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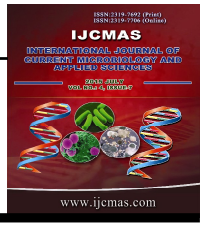

Original Research Article

Optimization of Process Variables for Bacterial PUFA Production by OFAT Method

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A B S T R A C T

Polyunsaturated Fatty Acids (PUFA) have several therapeutic effects and find applications in human health as nutraceuticals, pharmaceuticals and pharmaceutical precursors. Development of technology for Single cell oil (SCO) is nowadays focussing on production of these valuable long chain PUFA from microbial cells especially from bacteria. The single cell organisms can be easily cultivated in simple media and have short production times. An investigation on the effect of some growth variables on the production of PUFA on 5 bacterial strains isolated from marine and fresh water sources is presented. Optimization of PUFA production was attempted using the one factor at a time (OFAT) approach in shake flask fermentation. A range of factors like duration of incubation, temperature of incubation, aeration/mixing speed, pH and C/N ratio of culture medium were optimized. All the isolates studied produced maximum PUFA at lower temperature (20^{o}C) and less mixing speeds (100/180 rpm) from the range tested. Salt water isolates gave best results at the unaltered pH of 5.8 while the fresh water isolates at the extreme of pH (4.0/9.0). Considerable increase in PUFA percentages was achieved after the optimization of the process parameters coupled with finding the right C:N ratio for production medium. C/N ratio of 40:1 was found to be optimum. The data obtained in this study will be of value for further optimization by statistical methods or by strain improvement programs and scale up.

Introduction

PUFA constitute a large group of fatty acids containing long chain carbonic molecules that include ω-3 and ω-6 fatty acids, a few of which are Linoleic Acid (LA) 18:2 ω -6, Alpha-Linolenic Acid (ALA) 18:3 ω-3, Eicosapentaenoic Acid (EPA) 20:5 ω-3, Arachidonic Acid (ARA) 20:4 ω-6 and Docosahexaenoic acid (DHA) 22:6 ω-3 (Figure 1). PUFA play varied beneficiary roles in the maintenance of 'Healthy' state of an individual. The human health benefits of PUFA have been extensively evaluated through a wide range of clinical studies (Seppanen-Laakso *et al.,* 2002; Rossi *et al.,* 2011; Gupta *et al.,* 2012).

PUFA are found in blue backed fish and certain plant sources like olive and flax seed. Due to problems of fast depleting conventional sources, foul fishy smell and scarcity, nowadays focus has turned to acquiring PUFA from newer sources like microorganism having the ability to produce SCO. PUFA is being obtained from SCO produced by algae, fungi and more recently bacteria. Thus there is an urge to improve and optimize the production processes in these microorganisms (Papanikolaou *et al.*, 2004).

Because of the complexity of growth and product formation processes, protocols used to optimize the production of PUFA cannot be directly applied from one to another organism. The dynamic interactions among the growth, fatty-acid synthesis and nutrient limitation result in significant variations in the fatty-acid profile and PUFA production (Wu *et al.*, 2005). Therefore, the optimization of temperature, mixing/aeration, pH, carbon to nitrogen ratio variables has to be performed in order to evaluate the potential for growth, lipid yield, and PUFA percentage for any newly isolated microbes.

In conventional OFAT approach, the nutritional/cultural factors are optimized by changing one factor at a time. This approach is simplest to implement, and primarily helps in selection of significant parameters affecting the product yield (Singh *et al.*, 2011). Keeping the above in view, the conventional OFAT approach was adopted for optimization of process parameters to enhance PUFA production in shake flask fermentation.

Work by many a scientist in the field of microbial PUFA production indicate that different incubation and culture cultivation conditions are needed for increasing PUFA production (Yano *et al.*, 1994; Yaguchi *et al.*, 1997; Dyal *et al.*, 2005). Therefore, it is necessary to find out optimum conditions for each culture during the initial studies which are normally done at shake flask level. Shake flask system is a closed culture system which contains an initial, limited amount of nutrients (Stanbury and Hall, 1997). The control of process parameters in shake flask fermentation is only partial as the oxygen concentration, pH changes and nutrient availability cannot be monitored and controlled. Yet the shake flask system is preferred for most laboratory scale process development and strain improvement programs, owing to the ease with which it can be handled and studied. Shake flask also serves as a rapid method for screening a large number of cultures simultaneously. Shake flask cultures also give quick and relevant information that can be in most cases, used to predict the behaviour of the studied strain in further fermentation studies (Pujari, 2014).

As for any fermentation, the culture performance is affected by external variables like temperature, pH, C/N ratio of production medium, concentration of substrates, trace elements, extent of agitation, and dissolved oxygen. Optimization of fermentation conditions in shake flask system has been used to substantially enhance yield and productivity of many bioprocesses. Conventionally, fermentations are optimized empirically by using one-at-a-time variation of process parameters (Grothe *et al.*, 1999).

Fatty acids are major cellular components of microorganisms. Growth and fatty acid formation are affected by key medium components and environmental conditions (Jiang and Chen, 2000). Considering that PUFA production is growth associated, obtaining higher biomass without sacrificing

on the lipid accumulation was part of this optimization study (Iida *et al.*, 1996).

This paper describes the optimization of PUFA production by five select superior isolates under variations of environmental and nutritional factors. The important fermentation process parameters i.e. temperature, aeration/mixing, initial pH of culture broth and carbon to nitrogen ratio using the conventional 'one-factor-at-a-time' approach (OFAT) is presented. Five PUFA producing cultures isolated from various sources were selected for shake flask optimization. These cultures are listed in table 1.

Materials and Methods

Preparation of media: The liquid Semi-synthetic minimal medium MI was used for all shake flask studies (Composition as described by Masurkar *et al.*, 2015). All experiments were performed in 250cm^3 Erlenmeyer flasks containing 50cm^3 of MI growth medium sterilized at 121^{o}C for 20 min. Each subsequent experiment was based on previous optimal results obtained. In each experiment and for each condition, the cultures were allowed to grow up to 120hr then the broth was filtered, biomass dried and amount of biomass, lipid yield, and fatty acid profile were recorded. All important experiments were carried out in triplicate.

Preparation of inoculum: The inoculum was prepared using a loopful of growth from actively growing young slant cultures of 18-20 hr age. The seed flask was incubated at 25^{o}C, on an orbital shaker set at 180 rpm, (stroke 25mm, make Orbitek, Scigenics Biotech, India). At the end of incubation period the Optical Density (O.D) of the actively growing culture was read at 530nm (Equiptronics EQ-650, India), against saline blank. O.D of the culture suspension was adjusted to 1.00 at 530nm using saline diluents.

Inoculum: 5 % inoculum of 1.00 OD was used to inoculate the production medium flask (50 cm^3 medium/250 cm^3 Erlenmeyer flask). The flasks were incubated at required rpm and temperature for usually 120 hr and then harvested and contents analyzed.

Assessment methods for optimization studies:

Growth assessment: Biomass was assessed as indicator of growth by measuring O.D at 530_{nm}. Wet weight (w.w) and DCW measurements were also done as additional tests to assess growth (Mills and Lee 1996; Tripathi *et al.*, 2009; Tapia *et al.*, 2012).

a) O.D of 120hr fermented broth- Aliquots of 2cm^3 were used to determine the O.D at 530nm on a colorimeter (Equiptronics EQ-650, India) against a sterile MI medium blank.
b) Determination of w.w- Aliquots of 1cm^3 of cultures were taken in pre-weighed 1.7cm^3 capacity Eppendorf tubes and centrifuged at 8,000 g for 10 min. The supernatant was discarded and w.w. of the pellet was determined.
c) Determination of dry cell weight (DCW)- Aliquots of 1cm^3 of culture were taken in pre-weighed 1.7cm^3 capacity Eppendorf tubes and pelleted by centrifugation at 8,000 g for 10 min. Supernatant was discarded and pellet were dried in a microwave oven at 450 watts/120^{o}C for 2min with 30 seconds intervals. The tubes were left overnight at room temperature (R.T.) for complete drying.

