International Journal of Advanced Research in Biological Sciences
ISSN : 2348-8069
www.ijarbs.com

Research Article

Production and characterization of lipase from zygomycetous fungi *Rhizopus japonicas*

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Abstract

The present study has been carried out to find out the lipase enzyme production by the fungus, *Rhizopus japonicas*. In commonly available natural waste such as, Caster oil cake, Gingilly oil cake and groundnut oil cake collected from oil lotteries. The oil cake materials were diluted in the proportion of 11 grams of cakes dissolved in 100ml of water for the experimental work. Among the three natural waste employed, the groundnut oil extract showed highest lipase activity at 48hrs of incubation. Hence, groundnut oil waste was then preferred for further studies to design the production medium by supplementing different carbon, nitrogen, micro and macro nutrients, inducer compounds and also by substituting different with all these combination of gum Arabic with olive oil was also supplemented. The experiments were also carried out at different pH (2-9) and temperature (0ºC-60 ºC) levels. The interesting observation in this line revealed that, in Groundnut oil when different above-mentioned sources employed, the nitrogenous source like peptone at 2.5% level, at 7.0pH under 38 ºC and at 48 hours of incubation exhibited maximum lipase production using the fungus *Rhizopus japonicas* When the enzyme activity is analyzed or its stability at different pH (6.3-9.3) and temperature (0ºC-60 ºC), using Groundnut oil extract was done in the initial phase of study, showed maximum stability at pH 7.3 and 40ºC respectively.

Keywords: lipase enzyme, , *Rhizopus japonicas*, Groundnut oil extract.

Introduction

Enzymes are biocatalyst synthesized by living systems, which are important in synthetic as well as degradative processes. The study of enzymes is an important area, because it exists just on the borderline where the biological and physical sciences meet. Life depends on the complex network of chemical reactions carried out by specific enzymes may have far reaching consequences for the living organism. (Dixon and Web, 1964).

Enzymes as systems

Most of the industrial enzymes are of microbial origin. The development during the last 25 years has taken place primarily within this group, presumable because the variation in microbial enzymes is wide and because microorganisms can be easily and rapidly cultivated thus forming an unlimited enzyme source. (Alfored *et al*., 1964).

The preparation and properties of various microbial enzymes, described in various publications form an inspiring catalog of technological possibilities. The present primary concern of enzyme manufacture is safety, quality, reliability and economy. Till date approximately 3000 enzymes have been characterized and 300 of them are available commercially including those in small quantities for analytical and research development purpose. Only 30 enzymes are available in huge quantities for industrial application, enzymes have found numerous applications in food, medical and chemical processing and pharmaceutical industries.

The commercial use of enzymes in the process of pharmaceutical industries has so far limited with a possible exception of penicillin amylase for the synthesize of penicillin derivatives. While a number of processes can use enzymes, their large-scale availability with reasonable cost has often limited scope. However, significant potential, organic synthesis, drugs and pharmaceuticals and polymer synthesis is emerging in near future. This would necessitate large-scale enzyme production facilities (Macrae and Hammard, 1985).

Microbial sources of enzymes

Microbial cells produce a variety of enzymes and help in microbial growth and respiration including other cellular activities. At times, these enzymes may themselves become fermentation products, so that one of them is specifically interested in obtaining high-level activity of enzymes (Bell *et al*., 1972).

In general, enzymes produced by microorganisms are of two types. There are extra cellular and endocellular enzyme. Extra cellular enzymes, which include most of the enzymes, produced by microbial fermentation for commercial usage, are also produced by microbial cells, but in addition are liberated to the fermentation medium so that the enzyme hydrolytically attack and degrade. Such enzymes are produced when substances are too large or insoluble to pass through the microbial cell wall. Examples of exocellular enzymes are amylases attacking starch and protease attacking protein. An endocellular enzyme is produced within the cell or at the cytoplasm membrane and normally does not find its way in to the fermentation medium surrounding the cell.

