Use of Ghee Residue As A Substrate For Microbial Lipase Production

Janhavi Sahasrabudhe, Shantanu Palshikar, Arun Goja,Chandrashekhar Kulkarni

ABSTRACT :- Microbial lipases are commercially important and can be produced by Solid State Fermentation (SSF) as well as by Submerged Liquid Fermentation (SLF). In this study ghee residue, which is one of the largest by-product of dairy industry was used as a substrate. The main objective of the study was to determine whether ghee residue could be used as a substrate over the conventional synthetic substrate for efficient lipase production. Two organisms were used for lipase production, one of them was a known lipase producer – Bacillus subtilis (NCIM 2063) and the other organism belonging to the Proteus spp, was isolated from the soil contaminated with oil. The production of lipase was significantly higher in SSF (ghee residue as substrate) than in SLF (tributyrin oil as substrate). The lipase yield was observed to be 41.27 U/mg by Proteus spp and 35.93 U/mg by B. subtilis in SSF using ghee residue as substrate. In SLF using Tributyrin as substrate, the lipase production by Proteus spp was 20.78 U/mg and 28.63 U/mg by B. subtilis.

Keywords:- Lipase, ghee residue, Tributyrin oil, SSF, SLF

1. INTRODUCTION

Lipases are triacyl glycerol acylhydrolases which hydrolyzes fats and oils in steps into the substituted glycerides and fatty acids. They carry out esterification and trans-esterification reactions only at oil-water interface. Lipases belong to the class of Serine hydrolases, which do not require any cofactors [1]; therefore they have been of great interest to the researchers. Lipases occur widely in nature; however microbial lipases are preferred for commercial application because of their multifold properties and easy extraction procedure [1]. Lipases are widely used in the processing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture, production of cosmetics, and pharmaceutical[2]. But, high cost of production proves to be a major problem in the industries. To overcome this problem, attempts are being made to use waste as a raw material for lipase production. Research has been carried out for making use of various oil cakes such as groundnut, coconut, mustard, castor etc. Also, agricultural by- products such as wheat bran, rice husk, almond meal, cotton seed meal, mustard meal etc, have been used as a substrate for lipase production[3]. Thus, with its wide range of industrial applications it is desirable to look out for an alternative economical substrate for its industrial production. Attempts are being made to utilise the dairy by-products for lipase production. As most of them have high nutritive value, they are usually channelised to food industries [4].

In the present study, Ghee residue (one of the largest by-product of dairy industry) was used for enzyme production. Till today, the Ghee residue has been used in food industries for making sweets, bakery products, as a flavour enhancer etc[4]. Most of the times, it is considered as a waste at the industry level as well as at the domestic level. As ghee residue contains 32–70% of fats, it could be used as a poential substrate for production of lipase [5]. In the present study, solid state fermentation was used for bacterial lipase production with ghee residue as a substrate [6]. The project also encompasses a comparative yield of lipase obtained by SSF using ghee residue and that obtained by SLF using a synthetic substrate like Tributyrin.

2. MATERIALS AND METHODS

2.1 Sample collection

Soil sample polluted with engine oil was collected from the parking area at Shivajinagar Bus stand, Pune, Maharashtra, India. The collected sample was transferred to a sterile screw-capped bottle and taken immediately to the laboratory and maintained at 40C for further studies.

2.2 Bacterial isolation and identification

1 gm of soil sample was mixed with 9 ml sterile saline. Serial dilutions were performed and 0.1 ml was plated on Tributyrin agar plates. The appearance of zone of clearance around the colony was indicative of the lipolytic activity [7]. The colony showing significantly large zone of clearance was selected for further studies [8]. The isolate was then identified using biochemical tests as described in Bergey's manual of Determinative Bacteriology. The lipase production by this strain was compared to standard strain of *Bacillus subtilis* (NCIM 2063) *[9].*

2.3 Fermentations

a) Inoculum development

The isolated bacteria were enriched in a media having composition of (g/l) – Peptone 2.00g, Diammonium hydrogen phosphate 1.00g, Sodium chloride 2.50g, Magnesium sulphate heptahydrate 0.40g, Calcium chloride 0.40g; Final pH 7.0±0.2 (at 25°C) [10]. A loop full of the standard as well as the newly isolated cultures were inoculated into the enrichment medium and incubated for 24 hrs. After 24 hrs of incubation, the O.D. was checked at 620nm and the CFU count was taken. Approximately 5% of the inoculum was inoculated in the fermentation medium [11].