Lipid accumulation: The extent of lipid accumulation was estimated by determining the Lipid content and it was expressed w.r.t w.w. and DCW as described earlier (Masurkar *et al.,* 2014).

1. **Physiological parameters:** The parameters indicating the extent of the physiological processes and characteristic of the cells were studied by measuring the pH of spent medium. While the substrate for fatty acid synthesis (glucose) was estimated in the spent medium.

    a) Measurement of pH- The broth culture (20 cm^3) was centrifuged at 8000g for 10 min and the pH of the supernatant was measured using a Lab India PICO Series Digital pH meter.
    b) Measurement of Residual Glucose- The amount of glucose in the spent culture medium was measured by the DNSA colorimetric method (Miller, 1972). Twenty µl of clarified culture supernatant was appropriately diluted and used as a sample for analysis.

2. **Lipid profile by GC:** Lipid was extracted and Fatty acid methyl esters (FAME) were prepared and analysed by GC-FID. The lipid profile showing the fatty acid composition was determined as per the GC Analysis method described in the paper by Masurkar *et al.* (2014). PUFA peaks were identified by matching with the retention times of samples with that of standards. Concentration of PUFA was estimated using the peak area percentage after GC analysis.

Optimization studies for Physical parameters

a. Duration of incubation: The optimum duration of incubation was studied by incubating cultures at 25^{o}C–180 rpm up to 168 hr. Sampling was done at 24 hr intervals for assessing the amount of lipid accumulated. The cells were stained by Sudan Black B as described by Patnayak and Sree (2005). The presence of lipid within the cells was checked qualitatively using a microscope and 1000X magnification. Since lipid accumulation was judged qualitatively under microscope the experiment was repeated twice to confirm the trend of lipid accumulation.

b. Temperature of incubation: The 5 cultures under study were subjected to different incubation temperatures (20^{o}C, 25^{o}C and 30^{o}C) - at fixed rpm of 180 (Jang *et al.,* 2005). A set was also run with an initial higher temperature (30^{o}C) for 24hr to enhance biomass production and then at a lowered temperature of 20^{o}C from 24hr of incubation to 120hr to enhance PUFA production (30–20^{o}C).

c. Aeration/Mixing: After optimum temperatures were determined for each culture the cultures were grown at 100 rpm, 180 rpm and 250 rpm at the optimized temperature.

Optimization studies for nutritional parameters

a. Initial pH of culture medium: The 5 selected bacterial strains were cultivated in the semi-synthetic MI medium over a pH range of 4.0–9.0 for 120hr (Komazawa *et al.,* 2004; Jiang and Chen, 2000). Each

culture was incubated in triplicates at their earlier established optimum incubation temperature and shaking speed (100 rpm, except for GN/PA/LR17 incubated at 180rpm) for a period of 120hr. The original semi-synthetic MI medium with pH= 5.3 and 5.8 was prepared first and the pH of this medium was adjusted prior to autoclaving to pH 4.00, pH= 7.00, pH= 8.00, pH= 9.00.

b. Carbon/nitrogen ratio: The three C/N ratio studied were: C/N= 60:1 (Original C/N ratio), C/N= 40:1 and C/N= 30:1. The calculations for the glucose (Carbon source), Yeast extract and Ammonium Nitrate (Nitrogen sources) are given in table 2.

As per the conventional OFAT optimization approach, critical physical and physiological parameters were optimized by varying a single factor at a time. Each subsequent factor was examined after taking into account the previously optimized factor(s). The highest yielding variable in each study was carried forward for the optimization of the next parameter.

Results and Discussion

Physical parameters:

a. Duration of incubation: The optimum duration of incubation for lipid accumulation was determined qualitatively by Sudan Black B staining. The results are given in table 3.

As shown in table 3, except isolate No. GN/PA/H6, all cultures showed beginning of lipid accumulation as early as 24 hr. All 5 cultures showed maximum lipid accumulation at 96 hr with no further increase beyond 120 hr (Data for 144 hr and 168 hr not shown). The lipid accumulation and fatty acid modifications like elongation and desaturation progress through the late log phase and the stationary phase of the culture (Wynn and Ratledge, 2005). The cells were incubated up to 168 hr to check the effect on lipid accumulation up on extended incubation period. The log phase of growth lasted till 48 to 72hr of incubation in the nitrogen limited semi-synthetic medium.

A comparison between the fatty acid profiles of isolate No. GN/PA/H8 studied after 120hr and 168hr incubation is shown in figure 2, where the reduction in the fatty acids was observed. Also the longer chain fatty acids like linoleic acid observed at 120hr are absent at 168hr.

PUFA are primary metabolites and the yield is expected to be associated with the cell growth but the time for maximum yield of PUFA depends on the kind of fatty acid (Jang *et al.,* 2005). It was observed by Jang *et al.* (2005) that *Mortierella alpine*, a fungus gave a maximum yield at 72 h for α-linolenic acid, 120hr for linoleic acid and 144hr for total PUFA and arachidonic acid.

However, production of PUFA decreases gradually in prolonged cultivation due to cell lysis (Bajpai and Bajpai, 1992). Also, that accumulated lipids and fatty acids within oleaginous microorganisms are, dynamic storage materials and are produced in times of plenty when carbon is sufficient and utilized in times of starvation when carbon is deficient may further reduce lipid yields (Wu *et al.,* 2005). A similar result was observed in the present study where the relative percentage of fatty acids decreased on prolonged incubation i.e. 168hr or 7days (Figure 2).

b. Temperature of incubation: The isolates studied showed an increase in biomass with an increase in incubation temperature from 20^{o}C to 30^{o}C. The effect of temperature of

incubation on DCW, Lipid and PUFA production by the 5 selected cultures is shown in table 4 and figure 3 and 4.

Ambient temperatures support cell growth while PUFA are low at higher temperatures. Microorganisms are reported to produce PUFA at low temperature environments to maintain cell membrane fluidity (Yano *et al.,* 1994; Dyal *et al.,* 2005). But the biomass production may be retarded at lower temperatures. Komazawa *et al.* (2004) has suggested that the culture temperature is controlled in such a way that the fat and oil composition of interest can be produced at a higher level.

A drop in pH of the spent culture broth was observed in cultures GN/PA/H8 and GN/PA/SL1. While isolate No. GN/PA/H6, GN/PA/LR17 and GN/PA/N4 showed an increase in broth pH up to 6.0-7.0 from an initial pH of 5.3. The pH of spent culture broth decreased from 5.8 to up to 4.0-3.0 after 120hr incubation for isolate No. GN/PA/H8 and GN/PA/SL1 (Table 4, 5, 6, 7).

Concentrations of residual glucose in spent culture medium varied from culture to culture. The starting concentration of glucose in the medium was 40g/L. Average glucose utilized by cultures ranged from 2-10g/L at various temperatures, rpm, pH and C/N ratio of medium (Table 4, 5, 6, 7). Minimum residual glucose of 21.44g/L at 20^{o}C-100rpm was found in the spent medium of the culture No. GN/PA/LR17 (Table 6).

It was observed that the highest amount of PUFA was produced by 3 selected marine isolates viz. GN/PA/H6, GN/PA/H8 and GN/PA/SL1 at 20^{o}C-180rpm. The fresh water isolates viz. GN/PA/LR17 and GN/PA/N4 showed maximum PUFA at 25^{o}C-180rpm. Although a considerable percentage of PUFA was obtained at 20^{o}C-180 rpm as well. No PUFA was produced by the isolates No. GN/PA/SL1 and GN/PA/LR17 at 30^{o}C. A small percentage of PUFA was seen in isolates No. GN/PA/SL1 and GN/PA/N4 by changing the incubation temperature from 30^{o}C-20^{o}C after 72hr of incubation (Table 4 and Figure 4). This shows that PUFA production may be induced in the said cultures due to lower incubation temperature.

