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Biodegradation of waste grease by *Penicillium chrysogenum* for production of fatty acid

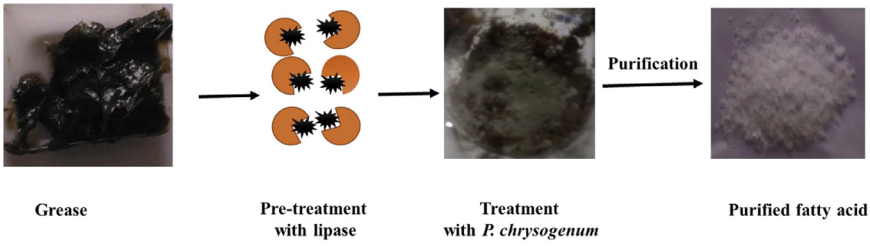
Arti Kumari [a], Razi Ahmad [a], Sangeeta Negi [b], Sunil Kumar Khare [a,*]

[a] Enzyme and Microbial Biochemistry Lab, Department of Chemistry, Indian Institute of Technology, New Delhi 110016, India
[b] Department of Biotechnology, Motilal Nehru National Institute of Technology, Allahabad, UP 211004, India

HIGHLIGHTS

- Pre-treatment is needed to bio-remediate grease waste.
- *Penicillium chrysogenum* can utilize pre-treated grease as a sole carbon source.
- Fatty acid was purified by crystallization methods and analysed by GC–MS.

GRAPHICAL ABSTRACT



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ABSTRACT

The aim of present work was to effectively remediate grease waste by *Penicillium chrysogenum*. For efficient degradation, grease waste was pre-treated using various lipases, among them lipolase was the best. The pretreated grease was used as a substrate by *P. chrysogenum* resulting into the production of fatty acids. Process was optimized by response surface methodology (RSM) using four variables *viz*; $FeCl_2$ (mM), spore concentration (spores/ml), time period (days) and amount of grease (g). The optimized conditions *viz;* $FeCl_2$ 1.25 mM, culture amount 5×10^{11} spores/ml and time period 16 days led to the production of 6.6 mg/g fatty acid from 10.0 g of pre-treated grease mixed with 5.0 g wheat bran in 10.0 ml czapek-dox medium under solid state fermentation. The fermented media was extracted with hexane and subjected to GCMS analysis, which showed the presence of higher amount of palmitic acid. It was purified by crystallization method and 2.8 g of palmitic acid was recovered from 1.0 kg grease waste in tray fermentation.

1. Introduction

Grease is widely used as a lubricating agent in almost every machine to reduce the friction generated between the two moving parts (Lundberg and Hoglund, 2000). It consists of thickening agents such as metal soap (5–20%), base oils (80–95%) and additives (0–10%). Lubricating property of grease is due to presence of oil that reduces the friction, while thickeners avert loss of lubricant (Lundberg and Hoglund, 2000).

Disposal of grease waste poses a serious problem and becomes a threat to the ecosystem as it is non-biodegradable. In most of the countries, waste grease has been dumped in the litter site or in the sewages without any pretreatment leading to severe environmental issues (Pilusa et al., 2013). In South Africa, there is a special landfill site where hazardous grease is disposed in 1:1 ratio of grease: ash blending (Pilusa et al., 2013). Grease waste in effluents causes reduction in the cell-aqueous phase transfer rates, reduced sedimentation, and formation of floating sludge, clogging and the emergence of unpleasant odors (Rincon et al., 2007). This can be tackled by carrying out improved biodegradation of grease waste. Therefore, effective bioremediation of grease is highly desirable. The most commonly methods employed for treatments of fats, oils,

* Corresponding author.
E-mail address: skhare@rocketmail.com (S.K. Khare).

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and grease (FOG) are physical removal (cleanouts) or chemical treatment with bacteria, enzymes, caustics and solvents (Murty et al., 2002). Utilization of grease as a substrate opens up new strategy for product development vis-a-vis its remediation (Kumar et al., 2011). Kumar et al. showed the utilization of grease waste by *P. chrysogenum* SNP5for the production of lipase, suggesting that this strain can be used for grease remediation (Kumar et al., 2012). Recently, Kumar and Negi developed an economical process for the production of fatty acids from repeatedly used cooking oil (Kumar and Negi, 2015).

In current study, a process for the bioremediation of grease waste was standardized in succession with pre-treatment by lipase followed by degradation from *Penicillium chrysogenum* leading to the successful production of fatty acid.

2. Material and methods

Penicillium chrysogenum SNP5 is an isolated strain and identified by Microbial type Culture Collection and gene bank Chandigarh India (Kumar et al., 2012). *Rhizopus oryzae*, *Aspergillus oryzae* (lipolase), *Candida rugosa* and *Thermomyces lanuginose* was purchased from Sigma Aldrich, Surfachem, Amano and Novozyme, respectively. Grease was locally collected from loco shed area Indian Railway, Allahabad (25.4457° N, 81.8302° E), INDIA. Wheat bran was purchased from the local market. Other medium ingredients and czapek-dox medium were sourced from SRL (Sisco Research Laboratories Pvt. Ltd.) Mumbai, India.