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b) Solid State Fermentation (SSF)

For SSF, 10 gm of ghee residue was over layered with 50 ml of the fermentation medium [composition in g/l– Peptone 2.00g, Diammonium hydrogen phosphate 1.00g, Sodium chloride 2.50g, Magnesium sulphate heptahydrate 0.40g, Calcium chloride 0.40g, 1–2 drops of Tween 80; Final pH 7.0±0.2 (at 25°C)] [10]. Three sets of two screw capped bottles, containing fermentation medium were inoculated with enriched cultures of *B. subtilis* and *Proteus spp* respectively. The bottles were incubated at static conditions at 37^0C for 5 days.

c) Submerged Liquid Fermentation (SLF)

Three sets of two 250 ml Erlenmeyer flasks, each containing 100 ml of the fermentation medium [composition in g/l– Peptone 2.00g, Diammonium hydrogen phosphate 1.00g, Sodium chloride 2.50g, Magnesium sulphate heptahydrate 0.40g, Calcium chloride 0.40g, 0.5 ml Tween 80 and Tributyrin (Carbon source) 1% of the fermentation medium; Final pH 7.0±0.2 (at 25°C)] [10]; were inoculated with enriched cultures of *B. subtilis* and *Proteus sp.* respectively. The flasks were incubated at 37^0C rotary shaker for 48 hrs.

2.4 Extraction of the extracellular lipase

The extraction of crude lipase was carried out by centrifugation method [12]. As lipase is an extracellular enzyme, the supernatant obtained was used as crude enzyme. Different procedure of extraction was followed for SSF and SLF respectively. The SSF was initially filtered by an assembly made up of a funnel and a muslin cloth and the Filtrate obtained was centrifuge [13], while the SLF was used directly centrifuged.

2.5 Estimation

The crude extracellular lipase obtained after centrifugation was estimated by titrimetric method. Titrimetric method measures the rate of neutralization of sodium hydroxide by the released free fatty acid as a function of time [1]. In this reaction phenolphthalein was used as pH indicator [3]. The lipase activity was calculated using the following formula [14] –

$$\text{Activity} = \frac{\text{Vol. NaOH consumed } \mathbf{X} \text{ N (NaOH) } \mathbf{X} \text{ Vol. of reaction mixture titrated } \mathbf{X} \text{ 1000}}{\text{Time of incubation}}$$

Where,
Vol. of NaOH consumed = Burette reading of Test – Burette reading of Blank
N (NaOH) = Normality of NaOH
1000 = Conversion factor of moles to µmoles

The standardized assay conditions are as follows–

Submerged Liquid Fermentation (SLF)		
Parameter	***B. subtilis***	***Proteus sp***
E: S ratio	2:1	2:1
Time of incubation	30 min	30 min
Substrate Concentration	5%	5%
Temperature	37^0C	4^0C
pH	6.2	6.2

Solid State Fermentation (SSF)		
Parameter	***B. subtilis***	***Proteus sp***
E: S ratio	1:2	1:1
Time of incubation	10 min	10 min
Substrate Concentration	5%	5%
Temperature	4^0C	37^0C
pH	6.2	6.2

2.6 Protein estimation by Folin-Lowry's method

The total extacellular protein was obtained from culture filtrate was estimated by lowry's method. The phenolic group of tyrosine and trytophan residues in a protein produces a blue purple color complex, with maximum absorption in the region of 660 nm wavelength, with Folin-Ciocalteau reagent. This method is sensitive to even low concentrations of proteins and is probably the most widely used for protein assay [15].

