Analysis of Lov D homologs in bacteria using sequence similarity:

To find out the fungal analog in bacterial genome, we translated the whole bacterial genome using the translate tool of EXPASY tools. The bacterial proteome obtained after the translation was searched for the presence of amino acid sequence similar to fungal lovD protein. The sequence thus obtained was used in BLAST for validating the presence of a particular protein in bacteria of interest and identified a unique protein in *E. faecalis* , BG17, that encodes a 202-amino-acid protein. The amino acid sequence is around 30% identical to the protein of Lov D protein of *Aspergillus terreus* with significant E-value. This 202 amino acid protein was found to be belonging to transpeptidase family coding for beta-lactamase protein.

```
>lcl|Query_64083:4726957-4727159 ENTEROCOCCUS BACTERIA
VASLTKVVGTTTVILQLVEEGKIILDQSLQTYYPSFQDSNITIRHLLTHTANLQGYIPNRDQLNAQELKD
AYNHSFHAGKAIGKKVVYTDAGTILLGFMLEEMFQKSMIEILSERVLLPLGMNESTFLPKNPLDCVPTEL
HEQRGLIRGATHDPKAFVLREHAGNAGLFSNVYDLTKFVRMYLNRGSYHNLQFLKKETIDLLL
>lcl|Query_64083:4975406-4975435 ENTEROCOCCUS BACTERIA
LPLKHLMRHLSGLLLVWYHPFYQTLMQLQH
>lcl|Query_64083:1910198-1910225 ENTEROCOCCUS BACTERIA
LCRTIFNTHRNYGIRRLRYERSFYFYDD
```

```
compositionally adjusted substitution matrices", FEBS J.
272:5101-5109.


RID: M4977T6H01R


Database: All non-redundant GenBank CDS
translations+PDB+SwissProt+PIR+PRF excluding environmental samples
from WGS projects
           65,903,795 sequences; 23,612,046,542 total letters
Query= lcl|Query_64083:4726957-4727159 ENTEROCOCCUS BACTERIA

Length=203
                                                                      Score     E
Sequences producing significant alignments:                          (Bits)  Value

ref|WP_002411331.1|  beta-lactamase [Enterococcus faecalis]           421     5e-148
ref|WP_002388372.1|  beta-lactamase [Enterococcus faecalis]           415     5e-146
ref|WP_002407212.1|  beta-lactamase [Enterococcus faecalis]           414     2e-145
ref|WP_010712723.1|  beta-lactamase [Enterococcus faecalis]           414     3e-145
gb|EPI30890.1|  beta-lactamase [Enterococcus faecalis WKS-26-1...     413     4e-145
ref|WP_002397996.1|  beta-lactamase [Enterococcus faecalis]           413     4e-145
ref|WP_002384157.1|  beta-lactamase [Enterococcus faecalis]           413     4e-145
ref|WP_010710639.1|  beta-lactamase [Enterococcus faecalis]           413     4e-145
ref|WP_002374242.1|  beta-lactamase [Enterococcus faecalis]           413     4e-145
ref|WP_010777864.1|  beta-lactamase [Enterococcus faecalis]           413     5e-145
ref|WP_002387144.1|  beta-lactamase [Enterococcus faecalis]           413     6e-145
```

Figure 4.36: Bacterial homolog of fungal Lov D

4.4.4.2 Structural Similarity and Putative Function:

Based on the BLAST results the structure of bacterial beta-lactamase protein was retrieved from PDB database, PDB Id: 3CJM-beta-lactamase. Similarly, structure for fungal lovD was taken from PDB database, PDB Id: 3HLD. To understand the structural relationship between fungal and bacterial protein PDB Id: 3CJM, structural alignment was performed using SALIGN. The RMSD between the aligned structures was calculated using CHIMERA (Figure 4.37) which indicates their divergence from one another. The RMSd for two proteins was found to be 2.062.