The effect of different incubation temperatures was best seen in the lipid profile of isolate No. GN/PA/LR17 and is shown in figure 5. The comparative chromatogram shows that a higher number of fatty acids at 20^{o}C-180rpm than at 25^{o}C-180rpm. Also, at 30^{o}C-180rpm and 30^{o}C-20^{o}C-180rpm a noteworthy decline in fatty acids were observed. Thus the temperature of 20^{o}C was chosen for consequent studies for this culture.

Because the lower temperature i.e. 20^{o}C did not affect the biomass production to a great extent and gave maximum PUFA it was considered as the optimum temperature of incubation for the isolates No. GN/PA/H6, GN/PA/H8 and GN/PA/SL1. These results are in agreement with findings of Komazawa *et al.* (2004) who reported an optimum temp as 20^{o}C for a *Thraustochytrium* strain for maximal production of DHA.

While, as isolate No. GN/PA/N4 and GN/PA/LR17 showed similar results w.r.t. to PUFA at both 20^{o}C and 25^{o}C but a considerably higher lipid accumulation in biomass and MUFA with only a marginal decrease in biomass at 20^{o}C, 20^{o}C was also considered optimum for further aeration/ mixing optimization studies for both these cultures.

Taking in view all the factors a low temperature of incubation stimulated the production of higher yields unsaturated fatty acids by all the 5 selected cultures. Similar reports have been made by Jang *et al.* (2005), where *Mortierella alpine* showed a total high PUFA yield at lower temperatures and produced PUFA such as arachidonic acid, eicosapentaenoic acid, linoleic acid and α-linolenic acid. While the PUFA produced decreased when incubation temperature was higher than 20^{o}C.

c. Aeration and Mixing: After the optimum temperature was determined for each culture i.e. 20^{o}C, a range of mixing and aeration were studied (i.e. RPM 100, 180 and 250 rpm). The critical effect of aeration and mixing speeds (RPM) on DCW, Lipid and PUFA production by the 5 select isolates is shown in table 5 and figure 6 and 7.

At flask level fermentation process, only agitation can be regulated (Singh *et al.*, 2011) and it results in limited aeration, and hence reduced yield. This could be due to less availability of dissolved oxygen (DO), which may adversely affects the cell growth and product yield. However, hhigher levels of lipid accumulations are reported at lower oxygen levels (Wynn and Ratledge, 2005; Jakobsen *et al.,* 2008). A culture's demand for oxygen is very much dependent on the source of carbon in the medium. Carbohydrate substrates like glucose due to their reduced state generally have a lower oxygen demand in biomass production as compared to other hydrocarbon substrates (Stanbury and Hall, 1997). As shown in figure 7 maximum PUFA production was seen at 20^{o}C-100 rpm) for four of the cultures under consideration. While isolate No. GN/PA/LR17 worked best at 20^{o}C-180rpm.

A lower mixing speed like 100rpm and glucose as carbon source in the culture medium may have supported a higher PUFA production. Moreover, oxygen intoxication at higher operating temperatures decreases the level of DO. Thus at lower temperature (20^{o}C) more oxygen was available for microbial metabolism. Similarly at 250rpm the oxygen tension may have been far greater than that required to support lipid accumulation and PUFA production. Very high agitation may cause high shear forces, which may damage the cell wall and cause cell rupture (Stanbury and Hall, 1997). Thus, a reduced or no production of PUFA was observed at 250rpm by all cultures. Representative GC chromatograms comparing the effect of culturing RPM are shown in figure 8.

As shown in the figure 8 the gas chromatograms markedly display the effect of RPM on the lipid profile of isolate No. GN/PA/SL1 cultured at various RPM. Approximately a total of 15.58% Linoleic acid and Alpha Linolenic acid was produced by isolate No. GN/PA/SL1 at 100rpm while, only 8.46% of Linoleic acid was produced at 180rpm. No PUFA was produced at 250rpm. In view of the overall results obtained from the optimization studies for physical parameters like temperature, aeration and mixing speed a set of culture conditions were determined for enhanced PUFA production. These were utilized for further studies in determining the optimum physiological parameters.

Nutritional parameters:

a. Initial pH of Culture Medium: The effect of initial culture medium pH on growth, lipid and PUFA production is shown in table 6 and figure 9 and 10.

After PUFA productivity was compared with the original pH of the Semi-synthetic Medium i.e. 5.8 and 5.3 for Salt water containing and Fresh water media respectively, highest PUFA yield was observed at the two extreme pH for 2 bacterial strains obtained from fresh water sources (*viz*. GN/PA/LR17 and GN/PA/N4) (Figure 10). This finding is similar to the reports by Dyal *et al.* (2005) where higher PUFA was observed in microbes in response to extreme pH environments probably to avert adverse effects due to high H^+/OH^-. On the other hand, the 3 bacterial strains from salt water sources viz. isolate No. GN/PA/H6, GN/PA/H8 and GN/PA/SL1 gave best results when the initial pH of the medium was unaltered.

Jiang and Chen (2000) have reported highest DCW and DHA content of total fatty acids at unaltered optimal medium pH of 7.2 by the alga *Crypthecodinium cohnii*. In this study the growth of *C. cohnii* was most readily initiated at pH 7.2, but it would grow reasonably well over a wide range of pH (i.e. from 5.5 to 9)**.**

The cultures reported in the present study also gave a comparable biomass and lipid over a range of pH from 7.0-9.0 (Table 6). While in isolate No. GN/PA/H6 and GN/PA/H8, the percentage of PUFA did not vary significantly over the range of pH. Thus, other factors like biomass production, percentage lipid content in biomass, type/s of PUFA produced (ω-3/ω-6) and percent Monounsaturated Fatty acids (MUFA) produced were also taken into consideration while selecting cultures for future development.

Komazawa *et al.* (2004) reported that for a *Thraustochytrium* strain, pH between 5.0 and 8.0 was optimum. Similarly, the cultures studied here worked best between a pH of 5.3/5.8 and 7.0. The amount of PUFA as well as lipid accumulation dropped at pH 8.0. Isolate No. GN/PA/SL1 did not produce any PUFA at pH 8.0 (Figure 10). Also, isolate No. GN/PA/N4 produced only negligible amount of lipid at pH 8.0 (Figure 9).

Similar results were obtained by Wu *et al.* (2005) when the initial culture pH was varied between pH 5 and 8, a maximum biomass and DHA yield was obtained from *Schizochytrium* sp. S31 at pH 7.0. But it was observed that this strain did not grow well at an initial culture pH of 8.0.

At an initial pH 4 all the 5 isolates exhibited poor growth and lipid accumulation. The biomass and lipid at initial pH 7.0 and 9.0 yielded comparable biomass indicating a relative insensitivity of the strains to these pH. On the other hand pH= 8.00 resulted in sharp decrease in biomass and lipid content in isolate No. GN/PA/SL1 AND GN/PA/LR17. The maximum biomass and lipid was observed for all 5 isolates at the unaltered pH of the medium i.e. 5.3/5.8 (Table 6 and Figure 9).

At the lower initial pH 4.0 used for cultivation, the bacterial strains consumed the carbon source glucose far less than that at pH 7.0. The production of biomass and lipid production by all the 5 isolates incubated at two extreme initial pH 4.0 and 9.0 was low (Table 7, Figure 9). This indicated that some carbon source was used to maintain physiological condition at the less than optimal pH instead of cell growth, lipid and fatty acid production (Wu *et al.,* 2005).

Gas chromatograms showing the lipid profile of 5 selected isolates cultured at various pH is shown in figure 11.