2.1. Growth of P. chrysogenum on grease

A single colony of *P. chrysogenum* was picked from PDA plate and inoculated in czapek-dox medium and dry cell weight was estimated after regular interval of time period. The cell free supernatant was washed with sodium phosphate buffer (0.1 M, pH 7.0) and kept for drying in hot air oven (Scientific Co, Delhi, India). The dry cell weight was determined after drying 1 ml pelleted culture at 50 °C for 24 h and dry cell weight (DCW) was determined gravimetrically using Sartorius (BSA2245-CW) weighing balance. Similarly, czapek-dox medium containing 5.0 g grease was taken and inoculated with a single colony of *P. chrysogenum* to monitor growth at regular time period. The growth curve of *P. chrysogenum* was plotted between biomass (g/L) vs time (days) and growth rate was calculated from the slope of growth curve (Sutton and Starzyk, 1972).

2.2. Pre-treatment of grease

Pre-treatment of grease was given with the commercial lipases *viz*., *Rhizopus oryzae*, *Aspergillus oryzae* (lipolase), *Candida rugosa* and *Thermomyces lanuginose* lipases. Grease waste (5 g) was dissolved in 9.0 ml hexane and mixed with 50.0 U of lipase in 1 ml sodium phosphate buffer (0.1 M, pH 7.0). The mixture was incubated at 40 °C for 3 h. The resulting sample was used as a substrate to grow *P. chrysogenum* after evaporating hexane by rota vapor (BUCHI India Pvt Ltd).

Pre-treatment of grease was optimized by varying enzyme concentration (50.0 U, 100.0 U and 150.0 U), varying the solvents (hexane, acetonitrile, 1,4-dioxane and *iso*-propanol) and time period of incubation (3 h, 5 h, 8 h and 12 h).

2.3. Solid state fermentation (SSF) of pretreated grease

A 5.0 g sample of pretreated grease waste was mixed with 5.0 g of wheat bran and 10.0 ml of czapek-dox medium containing $FeCl_2$ (1.0 mM) and gum acacia (0.2 g) in 250 ml Erlenmeyer flask (Bushnell and Haas, 1941; Negi and Kumar, 2012). This medium was inoculated with varying concentration of spores from *P. chrysogenum* in order to get the maximum grease degradation and fatty acid production. The spore were prepared on the slants of PDA Agar for seven days and dissolved in 1 ml of solution containing 1% tween 80 and 0.1% NaCl solution.

2.4. Fatty acid estimation and lipase assay

Fatty acid was estimated by titration method as mentioned by Saifuddin et al. (2009), as well as by GC as described by Kumar and Negi (2015). The samples of GC were analyzed from Perkin Elmer GC Model-Autosystem XL in which OV-1 packed column and FID detector was used. OV-1 column was packed with 100% methyl gum as stationary phase. Nitrogen gas was used as mobile phase at rate of 14 ml/min. The injector temperature was set at 250 °C and the detector temperature was 260 °C. Fatty acid concentration of sample was calculated by comparing the peak area of standard palmitic acid (1.0 mg/ml).

Simultaneously, lipase activity was also determined using *p*-nitro phenyl palmitate as a substrate (Winkler and Stuckmann, 1979).

2.5. Process optimization by response surface methodology (RSM)

A mathematical approach RSM was employed for process optimization to maximized fatty acid production from grease using four different parameters: $FeCl_2$ (1.0–2.0 mM), spore concentration (10^7–10^{12} spores/ ml), time period (9–30 days) and amount of grease (5.0 g–20.0 g) at five different levels (−2, −1, 0, 1, 2) using central composite design (CCD) (Table 1). MINITAB 16 was used to design the experiment and a set of 30 experimental runs were predicted. The model was analyzed by ANOVA. Contour plots were generated to analyze the interactive effect and to determine their optimum concentration of these factors for the maximum production of fatty acid.

The optimized solid state fermentation process was done in tray using 1.0 kg grease. A surgical tray (35 × 25 × 5 cm), bed 1.5 cm with all the controlling units for humidity and temperature (30 °C) was set up (Mohseni et al., 2012). The parameters standardized by RSM was followed accordingly for the over production of fatty acid from 1 kg grease waste.

2.6. Fatty acid purification and characterization

Fatty acid was purified by crystallization procedure as described by Rubin and Rubin (1988) with slight modification. The solid state fermentation media was mixed with hexane and filtered by muslin cloth. The filtrate was centrifuged and the supernatant was recovered. The solution of free fatty acids in hexane was mixed with the methanol in 9: 1 ratio. The following mixture was then incubated at −20 °C for 12 h resulting in the formation of fatty acid crystals. These crystals were recovered in the form of pellet by centrifugation at 8000*g* for 15 min. The liquid phase was decanted and the pellet was dissolved in hexane and subjected to Gas chromatography–mass spectrometry (GC–MS) analysis as described above.

2.7. Statistical analysis

All experiments were repeated three times in duplicate. Data was plotted with mean ± SD. Mean and SD was calculated using Excel-2013.

Table 1
Experimental range of four different parameters used in central composite design of RSM.

Component	−2	−1	0	1	2
$FeCl_2$ (mM)	0.5	0.875	1.25	1.625	2.00
Culture amount (spores/ml)	10^7	2.5×10^{11}	5×10^{11}	7.5×10^{11}	10^{12}
Time period (days)	3	9	16	23	30
Grease (g)	5	7.5	10	12.5	15

3. Results and discussion

Worldwide approximately, 10 lakh tons of grease has been produced globally and in presence of improper disposable, waste grease is turning out to be a serious problem for the environment (Mang and Dresel, 2007). Waste grease encompasses considerable quantity of base oil and it is quite worthy to recover some of the valuable products such as fatty acids by biological treatment vis-a-vis its degradation.