Industrial production of microbial enzymes

The first stage of the development of a fermentation process is to isolate a strain with the potential capability of producing the particular product (enzymes) in commercial yields. It involves designing rapid screening procedures capable of testing a large number of strains easily for enzyme production. Screening and selection of an organism for industrial exploitation should consider the following criteria.

1. The organism must produce the enzyme in good yield in a relatively short time and ideally in-submerged culture.
2. The organism must grow and produce enzyme on inexpensive readily available nutrients.
3. The organism should be non-pathogenic and unrelated phylogenetically to a pathogen.
4. It should be easily removable from the fermentation liquor.
5. The enzyme produced should preferably be extra cellular can be isolated easily from the fermentation liquor
6. Ideally the organism should not produce toxins or other biologically active materials.
7. The organism should be genetically stable and not susceptible to bacteriophages.

Generally approaches to strain isolation have been discussed (Bulock *et al*., 1982). Having isolated the best strain further increase in production capacity can be achieved by optimization of the medium. Further improvement of the potential of the organism can be made mutation selection programmes and other genetic manipulation techniques.

Industrial application of enzymes

The use of enzymes in food preservation and processing predates modern civilization. Fermentation of common substrates such as fruits, vegetables, meat and ilk provide a diverse array of food in the human diet. Beer, wine, pickles, sausage, salami, yogurts, cheese and buttermilk are all fermented products. Irrespective of their origin, these fermented food products are, in fact, result of the enzymatic modification of constituents in the substrate.

Microbial lipases

Lipases are widely distributed among yeast, fungi and bacteria. Short chain triglycerides are also very good substrates for lipases. Enzymes such as proteases and amylases have dominated the world market owing to their hydrolytic reactions for proteins and carbohydrates. However with the realization of the biocatalytic potential of microbial lipases in both aqueous and non-aqueous media in the last one and a half decades, industrial fronts have shifted towards utilizing these enzymes for a variety for reactions of immense importance. It is in the last decade that lipases have gained importance to a certain extent over proteases and amylases, especially in the area of organic synthesis. The regioselective nature of lipases have been utilized for the resolution of chiral drugs,

fat modification, synthesize of cocoa butter constituents, biofuels and for synthesize of personal care products and flavor enhancers. Thus lipases are today the enzyme choice for organic chemist, pharmacists, biophysicists, biochemical and process engineers, biotechnologist, microbiologist and biochemist.

Fungal lipases

Fungal lipases have been studied since 1950s. Lawrence (1967) Brockerhoff and Jenson (1974) have presented its comprehensive reviews. These lipases are being exploited due to their low cost of extractions, thermal and pH stability, substrate specificity, and activity in organic solvents. The chief producers of commercial lipases are *Candida cylindracea, Humicola lanuginose, R.delemar, R.japonicus, R.niveus and R.ryzae* (Godfredson, 1990).

Industrial application of lipases

Lipases have wide applications as a good substitute for classical organic techniques in the selective transformation of complex molecules. The employment of lipases in the above reactions can reduce side reactions and easy the separation of molecules (Ashokpandey *et al*., 1999). In the present day industry, lipases have made their potential realized owing to their involvement in various industrial reactions either in aqueous or organic systems, depending on their specificity (John and Abraham, 1990; Kotting and Kibl, 1994).

Applications of Lipase enzyme

Lipases in food industry
Lipases as biosensor
Lipases in biomedical application
Lipases in leather industry
Lipases in pesticide
Lipases in environmental management
Lipases in detergents
Lipases in dairy industry
Lipases in oleochemical industry
Lipases in synthesis of triglycerides
Lipases in synthesis of surfactants
Lipases in synthesize of ingredients for personal care products
Lipases in pharmaceuticals and agro chemicals
Lipases in polymer synthesize

Aim and objective

Keeping in mind the importance of industrial application of fungal lipases, the present study has been undertaken with the following objectives.To estimate the lipase enzyme production from the *Rhizopus japonicus* Using different natural wastes by introducing changes in