Table.1 Selected isolates with good PUFA producing potential (Masurkar *et al.*, 2014, 2015)

Sr. No.	Isolates under study	Code No.	Sample Source	PUFA Produced
1.	*Bacillus* sp. R7-532	GN/PA/H6	Salt Water (Arabian Sea)	LA, ALA
2.	*Halomonas* sp. QY113	GN/PA/H8	Salt Water (Arabian Sea)	LA, ETA
3.	*Bacillus* sp. FPZSP13	GN/PA/SL1	Salt Water Fish *(Sardinia longiceps)*	LA
4.	*Bacillus thuringiensis* Bt407	GN/PA/N4	Fresh Water (Pindhari river, Uttaranchal)	LA
5.	*Bacillus endophyticus* strain SCSGAB0048	GN/PA/LR17	Fresh Water Fish *(Labeo rohita)*	LA, ALA

Key: LA=Linoleic, ALA=Alpha Linolenic Acid, ETA=Eicosatrienoic Acid

Table.2 Calculations for the addition of Glucose, Yeast extract and Ammonium Nitrate for preparing Semi-syntheic MI medium having different C/N ratio (Merck Index, 2009)

Sr. No.	C/N Ratio	Amount of glucose (g/L)	Amount of Yeast extract (g/L) [N-ye]	Amount of Ammonium Nitrate (NH_4NO_3) (g/L) [N-an]	Amount of Carbon (g)	Total Amount of Nitrogen		
						[N-ye]	[N-an]	=N(g)
1	1:60	40.0	1.50	0.286	**16.0**	0.166	0.100	**0.266**
2	1:40	40.0	2.25	0.430	**16.0**	0.250	0.150	**0.400**
3	1:30	40.0	2.99	0.569	**16.0**	0.330	0.199	**0.530**

Table.3 Qualitative assessment of lipid accumulation by Sudan Black B staining

Isolate	Lipid accumulation				
	24hr	48hr	72hr	96hr	120hr
GN/PA/H6	-	W+	+	++	++
GN/PA/H8	+	+	+	++	++
GN/PA/SL1	+	+	+	++	++
GN/PA/LR17	W+	+	+	++	++
GN/PA/N4	W+	W+	+	++	++

Key: (W+) = Weakly Positive, (+) = Positive, (++) = Strongly Positive, (-) = Negative

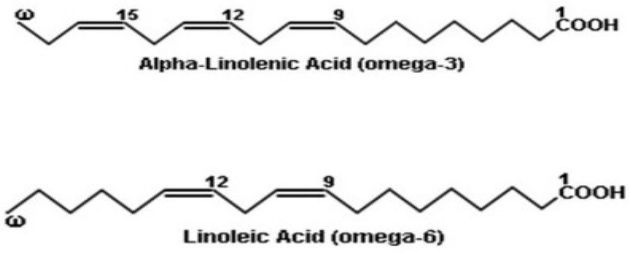
Table.4 Growth parameters and Lipid accumulation and PUFA production trends at different temperatures

Isolate	20^{o}C-180rpm						
	$O.D_{530nm}$	DCW ($mg/100cm^3$ of broth)	pH	Residual Glucose (g/L)	Lipid ($mg/100cm^3$ of broth)	% Lipid in DCW	% PUFA
Isolate	**25^{o}C-180rpm (Original culture condition)**						
GN/PA/H6	1.50	270	6.50	35.45	113.0	41.85	0.60
GN/PA/H8	1.60	300	3.85	32.50	100.5	33.50	0.91
GN/PA/SL1	1.72	450	4.90	28.50	185.4	41.20	5.06
GN/PA/LR17	1.54	210	5.70	30.10	160.6	76.48	1.63
GN/PA/N4	1.52	150	6.00	31.60	50.5	33.67	1.05
Isolate	**20^{o}C-180rpm**						
GN/PA/H6	1.40	240	6.50	30.80	98.6	41.08	1.24
GN/PA/H8	1.52	290	4.20	34.00	125.6	43.31	3.83
GN/PA/SL1	1.69	400	4.3	26.00	268.0	67.00	8.46
GN/PA/LR17	1.39	180	5.5	28.80	100.8	56.00	1.90
GN/PA/N4	0.95	100	6.0	30.40	65.4	65.40	0.60
Isolate	**30^{o}C-180rpm**						
GN/PA/H6	1.22	260	5.0	32.00	103.8	39.92	0.72
GN/PA/H8	1.69	430	4.0	28.00	88.4	20.56	0.62
GN/PA/SL1	1.69	450	5.0	28.40	365.2	79.16	0.00
GN/PA/LR17	1.39	200	5.0	30.00	127.8	63.90	0.00
GN/PA/N4	1.00	120	5.1	28.40	86.00	71.67	0.13
Isolate	**30^{o}C-20^{o}C-180rpm**						
GN/PA/H6	1.69	350	5.50	30.40	112.8	75.20	0.48
GN/PA/H8	1.52	350	4.80	28.40	104.6	29.89	0.39
GN/PA/SL1	1.52	360	5.80	28.80	187.8	52.17	0.24
GN/PA/LR17	1.39	180	5.50	28.80	61.8	34.30	0.00
GN/PA/N4	1.30	190	5.50	30.00	94.6	49.70	0.30

Table.5 DCW, Lipid other parameters and PUFA production at 20°C cultured at various RPM

Isolate	100rpm						
	O.D$_{530nm}$	**DCW** (mg/100cm^3 of broth)	**pH**	**Residual Glucose** (g/L)	**Lipid** (mg/100cm^3 of broth)	**% Lipid in DCW**	**% PUFA**
GN/PA/H6	0.95	360	6.40	36.50	126	35.00	7.54
GN/PA/H8	1.69	550	3.20	32.50	372	67.64	8.00
GN/PA/SL1	2.00	410	4.40	29.00	258	62.93	15.58
GN/PA/LR17	1.69	310	5.80	30.00	310	67.10	1.46
GN/PA/N4	1.30	260	6.70	33.00	104	40.00	1.48
Isolate	**180rpm**						
GN/PA/H6	1.40	240	6.50	30.80	98.6	41.08	1.24
GN/PA/H8	1.52	290	4.20	34.00	125.6	43.31	0.91
GN/PA/SL1	1.69	400	5.30	26.00	268	67.00	8.46
GN/PA/LR17	1.39	180	5.90	28.80	100.8	56.00	2.27
GN/PA/N4	0.95	100	6.00	30.40	65.4	65.40	0.6
Isolate	**250rpm**						
GN/PA/H6	1.69	440	6.50	32.00	308	70.00	1.42
GN/PA/H8	1.52	200	3.30	32.50	136	68.00	2.2
GN/PA/SL1	1.69	400	4.30	29.00	152	38.00	0.00
GN/PA/LR17	1.69	600	5.50	30.50	284	47.33	0.00
GN/PA/N4	0.22	140	6.70	33.00	76	54.29	0.6

Figure.1 Names and structures of two metabolically important PUFA



(Source: www.myamericanheart.org)

Table.6 DCW, lipid production, other parameters and PUFA production w.r.t. culture medium pH modification

Isolate	pH=4.00						
	O.D$_{530nm}$	**DCW (mg/100cm^3 of broth)**	**pH**	**Residual Glucose (g/L)**	**Lipid (mg/100cm^3 of broth)**	**% Lipid in DCW**	**% PUFA**
GN/PA/H6	0.20	50	3.20	39.20	41	82.00	6.40
GN/PA/H8	1.05	200	3.46	37.75	88.2	44.10	7.00
GN/PA/SL1	0.28	40	3.20	38.50	21.8	54.50	6.20
GN/PA/LR17	0.18	80	4.30	37.36	56.6	75.75	5.60
GN/PA/N4	1.00	190	3.22	38.02	129.2	68.00	6.90
Isolate	**pH=5.30/5.80***						
GN/PA/H6	1.66	380	6.50	36.50	133.6	35.16	6.50
GN/PA/H8	2.00	412	3.55	34.80	201	48.79	6.80
GN/PA/SL1	2.00	572	2.80	35.44	209.4	36.61	16.50
GN/PA/LR17	1.56	260	4.21	33.53	104	40.00	2.20
GN/PA/N4	1.82	324	6.90	30.50	174.4	53.70	1.53
Isolate	**pH=7.00**						
GN/PA/H6	0.56	100	7.18	35.55	82.8	82.80	5.80
GN/PA/H8	1.52	300	4.91	35.03	111.4	37.13	5.20
GN/PA/SL1	0.88	210	3.82	31.51	100.2	47.71	8.10
GN/PA/LR17	1.09	140	4.91	32.36	2.32	1.66	1.20
GN/PA/N4	0.82	380	5.09	28.19	118.6	31.21	2.32
Isolate	**pH=8.00**						
GN/PA/H6	0.72	160	7.41	35.76	46.4	29.00	3.30
GN/PA/H8	1.02	130	4.90	36.61	120	92.30	4.70
GN/PA/SL1	0.85	196	3.72	35.53	99	50.51	0.00
GN/PA/LR17	1.52	170	5.10	34.88	6.74	3.96	2.40
GN/PA/N4	0.90	210	4.69	32.40	122.6	58.38	6.74
Isolate	**pH=9.00**						
GN/PA/H6	0.85	190	7.30	36.20	165.4	87.05	4.80
GN/PA/H8	1.39	300	4.70	34.13	148.8	49.60	3.70
GN/PA/SL1	0.79	162	3.74	36.41	120.8	74.57	9.40
GN/PA/LR17	1.62	250	5.13	21.44	126.4	50.56	2.30
GN/PA/N4	1.39	270	4.93	32.29	101.4	37.56	7.40