An oil degrading strain *Penicillium chrysogenum* was screened for its growth on grease as sole carbon source. The growth curve showed that it was able to utilize grease as a sole carbon source without any delay in log phase (Fig. 1). This strain was previously isolated from Diesel loco shed area and is well known to degrade oils (Kumar et al., 2012). However, the growth rate (0.51) of *P. chrysogenum* was slow in grease containing medium in comparison to normal czapek-dox medium (0.68) (Fig 1). The probable reason behind this result is the presence of grease which is a complex carbon source (Cammarota and Freire, 2006). Therefore, pre-treatment of grease with some enzyme formulations is needed for the full grease bioremediation by *P. chrysogenum*.

It is considered that fatty acid chains in grease are extremely hydrophobic and align as soon microbes use them, making it difficult to act up on (Mohammad, 2011). Some common pre-treatment methods have been suggested to firstly hydrolyze lipid molecules/ fatty acid chain which prevent their reassociation. Consequently, microbes are better able to utilize it as carbon source (Cammarota and Freire, 2006). Various researchers have used NaOH, HCl and $Ca(OH)_2$ to reduce the size of fats and lipids present in the grease samples (Masse et al., 2001). However, it led to drastic reduction in the growth of microbes in subsequent step (Cammarota and Freire, 2006; Masse et al., 2001; Omil et al., 2003). The lipase pre-treatment has been more effective in this context, while remediating fats in waste water (Masse et al., 2001). We screened various lipases for the pre-treatment of grease waste *viz*., *Rhizopus oryzae*, *Aspergillus oryzae* (lipolase), *Candida rugosa* and *Thermomyces lanuginose* (Lipozyme TL IM). The efficiency of pre-treatment was determined by the amount of fatty acid (acid value) released. The untreated grease has an acid value of 1.9 mg/g of grease. For pre-treatment, the matching activity units of each lipase were used (Fig. S1). The results showed that lipolase was most effective in releasing high amount of fatty acid. Therefore, we have standardized the condition for the pre-treatment of grease by varying lipolase concentration, solvents and time period using one variable at a time. Fig. S2a shows that 100.0 U and 150.0 U of lipolase treatment gave almost similar acid value (indicative of high fatty acid value) of nearly 4.1 mg/g of grease, suggesting that 100.0 U of enzyme is enough for the pre-treatment. The solvent is used to dissolve the grease also affects the lipase activity. One ml lipolase containing100.0 U in sodium phosphate buffer (0.1 M, pH 7.0) was used over the grease (5.0 g) dissolved in various solvents (9.0 ml) and the mixture was stirred continuously at 150 rpm at 40 °C (Fig. S2b). Evidently, grease treated by 100.0 U of lipase in presence of hexane released highest acid value of 4.6 mg/g of grease followed by 1,4 dioxane (2.8 mg/g), acetonitrile (2.5 mg/g) and *iso*-propanol (1.8 mg/g) indicating that hexane was the best solvent for the grease pre-treatment. The time course study suggested that 5 h of pre-treatment under standardized conditions led to the highest acid value (4.6 mg/g), which decreased thereafter (Fig. S2c). The overall, optimized conditions for pre-treatment were 5.0 g grease in 9.0 ml hexane with 100.0 U of lipolase enzyme in 1.0 ml sodium phosphate buffer, pH 7.0 (total 10 ml volume) for 5 h at 40 °C with constant shaking at 150 rpm.

The pre-treated grease was used as substrate for the *P. chrysogenum*. The growth rate of *P. chrysogenum* was better in pre-treated grease with specific growth rate of 0.65 (Fig. S3). This may be due to presence of higher amount of fatty acid in comparison to untreated grease that could be easily accessed by *P. chrysogenum* as a carbon source.

The process was optimized for the maximum degradation of grease and recovery of maximum fatty acids. For this purpose, the response surface methodology was used, using four different parameters *viz*., $FeCl_2$ (mM), spore concentration (spores/ml), time period (days) and amount of grease (g). Final RSM results by CCD revealed that both observed and predicted values of each experiment were nearly similar as shown in Table 2. The regression analysis of data gave the following equation for fatty acid (Y) as a function of $FeCl_2$ (A), spore concentration (B), time period (C) and amount of grease (D):

$$Y = -24.4939 + 16.5334A + 0.6167C + 2.367D - 57926A^2 - 0.0151C^2 - 0.1023D^2 - 0.0247AC - 0.08AD - 0.0081CD$$

The coefficients of the model as determined by multiple linear regression analysis and ANOVA test are presented in Table 3. Quadratic model has a very low probability value ($P < 0.05$), representing a very high implication for the regression model. Similarly, the coefficient of correlation (R^2) for fatty acid production was