1. pH
2. Temperature
3. Carbon sources
4. Organic and inorganic nitrogen sources
5. Micro and macro nutrients level
6. Inoculum level and
7. Inducers level

Materials and Methods

Isolation of fungi from soil sample (Kamini *et al*., 1997). In order to initiate the proposed study, in the first phase, different fungi isolates were obtained from the soil sample by administering serial dilution technique. The selective medium for fungi, Czepak Dox and Rose Bengal Agar medium was used to isolate the fungi using 10^{-2}-10^{-7} dilutions, and poured into sterile petriplates. After plating, plates were incubated for 72 hours in room temperature at 28ºC.

Screening of lipase producing fungi (Kamini *et al*., 1997)

To screen the lipase producing from that of the isolates obtained in Czepadox agar plates, the colonies from these agar plates were then transferred to Tribytyrin agar, of which notably species *Rhizopus japonicas* and another fungus with remarkable morphological feature showed clear zone formation around the colonies to ensure with their ability .

Preparation of inoculation medium

In order to prepare the inoculam, the four days incubated fungal strain was taken. To these, 5ml sterile water was added; developed growth was scrapped with needle and transferred into 95 ml inoculation medium (simple nutrient broth) taken in 500ml Erlenmeyer flasks. These flasks were then incubated at 28ºC on rotary shaker at150rpm for 72 hrs. At this stage, 10% of inoculam was transferred to

the production medium incubated at 28ºC on rotary shaker at 150rpm for 72hrs.

Preparation of production medium

Cheaply available natural waste such as Groundnut, Gingilly, Caster wastes, were procured from a local oil mill, soaked in 50ml (5.5g waste) distilled water and autoclaved at 121ºC for 1 hr (Plate 5). After cooling, the liquid content of the cake was squeezed out using a muslin cloth by a hand mill. This extract was used as the raw substrate and was designated as medium M1. Different supplementations were made in the Groundnut oil to design the production media as given below. In all cases, the initial pH of the medium was maintained at 7.0.

Table: 1 Enzyme production media

Medium	Composition
M1	Ground nut waste extract
M2	M1+different carbon sources (1%)(CMC, Starch, Sucrose, Fructose, Glucose, Mannitol, Lactose, Glycerol) pH7
M3	M1+different organic/ inorganic nitrogen sources (1%)(Urea, Beef extract, Malt extract, Peptone, Yeast extract, Sodium nitrate, Ammonium nitrate, Ammonium chloride, Tryptone).
M4	M1+Various salts (1%)pH 7.0 (KCl, $MgSO_4$, $CaCl_2$, $ZnSO_4$, $MnSO_4$, $FeSO_4$, $MgCl_2$, $CuSO_4$
M5	M1+different inducers (1ml) (Coconut oil, Neem oil, Caster oil, Palm oil, sunflower oil, Groundnut oil, Gingillic oil, Olive oil)
M6	M1+ Inoculum concentrations (1%, 1.5%, 2%, 2.5% and 3.0%)
M7	M1+Olive oil (10%)+Gum Arabic (0.2-1%)
M8	M1+Different temperature (0ºC, 28ºC, 38ºC, 48ºC, 58ºC,68ºC)
M9	M1+ Different pH (2, 3, 4, 5, 6, 7, 8, 9)

Fermentation process (Shake – flask culture method):

After preparation of the production media 10% of inoculum was transferred to the production medium and incubated at 28ºC on a rotary shaker at 150rpm for 72hours.the flasks were removed at 24hr intervals, the mycelium was separated by filtration and filtrate was used for the enzyme assay.

Enzyme assay
Spectroscopy assay method

Lipase activity was assayed by the modified method Safarik (1999).