*pH= 5.80 for GN/PA/H6, GN/PA/H8, GN/PA/SL1 and pH= 5.30 for GN/PA/LR17, GN/PA/N4.

Table.7 DCW, lipid production, other parameters and PUFA production w.r.t. C/N ratio modification

Isolate	C/N Ratio = 60:1						
	Final $O.D_{530nm}$	**DCW (mg/100cm^3 of broth)**	**pH**	**Residual Glucose (g/L)**	**Lipid (mg/100cm^3 of broth)**	**% Lipid in DCW**	**% PUFA**
GN/PA/H6	1.09	190	6.39	32.2	76.8	40.42	6.54
GN/PA/H8	1.32	250	3.65	28.0	102.6	41.04	6.81
GN/PA/SL1	1.52	280	2.50	23.1	161.0	57.5	1.80
GN/PA/LR17	1.02	88	6.20	38.0	49.4	56.14	2.06
GN/PA/N4	1.58	320	6.50	31.4	180.8	56.5	2.63
Isolate	**C/N Ratio = 40:1**						
GN/PA/H6	1.52	250	7.00	35.0	103.45	41.38	5.18
GN/PA/H8	1.48	260	3.40	26.0	112.8	43.4	3.36
GN/PA/SL1	2.00	470	3.50	24.0	152.8	32.51	3.55
GN/PA/LR17	1.30	160	6.02	35.0	98.9	61.81	0.87
GN/PA/N4	1.69	395	7.50	34.5	197.1	49.9	2.80
Isolate	**C/N Ratio = 30:1**						
GN/PA/H6	1.30	290	6.56	37.0	90.0	31.03	3.46
GN/PA/H8	2.00	490	3.50	25.0	100.8	20.57	3.01
GN/PA/SL1	2.00	420	3.20	30.1	173.2	41.24	2.34
GN/PA/LR17	1.20	210	6.55	34.0	80.54	38.35	0.66
GN/PA/N4	2.00	580	7.01	36.2	210.4	36.28	2.06

*pH=5.80 for GN/PA/H6,GN/PA/H8,GN/PA/SL1,pH=4.00 for GN/PA/LR17 and pH=9.00 for GN/PA/N4.

Table.8 Optimum culture conditions for the 5 isolates

Isolates under study	**Code No.**	**Optimum Culture Conditions**				
		Duration of Incubation (hr)	**Temperature of Incubation (^oC)**	**Aeration/ Mixing (RPM)**	**pH**	**C/N Ratio**
Bacillus sp. R7-532	GN/PA/H6	120	20^oC	100	5.8	40:1
Halomonas sp. QY113	GN/PA/H8	120	20^oC	100	5.8	40:1
Bacillus sp. FPZSP13	GN/PA/SL1	120	20^oC	100	5.8	40:1
Bacillus thuringiensis Bt407	GN/PA/LR17	120	20^oC	180	4.0	40:1
Bacillus endophyticus strain SCSGAB0048	GN/PA/N4	120	20^oC	100	9.0	40:1

Figure.2 Gas chromatograms showing the lipid profile of isolate No. GN/PA/H8 at 120hr and 168hr

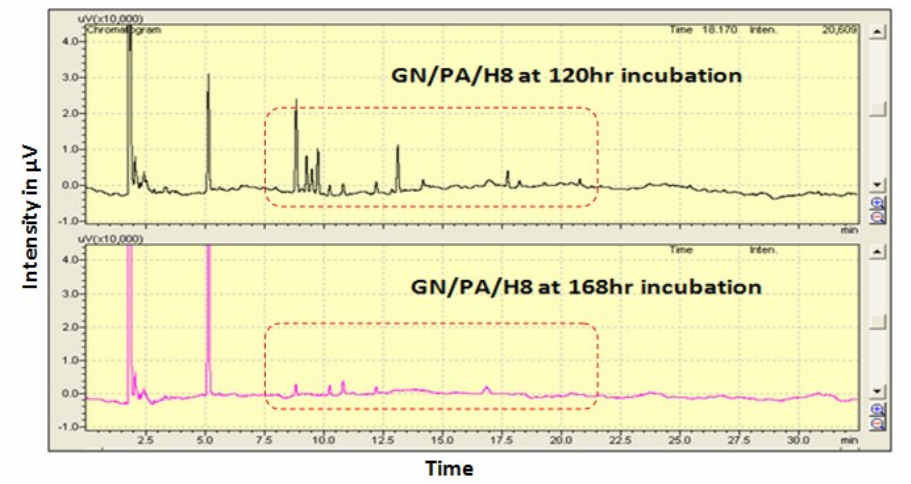


Figure.3 Effect of temperature of incubation on lipid content

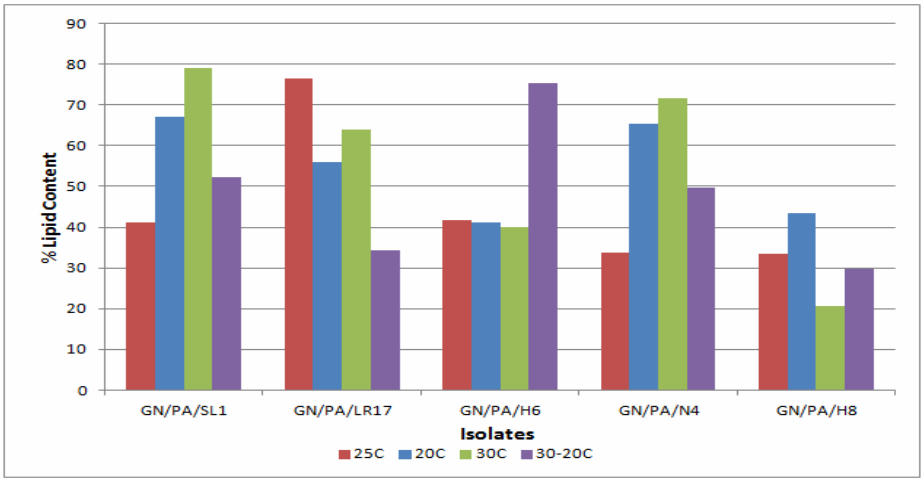
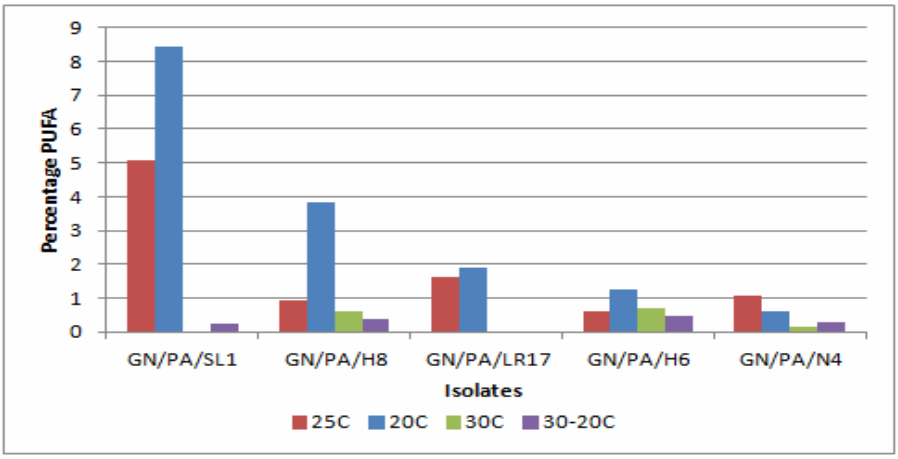