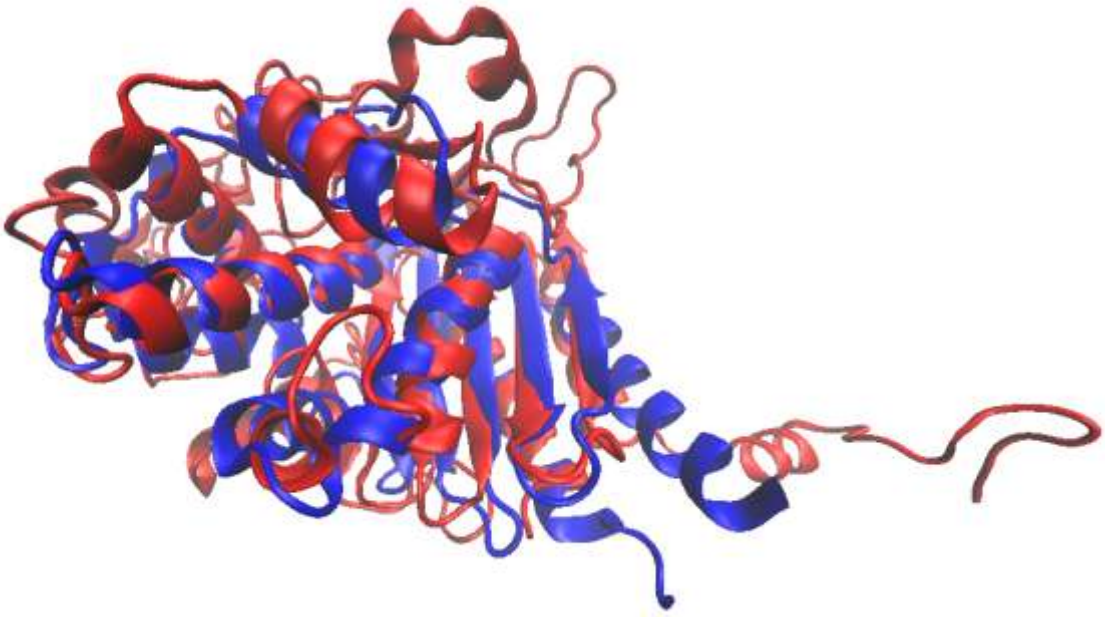

Figure 4.37: Structural similarity between bacterial and fungal protein (Red represents fungal protein and blue represents bacterial protein)

4.4.4.3 Phylogenetic Analysis:

Further Phylogenetic tree for bacterial protein and two fungal proteins was generated using MEGA 6 phylogenetic analysis software to find out the evolutionary relationship among Fungal transesterases/acyltransferases of *Aspergillus* (Lov D) and *Penicillium* (MlcH) with bacterial enzyme.

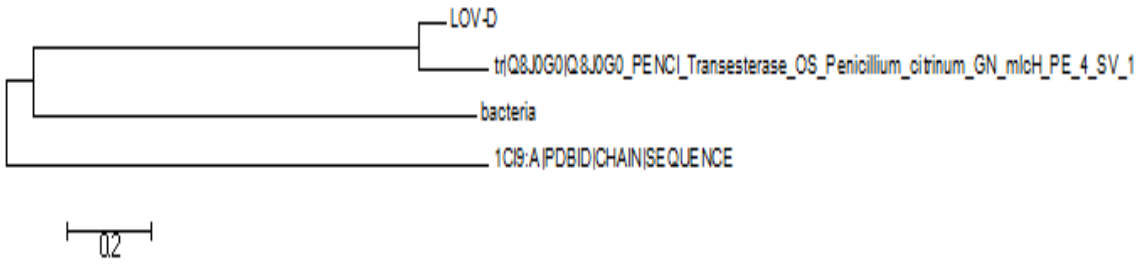

Figure 4.38: Phylogenetic relationship between bacterial and fungal proteins

4.4.5 Confirmation of presence of beta-lactamase gene in BG 17

4.4.5.1 Phenotypic Confirmation

The bacteria BG 17 was screened for its susceptibility to beta-lactam class of antibiotics in Kirby-Bauer disc diffusion assay, resistant to which shows the strong expression of beta-lactamase enzyme in the bacterium. When BG 17 was tested for penicillin, ampicillin and amoxicillin (antibiotics belonging to beta-lactam class), the bacteria was found completely resistant to these antibiotics. There was no inhibition of growth, the bacteria was able to survive and showed highly resistant growth profile (figure 4.39).

Figure 4.39: Bacteria showing resistance to beta lactam class of antibiotics.
(P-Penicillin; Amp-Ampicillin; Amx-Amoxycillin)

4.4.5.2 Genotypic Confirmation

PCR product of 546 bp could be amplified for beta-lactamase gene using following specific primers:

F- TTTGCGATGTGCAGTACCAGTAA
R- CGATATCGTTGGTGGTGCCATA

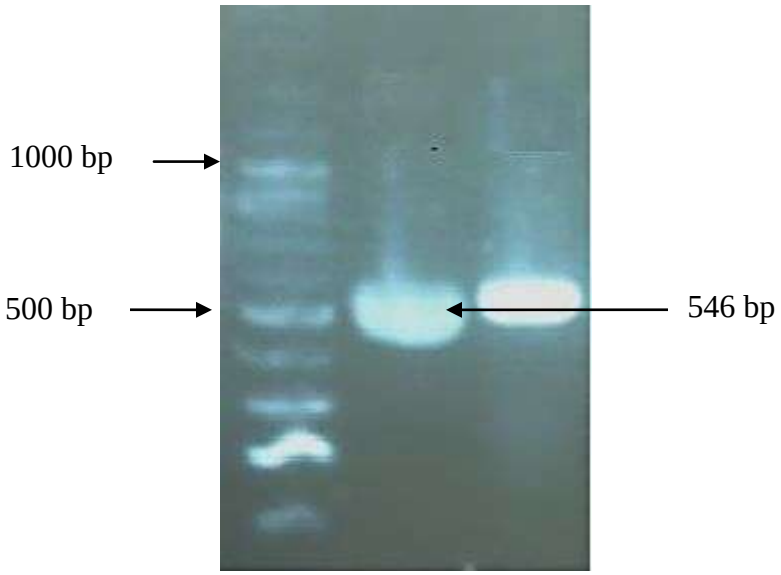

Figure 4.40: beta-lactamase gene amplified in BG 17