Determination of lipase activity:

For this, an aliquot of olive oil (20mg) was transferred into a test tube containing 2ml of phosphate buffer (pH 6.3) and 1ml of the sample was added to it. The mixture was vortexes for 15sec and incubated at 37ºC in water bath under static conditions for 30min. after stopping the reaction by adding 1ml of concentrated HCl and vortexing for 10sec, 3ml of benzene was added and after further vortexing for 90sec, the aqueous and organic phases were allowed to separate. From this 2ml benzene layer was withdrawn and transferred to a test tube containing 1ml aqueous solution of cupric acetate (5% concentration, pH adjusted to 6.2 using pyridine) and the mixture after vortexing for 90sec, was centrifuged at 5000rpm for 10 minutes at 5C to obtain a clear organic phase. The organic (Benzene) layer was removed and used to estimate the liberated free fatty acids by measuring the optical density (OD) against distilled water at 715nm using a spectrophotometer. One unit of lipase activity is equivalent to one µM free fatty acid liberated min^{-1} ml^{-1}, under the assay conditions and expressed μg^{-1} dry substrate (ds).

Estimation of protein (Lawry's method, 1951)

To 1ml of inoculated sample was taken and centrifuged at 10,000rpm for 20 minutes and supernatant was collected. To 0.1 ml of sample, 1.4 ml of 0.1N sodium hydroxide, 4.5ml of alkaline copper reagent were added and was allowed to stand for ten minutes. After incubation, 0.5ml of diluted folin's reagent was added to tubes with continuous shaking and allowed to stand for 30 minutes. The colour developed was read at 660nm. The amount of protein was expressed as mg/g.

Estimation of glucosamine (Sakurai *et al*., 1977)

The sample of varying concentration was taken for analysis. The solution were treated with 1ml of freshly prepared 2% acetyl acetone in 0.5M sodium carbonate in capped tubes and kept in boiling water bath for 15 minutes. After cooling in tap water, 5ml of 95% ethanol and 1ml of Ehrlich's reagent were added and mixed thoroughly. The purple red colour developed was read after 30 minutes at 530nm.

Estimation of total soluble sugar (Dubois, 1952)

2ml of the inoculation medium was taken and centrifuged. 1ml of the supernatant sample was taken in different test tubes. To each tube 4ml of the anthrone reagent was added along the side of the test tubes. A glass marble was placed on top of each tube to prevent loss of water by evaporation. The tubes were placed in a boiling water bath for 10 minutes. The tubes were then removed and cooled to room temperature in a water bath. A reagent blank was treated similarly. And the absorbency of the blue green solution was red at 625nm. The amount of sugars present in sample was calculated using standard curve prepared from glucose .

pH stability of the enzyme

The optimum pH of the enzyme was determined was using different pH buffer (i. e) using acetete (pH 4.6-5.5), Tris acetate (pH 6.0-7.0), Tris HCl (pH7.5-9.0) and Borate buffer (pH 9.0-15). The purified buffers was incubated in these 30 minutes, 60 minutes, 90 minutes and 120 minutes at 37ºC and then assayed for the residual activity for determining its pH stability.

Inoculated medium as centrifuged at 5000rpm and supernatant enzyme fluid were only taken. To the enzyme 1ml of 250mg Olive oil was to that different pH buffers were added in a series of test tubes and kept at 37ºC for 30 minutes the lipase producing strain were tested.

Thermal stability of the enzyme:

To determine the thermal stability of the lipase enzyme. The medium was centrifuged at 5000rpm and supernatant enzyme fluid was only taken. To the enzyme 1ml of 250mg olive oil was added and 2ml of Tris acetate buffer was added to make the pH 7. These preparation were taken in a series of test tubes and incubated at different temperature, from 30ºC to 90ºC and the incubation temperature various from 30minutes, 60minutes, 90 minutes and 120minutes etc. This test was done in lipase producing strain and the thermal stability of the enzyme was noted.

Growth curve

Groundnut oil waste extract was prepared in the medium. The fungal organism *Rhizopus japonicas* . was inoculated and kept in shaker at 28C. After 12 hours intervals O.D was measured at 600nm using spectrophotometer. The O.D value was measured up to 600nm using spectrophotometer. The O.D value was measured up to 72hours. Lipase activity was also analyzed in this sample.