Figure.4 Optimum temperature of incubation for PUFA production at 180 rpm



Percentages were calculated after GC analysis (Basis: Peak Area %)

Figure.5 Gas Chromatograms showing the lipid profile of isolate No. GN/PA/LR17 at various temperatures

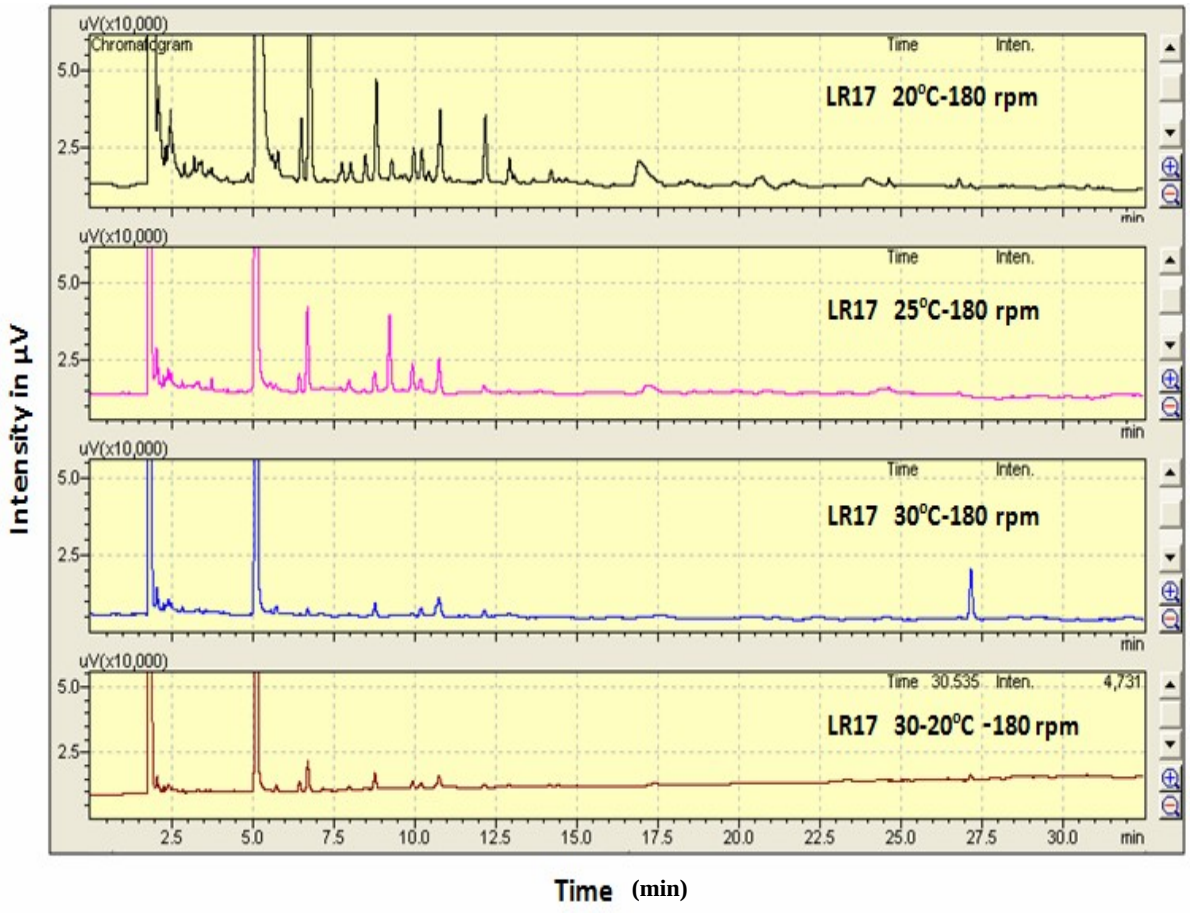


Figure.6 Effect of varying RPM on lipid content

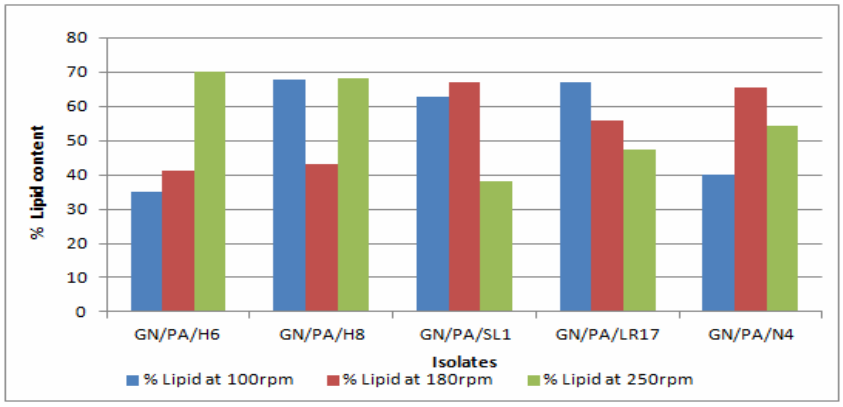


Figure.7 Effect of varying RPM on PUFA production at 20°C

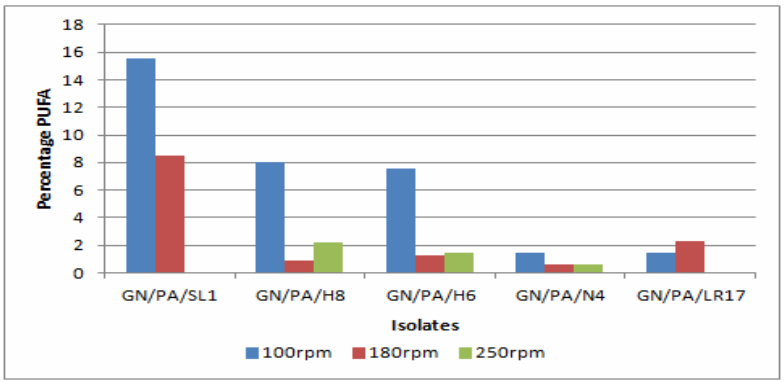


Figure.8 Gas Chromatograms showing the lipid profile of isolate No. GN/PA/SL1 cultured at various RPM