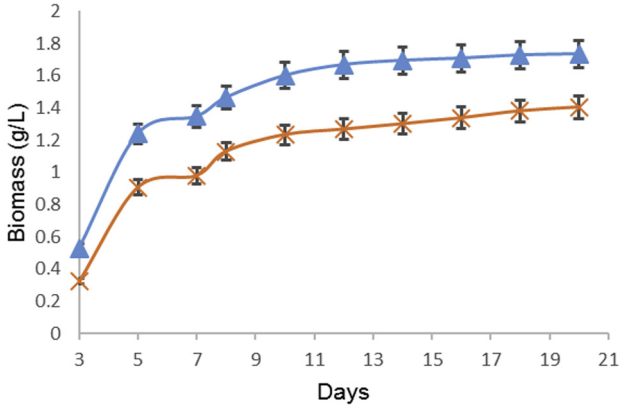


Fig. 1. Growth curve of *P. chrysogenum* in grease and grease free media. Line with triangles depicts the *P. chrysogenum* growth curve in czapek-dox media and with cross shows the growth curve in presence of grease.

Table 2
Experimental design of response surface methodology using four different variables in central composite design showing fatty acid release and lipase production by *P. chrysogenum*.

Run	PtType	Blocks	$FeCl_2$ (mM)	Culture amount (Spores/ml)	Time (days)	Grease (g)	Fatty acid*	
							O**	P#
1	1	1	1.625	7.5×10^{11}	23.25	12.5	3.3	3.39250
2	1	1	0.875	2.5×10^{11}	9.75	7.5	3.2	3.10917
3	1	1	0.875	7.5×10^{11}	9.75	12.5	4.4	4.39250
4	0	1	1.250	5.0×10^{11}	16.50	10.0	6.5	6.54667
5	1	1	0.875	2.5×10^{11}	23.25	7.5	3.5	3.47583
6	1	1	0.875	7.5×10^{11}	23.25	12.5	4.0	4.00917
7	1	1	0.875	7.5×10^{11}	23.25	7.5	4.3	4.37583
8	1	1	1.625	7.5×10^{11}	23.25	7.5	4.1	4.05917
9	1	1	1.625	7.5×10^{11}	9.75	12.5	4.0	4.02583
10	1	1	1.625	2.5×10^{11}	9.75	7.5	3.7	3.69250
11	1	1	1.625	7.5×10^{11}	9.75	7.5	4.0	4.14250
12	0	1	1.250	5.0×10^{11}	16.50	10.0	6.6	6.54667
13	1	1	0.875	7.5×10^{11}	9.75	7.5	4.2	4.20917
14	1	1	0.875	2.5×10^{11}	9.75	12.5	3.7	3.74250
15	0	1	1.250	5.0×10^{11}	16.50	10.0	6.5	6.54667
16	0	1	1.250	5.0×10^{11}	16.50	10.0	6.6	6.54667
17	1	1	1.625	2.5×10^{11}	9.75	12.5	4.1	4.02583
18	1	1	1.625	2.5×10^{11}	23.25	12.5	3.6	3.59250
19	1	1	0.875	2.5×10^{11}	23.25	12.5	3.7	3.55917
20	1	1	1.625	2.5×10^{11}	23.25	7.5	3.8	3.80917
21	0	2	1.250	5.0×10^{11}	16.50	10.0	6.3	6.30667
22	−1	2	1.250	1.0×10^{7}	16.50	10.0	3.5	3.64833
23	−1	2	2.000	5.0×10^{11}	16.50	10.0	3.1	3.03167
24	−1	2	1.250	5.0×10^{11}	16.50	15.0	3.7	3.73167
25	−1	2	1.250	5.0×10^{11}	30.00	10.0	3.4	3.41500
26	0	2	1.250	5.0×10^{11}	16.50	10.0	6.3	6.30667
27	−1	2	1.250	5.0×10^{11}	16.50	5.0	3.8	3.76500
28	−1	2	1.250	1.0×10^{12}	16.50	10.0	4.7	4.54833
29	−1	2	0.500	5.0×10^{11}	16.50	10.0	3.0	3.06500
30	−1	2	1.250	5.0×10^{11}	3.00	10.0	3.7	3.68167