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Results and Discussion

Plate assay

The fungal strain isolated from soil samples in our laboratory was identified as *Rhizopus japonicas*. Tributyrin was used as substrate for examining lipase production on Czapak dox agar plates. A halo zone of 5mm diameter around colony in the Tributyrin agar plates clearly indicated the production of lipase

In this study, the lipase production by the fungus *Rhizopus japonicas* was studied using cheap common natural waste (Oil cake) like Gingilly, Caster and Groundnut oil. The lipase activity was determined at 24h, 48h, and 72h intervals, the lipase activity increased up to 48h, after which it decreased (Table-2)

Table-2 shows the fermentation behavior of *Rhizopus japonicas* in Groundnut extract by shake flask culture.

The lipase activity at 24h, 48h, and 72h were10.43U/ml^{-1}, 12.23U/ml^{-1}, 09.0U/ml^{-1} respectively. Among the three durations, at 48h optimum lipase production was observed. The respective soluble sugar consumption at this stage was 8.05mg/ml, 09.53mg/ml, and 10.40mg/ml, as against the soluble protein content 20.70 mg/ml, 23.00 mg/ml, and 18.60 mg/ml, for 24 h, 48h and 72h durations.

There was very slight variation in the pH of media, which were 7.2, 7.3, and 7.4, at 24h, 48h, and 72h duration respectively. In view of high product titers in groundnut oil extract, further experiments were carried out with groundnut oil extract.

In order to determine the suitable carbon, nitrogen, inducers, salts etc, for optimum lipase production by *Rhizopus japonicas* Various nutrient sources like carbon, nitrogen, inducers, salts, phosphate and Gum Arabic were added individually to groundnut oil extract. More over the lipase activity has been checked at various temperature and pH level.

Effect of various carbon sources in Groundnut oil extract on lipase production by *Rhizopus japonicas* **in shake flask culture**

Several workers who use different carbon sources have reported an increased yield of alkaline lipase. In order to determine the suitable carbon source for maximum lipase production, different carbon sources were incorporated into the medium by replacing glucose. Maximum lipase production was observed with sucrose 26.06U/ml^{-1} and minimum with fructose 9.86 U/ml^{-1} (Table-3).

Effect of various Nitrogen sources in Groundnut oil on lipase production in *Rhizopus japonicas* **in shake flask culture**

To determine the suitable nitrogen source for maximum lipase production, different nitrogen sources were incorporated in to the medium, maximum lipase production (24.46U/ml^{-1}) was observed when peptone was used as the nitrogen source and minimum with ammonium nitrate (12.80 U/ml^{-1}), (Table-4)

Effect of various inducers in groundnut oil on lipase production by *Rhizopus japonicas* **in shake flask culture**

The lipase production by *Rhizopus japonicas* was studied in Groundnut oil by adding various inducers like coconut oil, neem oil, caster oil, Gingilly oil, Groundnut oil, palm oil, sunflower oil, and obtained data's confirmed that among the various types of inducers, olive oil was found to be most effective in inducing the lipase of *Aspergillus awamori* . Maximum lipase production was observed in olive oil 24.36U/ml^{-1} and minimum lipase production was observed in coconut oil 7.10U/ml^{-1} (Table-5)

Effect of various salts in groundnut oil on lipase production by *Rhizopus japonicas* **in shake flask culture**

In order to determine the suitable salts for maximum lipase production, different salts were incorporated into the medium. Maximum lipase production was observed with Ferrous Sulphate (Table- 6).

Effect of various inoculum concentrations in Groundnut oil on lipase production by *Rhizopus japonicas* **in shake culture**

The inoculum levels were also checked with various concenations to find out the correlation between inoculum level and lipase activity. Various inoculum levels (1% to 3%) were taken and tested. 10% inoculum promoted the higher rate of lipase yield (37.66U/ml^{-1}) (Table-7).