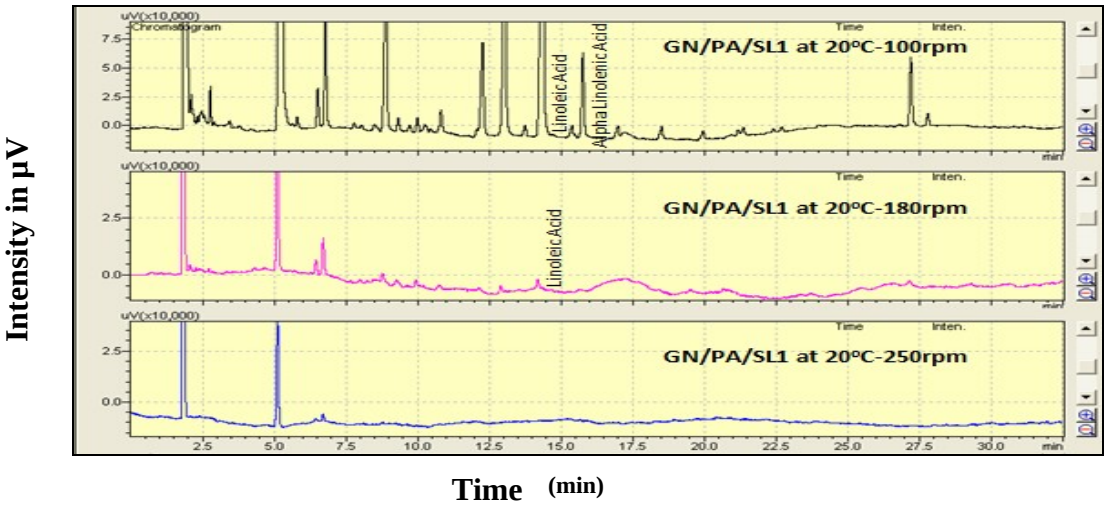


Figure.9 Effect of culture medium pH on lipid content

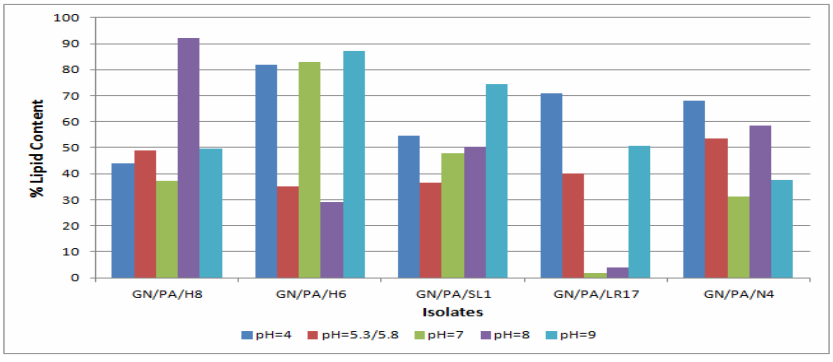
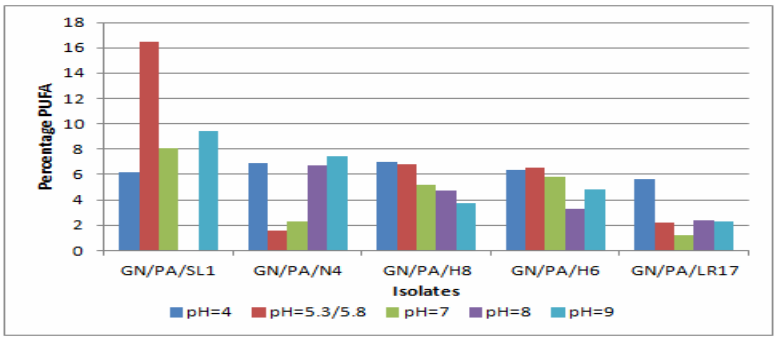


Figure.10 Effect of culture medium pH on PUFA production



Percentages were calculated after GC analysis (Basis: Peak Area %)

Figure.11 Gas chromatograms showing the lipid profile of 5 selected isolates cultured at various pH

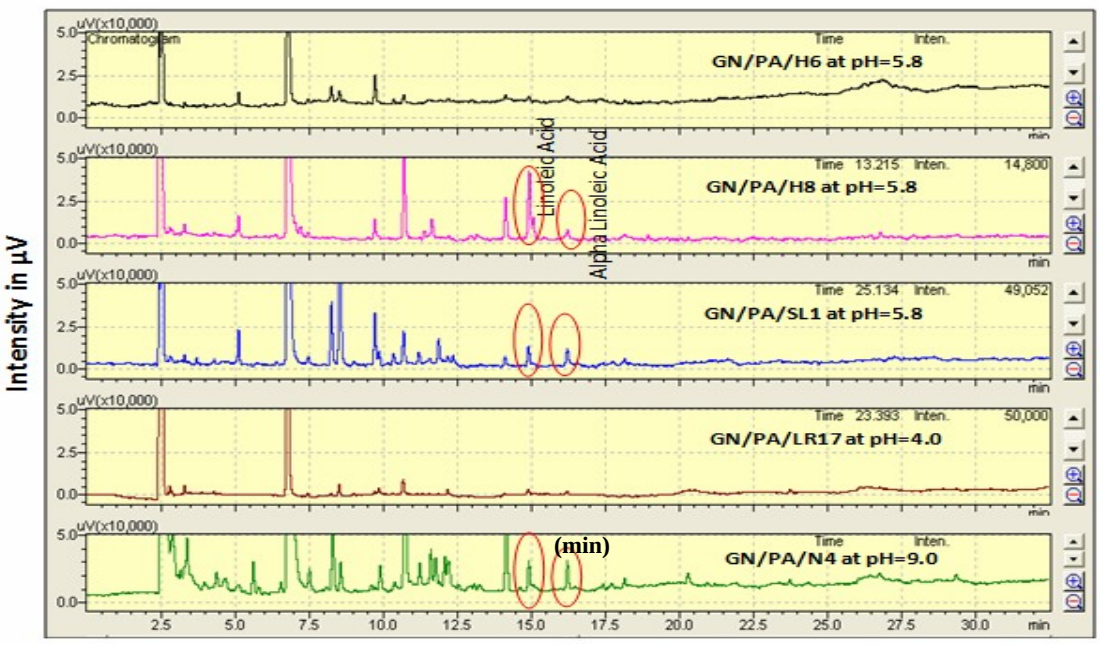


Figure.12 Effect of C/N ratio modification on lipid content

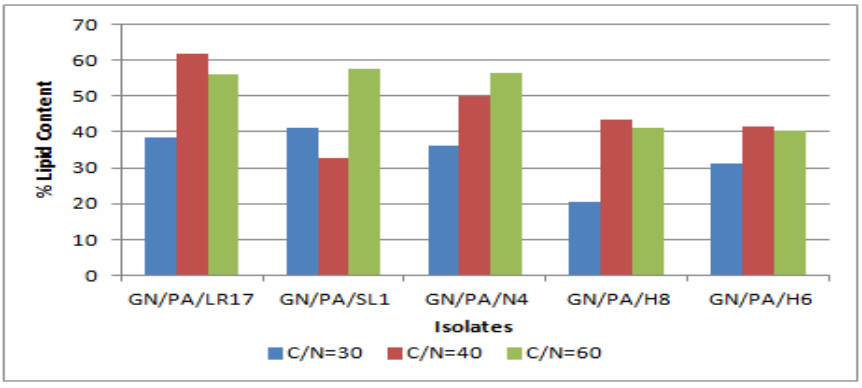
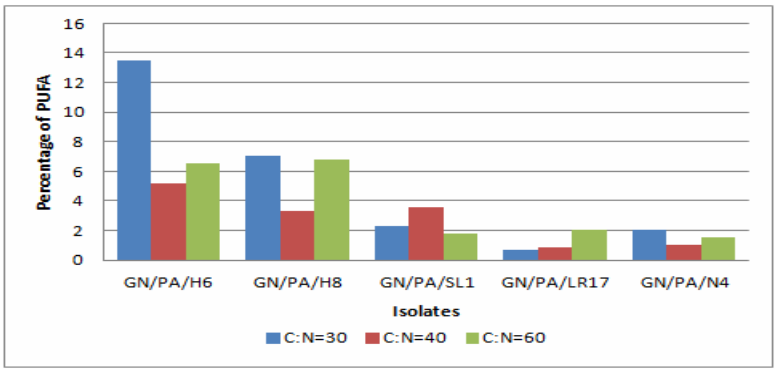
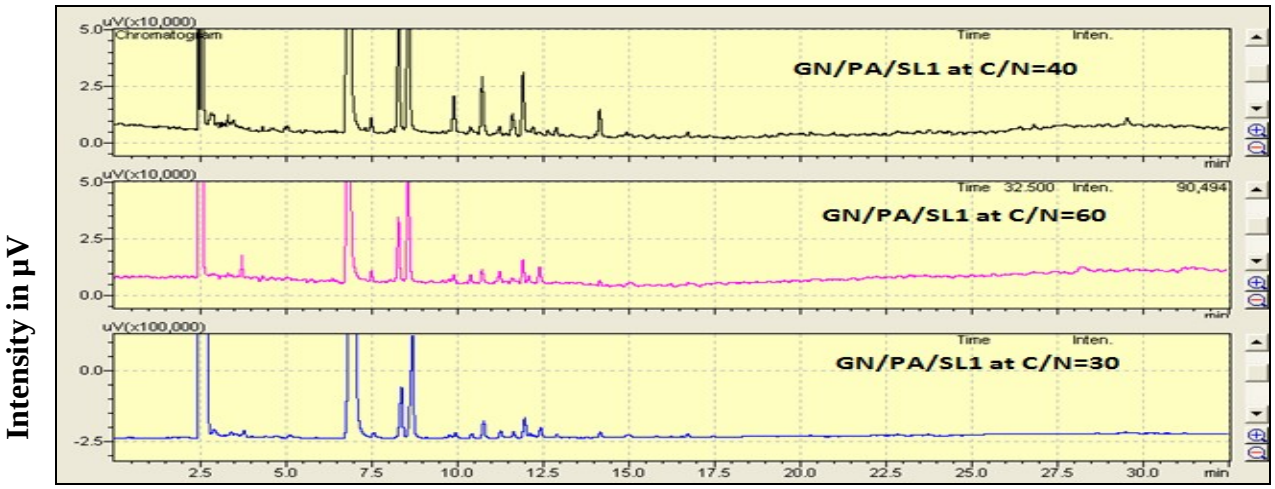