3. RESULTS AND DISCUSSION

3.1 Isolation and identification of isolate

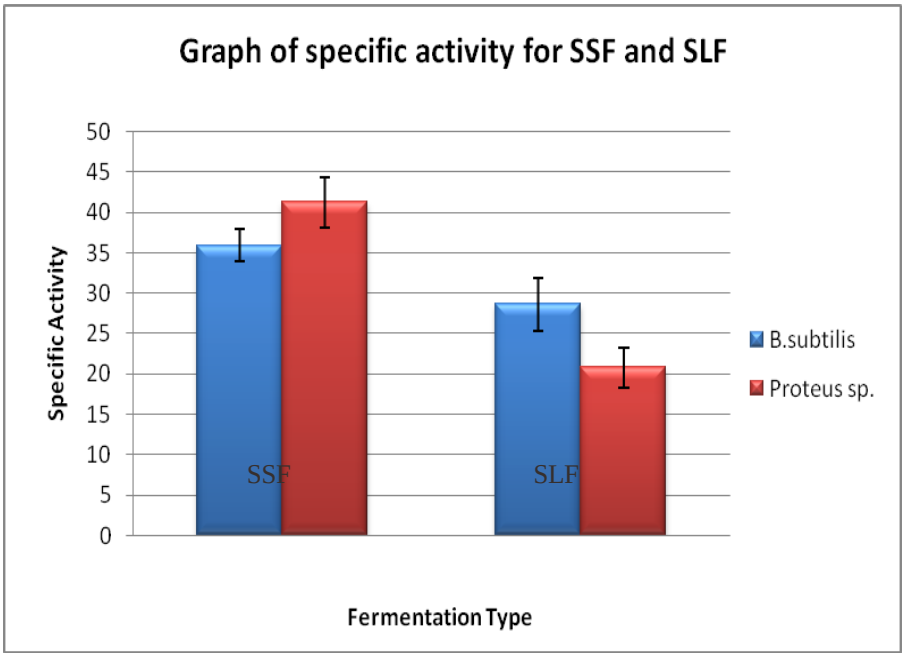
After studying the growth characteristics, and biochemical tests according to the Bergey's Manual of Determinative Bacteriology, it can be stated that the unknown organism isolated from soil sample polluted with engine oil belongs to the *Proteus spp*.

3.2 Fermentations

The comparative study of lipase production was done to check, whether Solid State Fermentation (SSF) or Submerged Liquid Fermentation (SLF) gives good yield. The average specific activity of the lipase produced in SSF and SLF were plotted and compared. The results obtained for two organisms were as follows:

Solid State Fermentation (SSF)								
Organism	**Fermentation**	**Average activity U/ml**	**Total activity U**	**Average protein conc. mg/ml**	**Total protein mg**	**Specific activity U/mg**	**Mean**	**SD**
B. subtilis	1	158.10	790.50	4.30	21.50	36.77	35.93	1.96
	2	164.30	821.50	4.40	22.00	37.34		
	3	155.00	775.00	4.60	23.00	33.69		
Proteus spp	1	151.90	759.50	3.40	17.00	44.68	41.27	3.30
	2	151.90	759.50	3.70	18.50	41.05		
	3	133.30	666.50	3.50	17.50	38.08		

Submerged Liquid Fermentation (SLF)								
Organism	**Fermentation**	**Average activity U/ml**	**Total activity U**	**Average protein conc. mg/ml**	**Total protein mg**	**Specific activity U/mg**	**Mean**	**SD**
B. subtilis	1	17.56	87.80	0.70	3.50	25.08	28.63	3.12
	2	23.86	119.30	0.80	4.00	29.82		
	3	18.59	92.95	0.60	3.00	30.98		
Proteus spp	1	25.83	129.15	1.20	6.00	21.52	20.78	2.41
	2	22.73	113.65	1.00	5.00	22.73		
	3	21.70	108.50	1.20	6.00	18.08		



From the graph it can be observed that the yield of lipase was found more in SSF than in SLF. Thus, it can be stated that ghee residue as a substrate for SSF gives better yield in comparison to conventional SLF. Ghee residue is produced in large quantity by dairy industries and most of the ghee residue goes to waste [4], which can be used as a substrate for large scale production of lipase enzyme. Also the production cost would be reduced for the lipase enzyme, which finds applications in wide range of industries.