Effect of Gum Arabic in Groundnut oil on lipase production by *Rhizopus japonicas* **in shake flask culture**

The results of the studies on the effect of supplementation of Gum Arabic at different concentration (0.2%, 0.4%, 0.6%, 0.8%, and 1%) in the presence of groundnut oil is presented in (Table-8)

Effect of various pH in Groundnut oil on lipase production by *Rhizopus japonicas* **in shake flask culture**

Table -2 Fermentation behaviour of *Rhizopus japonicus* on Groundnut oil extract

Sl.No	Time (hours)	Lipase activity (mg/ml)	Soluble sugar (mg/ml)	Soluble protein (mg/ml)	pH
1	24	10.43	08.05	20.70	7.2
2	48	12.23	09.53	23.00	7.3
3	72	09.00	10.40	18.60	7.4

Table-3 Effect of various carbon sources on lipase production by *Rhizopus japonicus*

Sl.No	Carbon sources	24h	48h	72h
1	CMC	11.40	26.16	20.08
2	Dextrose	12.87	18.67	14.35
3	Fructose	9.86	22.26	14.97
4	Starch	9.89	28.34	23.21
5	Lactose	11.54	15.87	12.34
6	Maltose	15.87	26.72	21.35
7	Sucrose	13.68	26.06	23.23
8	Mannitol	12.7	21.54	13.54
9	Glycerol	11.65	15.76	17.10

Table- 4 Effect of various Nitrogen sources in Groundnut oil on lipase production by *Rhizopus japonicus*

Sl.No	Nitrogen sources	24hrs	48hrs	72hrs
1	Urea	11.10	17.63	14.70
2	Beef extract	16.60	17.10	17.36
3	Malt extract	07.16	21.06	18.30
4	Tryptophane	18.76	21.83	16.66
5	Yeast extract	12.13	18.06	12.40
6	Sodium nitrate	13.93	15.36	10.46
7	Ammonium nitrate	09.00	12.80	12.20
8	Ammonium chloride	07.30	14.06	13.03
9	Peptone	09.23	24.46	1 5.70

Table -5 Effect of different inducers in Groundnut oil on lipase production by *Rhizopus japonicus*

Sl.No	Inducers	28hrs	48hrs	72hrs
1	Coconut oil	07.10	14.63	15.00
2	Olive oil	13.60	25.10	10.36
3	Neem oil	09.16	14.06	13.30
4	Caster oil	09.76	16.83	09.66
5	Palm oil	10.56	24.36	20.40
6	Groundnut oil	11.93	22.36	05.46
7	Gingillic oil	09.00	17.80	12.20

Table-6Effect of various salts in Groundnut oil on lipase production by *Rhizopus japonicas*

Sl.No	Salts	24h.	48h.	72hr.
1	KCl	07.80	14.66	15.86
2	$MgSO_4$	08.23	15.36	17.60
3	$CaCl_2$	10.03	17.79	15.10
4	$ZnSO_4$	08.58	13.90	11.98
5	$MnSO_4$	11.55	15.24	08.21
6	$FeSO_4$	11.25	21.54	23.98
7	$MgCl_2$	12.76	13.56	08.67
8	$CuSO_4$	07.78	13.67	12.56

Table -7 Effect of Inoculum concentrations in Groundnut oil on lipase production by *Rhizopus japonicus* in shake flask culture

Sl.No	Inoculum conc.	24h.	48h.	72h.
1	1.0ml	14.06	19.43	15.70
2	1.5ml	19.50	22.51	21.83
3	2.0ml	22.46	27.96	2530
4	2.5ml	30.05	34.50	35.78
5	3.0ml	29.98	37.66	32.76

Table -8 Effect of Gum Arabic concentration in Groundnut oil on lipase production by Rhizopus japonicus in shake flask culture

Sl.No	Inoculum conc.	24h.	48h.	72h.
1	0.2%	08.06	15.43	12.70
2	0.4%	12.50	23.51	17.83
3	0.6%	10.46	20.96	15.30
4	0.8%	08.05	17.50	10.78
5	1.0%	07.84	14.66	10.76