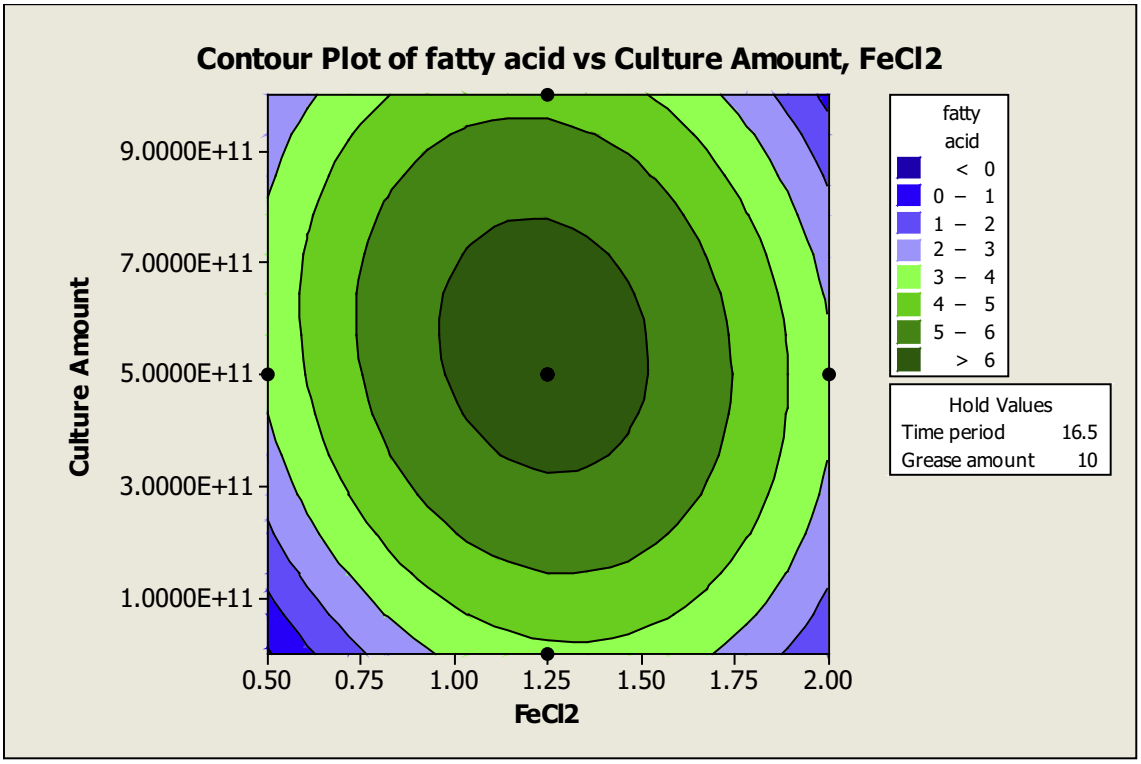
* Fatty acid was estimated in terms of fatty acid value. O** is observed value and P# is predicted value.

Table 3
ANOVA values of four factorial central composite design (CCD) used for the production of fatty acid.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Blocks	1	0.3840	0.3840	0.3840	38.35	0.000
Regression	14	38.4828	38.4828	2.7488	274.55	0.000
Linear	4	1.3250	19.2174	4.8044	479.86	0.000
$FeCl_2$	1	0.0017	11.6999	11.6999	1168.60	0.000
Culture amount	1	1.2150	4.6727	4.6727	466.71	0.000
Time period	1	0.1067	6.0648	6.0648	605.76	0.000
Grease amount	1	0.0017	9.4884	9.4884	947.71	0.000
Square	4	36.0378	36.0378	9.0095	899.87	0.000
$FeCl_2$ * $FeCl_2$	1	10.5609	18.2001	18.2001	1817.85	0.000
Culture amount * Culture amount	1	4.2367	8.3601	8.3601	835.02	0.000
Time period * Time period	1	10.0201	13.0430	13.0430	1302.75	0.000
Grease amount * Grease amount	1	11.2201	11.2201	11.2201	1120.68	0.000
Interaction	6	1.1200	1.1200	0.1867	18.64	0.000
$FeCl_2$ * Culture amount	1	0.4225	0.4225	0.4225	42.20	0.000
$FeCl_2$ * Time period	1	0.0625	0.0625	0.0625	6.24	0.026
$FeCl_2$ * Grease amount	1	0.0900	0.0900	0.0900	8.99	0.010
Culture amount * Time period	1	0.0400	0.0400	0.0400	4.00	0.065
Culture amount * Grease amount	1	0.2025	0.2025	0.2025	20.23	0.001
Time period * Grease amount	1	0.3025	0.3025	0.3025	30.21	0.000
Residual error	14	0.1402	0.1402	0.0100		
Lack-of-fit	10	0.1302	0.1302	0.0130	5.21	0.063
Pure error	4	0.0100	0.0100	0.0025		
Total	29	39.0070				

>0.75 indicating suitability of the model. The adjusted R^2 value for fatty acid production was 0.992. It helps to correct the sample size, the number of terms in the model and a high significance of the model. The coefficient of determination (R^2) was calculated to be 0.996 which implied that approximately 99% of experimental data of the fatty acid production was compatible with the data predicted by the model. The model was then subjected to optimization using *"Response optimizer"* of MINITAB 16 and the optimized fatty acid production was 6.2 mg/g. All the above results suggested the good adequacy of the regression model.

Response surface plots of all the four factors are helpful in understanding the individual and interactive effect of these factors