4. CONCLUSION

Ghee residue proves to be a better substrate for lipase production in comparison to synthetic counterpart. Also, SSF should be preferably employed for lipase production as it gives superior yields, requires no complex machinery and includes economical use of space. Use of Ghee residue for commercial lipase production would have the advantages like less investment in raw material, efficient use of a dairy waste.

REFERENCES:

[1] P.K. Ghosh, R.K.Saxena, Rani Gupta, R.P. Yadav, Sheba Davidson (1996) Microbial lipases: production and applications; Science Progress 79 (2), 119–157

[2] Rohit Sharma, Yusuf Chistib, Uttam Chand Banerjeea (2001) Production, purification, applications of lipases. Biotechnology Advances 19 627–662

[3] Tehreema iftikhar, Mubashir niaz, M. Anjum Zia, Rukhsana Jabeen3 and Muhammad Nauman Aftab (2011) Biosynthesis of extracellular lipases by *bacillus sp.* (mblb-3) in relation to the nutritional conditions 43(5): 2587-2591

[4] A.Y.Tamine. Dairy Fats and related Products (2009) Blackwell Publishing Ltd

[5] M.P. Arumugam, K. Vedhanayagam, K.A. Doraisamy, D. Narahari (1989). Chemical composition and nutritive value of ghee residue for chickens *Animal Feed Science and Technology* Volume 26, Issues 1–2, Pages 119–128

[6] Ashok Pandey, P. Selvakumar, Carlos R. Soccol and Poonam Nigam (1999) Solid state fermentation for the production of industrial enzymes Current Science, 77 (1). pp. 149-162

[7] E. Sirisha, N. Rajasekar and M. Lakshmi Narasu (2010) Isolation and Optimization of Lipase Producing Bacteria from Oil Contaminated Soils. Advances in Biological Research 4 (5): 249-252

[8] Anderson J A; (1939). The use of tributyrin agar in dairy bacteriology. Ber. 3. Int. Mikrobiol. Kongress, 3; 726-728

[9] Manoj Singh, Kumar Saurav, Neha Srivastava and Krishnan Kannabiran (2010) Lipase Production by *Bacillus subtilis OCR-4* in Solid State Fermentation Using Ground Nut Oil Cakes as Substrate Current Research Journal of Biological Sciences 2(4): 241-245

[10] Mobarak-Qamsari E, Kasra-Kermanshahi R, Moosavi-nejad Z. (*2011)***.** Isolation and identification of a novel, lipase-producing bacterium, *Pseudomnas aeruginosa* KM110 Indian Journal of Microbiology *Volume 3 Number 2, 92-98*

[11] William R. Gibbons and Carl A. Westby; (1986) Effects of inoculum size on Solid-phase Fermentation of Fodder Beets for Fuel Ethanol Production; Applied and Environmental Microbiology Vol. 52(4) 960-962.

[12] G.K.Sahu, M.Martin (2011) Optimization of growth conditions for the productions of extracellular lipase by bacterial strains from dairy industry effluents. Biotechnology Bioinformatics Bioengineering 1(3):305-311.

[13] Gwen Falony1, Janny Coca Armas1, Julio C. Dustet Mendoza1 and José L. Martínez Hernández. (2006) Production of Extracellular Lipase from *Aspergillus niger* by Solid-State Fermentation Food technology and Biotechnology 44 (2) 235–240

[14] T. Selva Mohan, A. Palavesam and G. Immanvel (2008) Isolation and characterization of lipase-producing *Bacillus strains* from oil mill waste African Journal of Biotechnology Vol. 7 (15), pp. 2728-2735

[15] Oliver H. Lowry, Nira J. Rosebrough, A. Lewis Farr, Rose T. Randau; (1951) Protein measurement with the Folin Phenol Reagent. The Journal of Biological Chemistry.193, 265-275.