Table-9Effect of different pH in Groundnut oil on lipase production by *Rhizopus japonicas*

Sl.No	pH	24h	48h	72h
1	2	4.10	7.46	5.26
2	3	4.50	7.72	7.73
3	4	6.20	7.20	6.72
4	5	7.23	10.26	11.35
5	6	10.30	13.80	9.52
6	7	12.78	14.26	12.26
7	8	10.24	13.90	10.40
8	9	9.40	11.32	8.40

Table-10 Effect of different temperature in Groundnut oil on lipase production by *Rhizopus japonicas*

Sl.No	Temperature	24h	48h	72h
1	0ºC	07.45	08.34	08.32
2	28 ºC	08.65	11.76	10.89
3	38 ºC	10.98	14.34	13.32
4	48 ºC	10.32	11.87	12.97
5	58 ºC	06.54	12.87	09.87
6	68 ºC	07.98	08.98	07.54

Table-11 Growth curve of ***Rhizopus japonicus***

Sl.No	Time	Growth rate	Lipase activity
1	12	0.125	07.35
2	24	0.390	12.00
3	36	0.716	15.80
4	48	0.820	16.40
5	60	0.690	11.95
6	72	0.515	08.20

Table-12 Thermostability of Lipase Enzyme

Sl.No	Temperature	30 minutes	1 hour	1.30 minutes	2 hours
1.	30 ºC	15.66	15.67	08.87	04.55
2	40 ºC	17.00	17.89	08.65	04.87
3	50 ºC	12.30	09.00	05.26	04.05
4	60 ºC	07.98	07.78	03.78	01.98
5	70 ºC	04.35	02.80	01.89	01.67
6	80 ºC	01.69	01.18	00.78	00.54
7	90 ºC	00.78	00.65	00.47	00.68

Table-13 pH stability of the lipase enzyme

Sl.No	pH	30 minutes	60 minutes	90 minutes	120 minutes
1	06.3	15.79	13.56	12.07	11.18
2	07.3	15.98	14.87	11.75	10.35
3	08.3	14.87	10.31	09.64	07.55
4	09.3	11.76	09.98	08.80	07.56
5	10.3	09.86	08.50	08.06	07.00
6	11.3	07.15	07.35	06.08	05.25
7	12.3	06.87	06.15	06.05	05.64
8	13.3	04.90	04.67	03.45	03.35

In order to determine the optimum pH for maximum lipase production, the organism was inoculated in media of varying pH. The enzyme functioned optimally at pH 7 after 48 h when compared with other pH values in waste extract (Table-9)Effect of various temperatures in Groundnut oil on lipase production by *Rhizopus japonicas* in shake flask culture. Optimum temperature for maximum lipase production was found to be 38ºC (Table-10).

Growth curve

Growth and enzyme production were determined at 12hours intervals. The cell growth increased up to 48h, after which it declined. In early log phase growth started and the maximum yield was obtained at late exponential growth phase. A fall in lipase production was observed in stationary and decline phase (Table - 11).

Enzyme characterization

Thermal stability

Thermal stability of lipase enzyme isolated from *Aspergillus awamori* was examined at various temperatures, (30 ºC, 40 ºC, 50 ºC, 60 ºC, 70 ºC, 80 ºC 90 ºC) along with various incubation periods, (30 minutes, 1 hour, 11/2 hours, 2 hours). The results are shown in (Table -12) enzyme was stable at 40 ºC for nearly 60 minutes.

pH stability

The optimum pH of the enzyme was determined using different pH buffer, Tris Acetate (pH 6.0-7.0), and Tris HCl (pH 7.5-9.0). The purified enzyme was incubated in these buffers for 30minutes, 60 minutes, 90 minutes and 120 minutes at 37 ºC and the residual activity was assayed for determining its pH activity. The enzyme was stable for 30 minutes at pH between 6.3 and 7.3. Similar results have been reported by Saxena *et al.,* (1999) for lipase from various fungi (Table-13).

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