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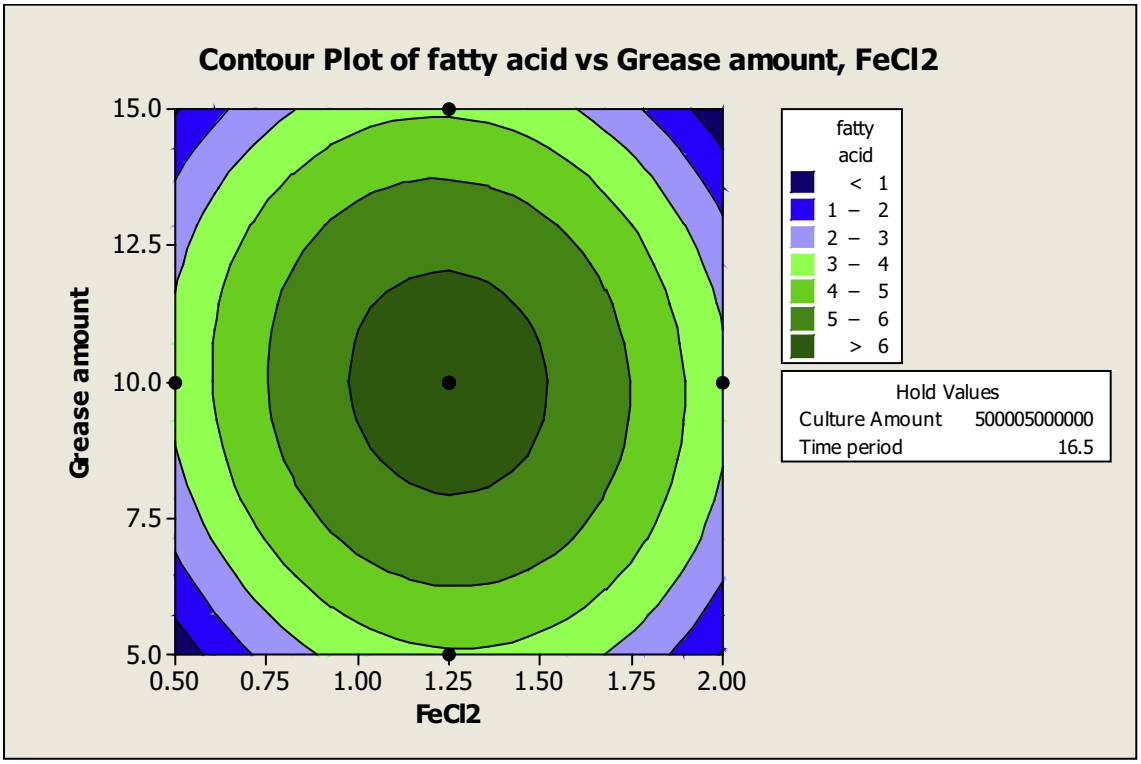
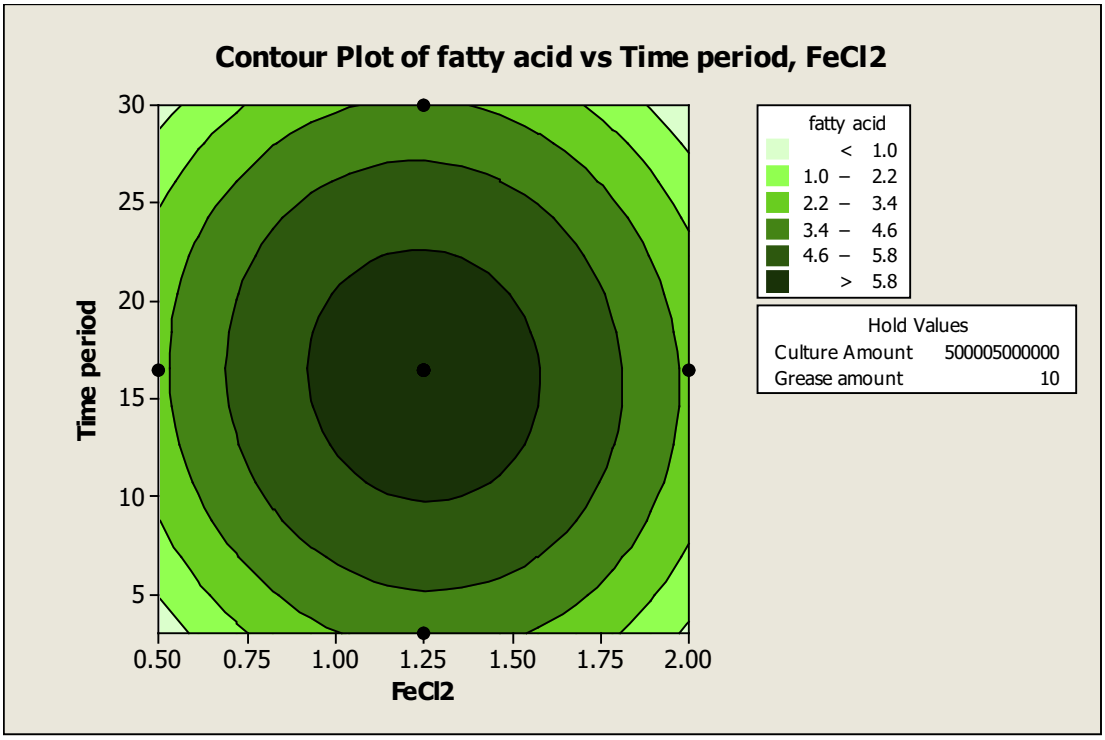


b

Fig. 2. Contour plots for fatty acid production; (a) Interaction of culture amount and $FeCl_2$; (b) Interaction of grease amount and $FeCl_2$; (c) Interaction of time period and $FeCl_2$; (d) Interaction of culture amount and time period: (e) Interaction of culture amount and grease amount; (f) Interaction of time period and grease amount.