Figure.13 Effect of varying C/N of culture medium on PUFA production



Percentages were calculated after GC analysis (Basis: Peak Area %).

Figure.14 Gas chromatograms showing the lipid profile of isolate No. GN/PA/SL1 cultured at 3 different C/N ratio



It is observed from figure 11 that all 5 isolates could produce Linoleic acid with isolate No. GN/PA/H6 and GN/PA/LR17 producing LA only in small amounts (very small peaks). Though the number of shorter fatty acids has reduced in both these cultures thus, increasing the relative percentage of PUFA in the total lipid. The results imply that there are some media–pH interactions that occur which can affect the growth and lipid production.

b. Carbon/Nitrogen ratio in medium: The aim of the present work was to further investigate the potential of lipid production by the 5 bacterial strains, in semi-defined nitrogen-limited media having various C/N ratio. Keeping in mind that higher C/N supports higher amounts of lipid accumulation but limits biomass production while lower C/N supports higher biomass production (Rossi *et al.*, 2011); the C/N ratio of the nutrient medium was modified in a range of 60 to30.

The effect of C/N ratio modification on DCW, lipid and PUFA production is shown in table 7 and figure 12 and 13.

All the five cultures showed a reduced PUFA percentage at C/N of 40 as compared to 60, expect for isolate No. GN/PA/SL1, a *Bacillus* spp. which showed an increase in PUFA. Assessing the effect of different C/N ratio on biomass and lipid production may be complex, because temperature, the concentrations of the carbon and nitrogen sources and other culture conditions may also play a role. Furthermore, in batch cultures the assimilated C/N ratio is continually changing throughout the cultivation and usually only the ratio of these nutrients as initially included in the medium is reported, which renders interpretation of the effect of C:N ratio on PUFA production in late log phase difficult (Immelman and Kilian, 1997).

The C/N ratio appears to have varying effects on the fatty acid composition of different microbes and no general conclusion in this respect can be drawn. Although work by many scientists has indicated that, a high C/N ratio favours high lipid content but compromises on the biomass production (Immelman and Kilian, 1997; Sattur and Karanth, 1989; Ykema *et al.*, 1988). This could be due to low protein and probably also a low nucleic acid content expected in cells grown under a nitrogen limitation (Immelman and Kilian, 1997).

To understand the effect of modified C/N ratio in entirety, all the factors considered in the temperature, aeration/mixing and pH study were taken into account when determining the optimum C/N ratio. Not only the percentage of PUFA produced but also the biomass, the lipid accumulation and percentage lipid content were compared to arrive at the optimum C/N ratio.

All 5 cultures showed increase in biomass and lipid levels when the C/N ratio was changed from 60 to 30. All the 5 cultures showed a higher biomass at the C/N ratio of 40 as compared to the original C/N ratio of 60 (Table 7). Also, most of the cultures accumulated similar amounts of lipid at both the C/N ratio of 60 and 40 it resulted in higher percentage of lipid in biomass (Figure 12). This may be because reduced nitrogen source (as compared to C/N=30) in the culture medium facilitated the shift from high biomass production to lipid accumulation. A higher intensity of lipid could also be observed in the GC lipid profile of the isolates. A representative comparison of Gas chromatogram from isolate No. GN/PA/SL1 cultured at 3 different C/N ratio is shown in figure 14.

As shown in figure 14 it was observed that a higher number and higher concentration of fatty acids were observed at C/N ratio of 40 as compared to those at C/N ratio of 60 and 30. The concentration of Linoleic acid a 18 carbon 2 unsaturations fatty acid was also reduced at C/N ratio of 60 and 30. The percentage of MUFA like Myristic acid, Palmitoleic acid, Oleic acid was observed to be higher at a C/N ratio of 40 as compared to other two C/N ratio.

To maximize the PUFA production per culture volume, it would be essential to obtain the maximum biomass concentration and intracellular lipid content (Immelman and Kilian, 1997). However, all the cultivations where a high biomass concentration was reached were characterised by a decrease in the lipid content which resulted in a low PUFA content. As the aim was to devise a culture strategy whereby a high cell concentration may be reached without sacrificing a high intracellular lipid and PUFA content simultaneously it was preferable to choose C/N=40:1 as the optimum carbon to nitrogen ratio.

The results in the present study are in agreement with the findings of Immelman and Kilian (1997) and who recommend a C/N ratio between 30 and 60 for maximum lipid and PUFA production. They have reported that a carbohydrate-carbon source an intermediate C/N ratio of 40:1 gave the best results balancing all factors under consideration in terms of Gamma Linolenic acid (GLA) production, lipid accumulation and biomass production by *Mucor circinelloides*. Ykema *et al.* (1988) have also suggested C/N=40:1 as the best intermediate ratio for lipid production by oleaginous yeasts in continuous cultures.

The results obtained in this section convey that varying the cultivation conditions profoundly affects cell growth and fatty-acid production and PUFA composition by these microorganisms. At the end of the study, a sturdy shake flask process was established supporting good PUFA production. All the studied optimum culture conditions for the 5 selected isolates are listed in table 8.

A series of experiments were conducted with the goal to optimize the growth conditions of the 5 bacterial strains for PUFA production. The optimization of duration and temperature of incubation, rate of aeration/mixing, media pH and carbon to nitrogen ratio were investigated. Options which could enhance the biomass, lipid and PUFA levels were chosen.

All the cultures produced higher amounts of PUFA when incubation temperatures were lowered. Most of the cultures produced greater lipid and PUFA at lower mixing speeds. A further investigation into the mechanisms involved in PUFA biosynthesis would allow one to better determine the contribution of oxygen to the synthesis of these high-value products. Isolates from Salt water produced maximum PUFA at the original pH of the medium i.e. pH=5.8. Fresh water isolates produced more PUFA at pH extremes of 4.0 and 9.0 as compared to the original slightly acidic culture medium. Thus, the PUFA production could be enhanced in fresh water bacterial species by altering the pH.

Although it is generally true that oleaginous microorganisms accumulate lipids when their nitrogen source has become limiting (Wynn and Ratledge, 2005; Dyal *et al.*, 2005), there appears to be an optimum carbon concentration at which this accumulation is maximized. Results from the experiments in this study indicate that these optimizations occur around C/N=40:1 as it exhibited finest results w.r.t PUFA productivity without compromising the lipid and biomass production.

Overall, the results of this study indicate that culture conditions and nutrient composition affect PUFA productivity in both salt water as well as fresh water bacteria. Thus, the modulation of process parameters can significantly help in improving PUFA production in these organisms. Moreover the data obtained here can be used for planning further process and media optimization by statistical methods. Also, the information could be utilized in scale up studies.

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