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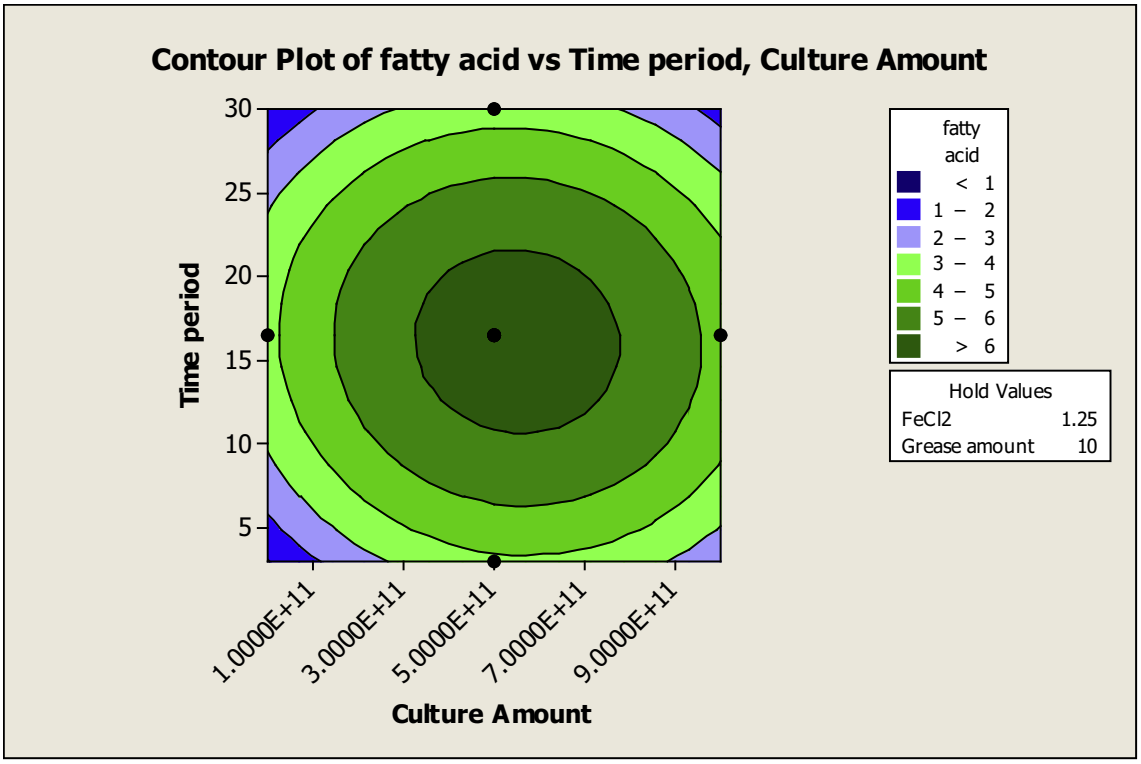
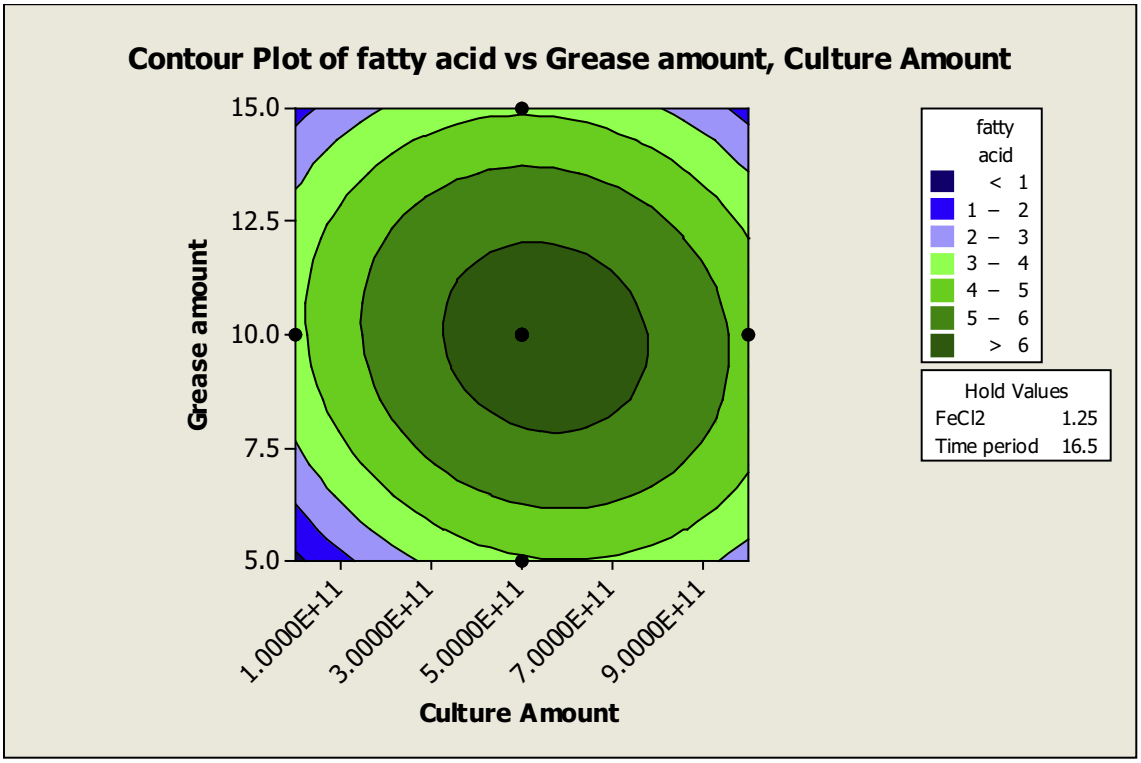


d

Fig. 2 (*continued*)

to determine the optimum level of fatty acid and lipase. The response contour plots for fatty acid production are shown in Fig 2(a–f). The shape of contour plot indicates the nature and extent of interaction. The prominent interactions of $FeCl_2$ (A), culture amount (B), time period (C), grease amount (D) and fatty acid (Y) are shown by the elliptical nature of the contour plots. From the



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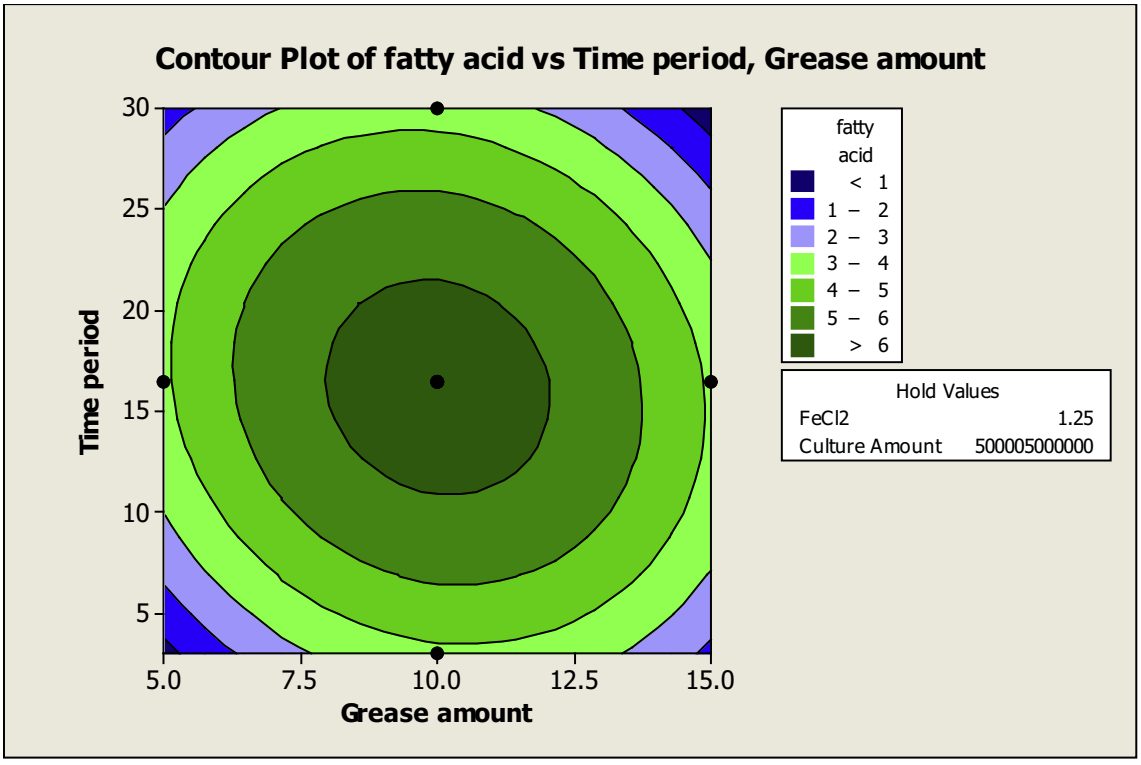

f

Fig. 2 (*continued*)

plots shown in Fig 2(a–f), it can be deduced that fatty acid production is maximum, when the $FeCl_2$ is between 0.5 mM and 2.0 mM, inoculum 1×10^{11} spore/ml–1×10^{12} spore/ml, grease amount is 5.0 g–15.0 g and time period 3 days–30 days. So the optimized values of the variables for maximum fatty acid production should lie in the above range.

Finally optimized conditions for highest fatty acid production were $FeCl_2$ 1.25 mM, culture amount 5×10^{11} spores/ml, time period 16 days and grease amount 10.0 g using wheat bran with 10.0 ml czapek-dox medium in solid state fermentation. However, till date no medium optimization has been performed for production of fatty acid from grease waste. Under optimized conditions the fatty acid value was 6.6 mg/g, which was in close agreement with the predicted value confirming the authenticity of model.

The pre-treatment of grease with optimized lipase followed *P. chrysogenum* by fermentation under standardized conditions showed no visible grease plaque in SSF medium. The same experiment was scaled up on tray fermentation by using one kg grease. The degraded grease contained fatty acid, ketone, alkane, silica compound and other compounds such as biphenyl, ethylene etc, (Table S1). However, predominant fatty acids were palmitic acid (C16) and oleic acid (C18), among them palmitic acid was highest. Therefore, an attempt was made to purify C16 by crystallization method (Rubin and Rubin, 1988).

The quantification of fatty acid was done by GCMS analysis by comparing with palmitic acid standard. One gram of untreated grease waste had 1.06 mg of palmitate. However, the degraded grease waste produced 5.3 mg palmitic acid from 1.0 g of grease and after purification 2.8 mg of purified palmitic acid was recovered shown in Fig. S4(a–d).

4. Conclusion

Grease waste is a serious threat to the environment and very difficult to remediate. Present study provides an effective way to bio-remediate it with the combination of lipase pre-treatment and *P. chrysogenum* fermentation. The recovery of fatty acid from degraded grease waste would be an added advantage. The results open up the new strategy for the effective grease management.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2016.12.006.

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