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ORIGINAL RESEARCH ARTICLE

Utilization Of Mixed Agroindustrial By - Products For Alkaline Lipase Production From *Penicillim chrysogenum*

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ABSTRACT

The aim of this work was to produce lipases by solid-state fermentation (SSF) using agroindustrial residues as substrate. For a group of fourty fungal strains received from Karpagam Microbial Culture Collection Center (KMCCC) were screened for exogenous lipolytic activity. Among the evaluated strains, the *Penicillim chrysogenum* was selected by presenting the best enzymatic production. Optimization of fermentation conditions such as substrate, temperature, pH, and moisture content and incubation period for maximum lipase production was examined under solid state fermentation. A comparative study on the production of extra cellular lipase by solid-state fermentation using *Penicillim chrysogenum* with various substrates has been made. Wheat bran showed the highest enzyme activity of 460 units per gram of dry fermented substrates (U/ gds), but with the combination of wheat bran and black gram husk (1: 1) resulted in a higher enzyme yield of 650 U/ gds after 7 days of incubation with 40 % of initial moisture content of the substrate at pH 9.

Key-Words: Agroindustrial residues, *Penicillim chrysogenum*, solid-state fermentation, Optimization.

INTRODUCTION:

Lipase (EC 3.1.1.3) hydrolyses triglycerides to fatty acids and glycerol and other certain conditions catalyses the reverse reactions, forming glycerides from glycerol and fatty acids. Some lipases are also able to catalyze transesterification and enantioselective hydrolysis reactions [1]. In the last decades, the interest in microbial lipase production has incresed. Due to the versatility of the molecular structure and catalytic properties, these enzymes have potential application in different industrial sectors such as food, waste water treatment, cosmetics, oleochemical, pharmaceutics, detergents [2, 3]and in the fuel sector, which applies lipase as catalyst for synthesis of esters and for trans esterification of the oil for the production of biodiesel [4, 5].

Lipolytic enzymes such as lipases can be found in animals [6], plants [7] and microorganisms [8, 9, 10]. Fungi are certainly the best lipase sources and are preferably used for industrial applications, mainly by the food industry [10]. Lipases from fungi were used in diverse range of industries like detergents, pharmaceuticals, beverages, dairy, etc. which make them commercially important enzyme [11, 12]. Lipases produced by fungi are typically extracellular and therefore relatively easy to recover after the fermentation [13].

As in any application that demands high enzyme quantities, such as treatment of oily wastewaters and biodiesel production, lipase utilization depends on the reduction of its costs to become economically feasible [13]. Solid-state fermentation (SSF) represents an interesting alternative to produce industrial enzymes at lower costs [14, 15] due to the possibility of using agro-industrial residues as culture media. Solid wastes from the production of vegetable oils (oil cakes) have been widely used for the production of industrial enzymes, antibiotics, biopesticides, vitamins and other biochemical because they are excellent supports for microbial growth as well as interesting sources of nutrients, requiring low or no supplementation. Furthermore, they are inexpensive and plentiful in countries like Brazil [16]. Solid state fermentation holds tremendous

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potential for the production of enzymes [17]. Lipase was characterized with respect to the optimal temperature and pH as well as to its stability [18].

This paper deals with the screening of potential fungi strains for production of lipases. Various agro industrial waste residues were used as substrate for lipase production by *Penicillim chrysogenum* in solid-state fermentation.

MATERIALS AND METHODS

Microorganisms:
A total number of 40 fungal isolates obtained from Karpagam Microbial Culture Collection Center, were examined for lipase production. The fungi were maintained on Potato Dextrose Agar (PDA) slants.

Screening method for lipase production:
The screening of the fungal strains for lipase production was studied by inoculating them on Rhodamine B medium [19]. Lipase production was detected by irradiating the plates with UV light at 350 nm. Fungal colonies that have lipolytic production showed orange fluorescent halo.

Screening of alkaline lipase production:
Selective agar medium (0.5 g yeast extract, 5.0 g (NH4)2SO4, 2.0 g (NH2)2CO, 1.0 g MgSO4.7H2O, 1.0g NaCl, 2.0 G bile salts, 10.0 ml olive oil, 20.0 g bacteriological agar, pH 7.0 (per litre)) for the isolation of alkaline lipase producing fungi was prepared using Colen et al. [20] and observed for clear hydrolytic halos.

Substrate:
Rice bran, wheat bran, coconut oil cake, gingelly oil cake, cotton seed cake, groundnut oil cake, sugarcane baggase and banana waste were used as substrates [16]. They were procured from a local market of Coimbatore, India and were dried at room temperature to reduce the moisture content and ground to the desired size.

Solid State Fermentation:
Ten grams of agro industrial waste (rice bran, wheat bran, coconut oil cake, gingelly oil cake, cotton seed cake groundnut oil cake, sugarcane baggase and black gram husk) was taken in 250 ml Erlenmeyer flasks, moistened with 10 ml of sterile distilled water in the ratio of 1:1, w/v and sterilized. After cooling, the flasks were inoculated with 1 ml of spore suspension (10^6 spores/ ml) and the contents were mixed and incubated at 30°C for seven days. Enzyme extraction was performed by adding 100 ml of distilled water to the solid moulding medium and shaking the mixture in a rotary shaker (100 rpm) for 1 hour. The extracts were squeezed through a muslin cloth and clarified by centrifugation at 10,000 x g for 5 minutes [17]. The supernatants were used as crude enzyme.

Enzyme assay:
According to Hasan *et al.*, 2009 [21] use of olive oil as substrate for titrimetric method have been reported by many scientists [22, 23, 24, 25].

Lipase activity was determined titrimetrically on the basis of olive oil hydrolysis [26]. One ml of the culture supernatant was added to the reaction mixture containing 1ml of 0.1M Tris-HCl buffer (pH 8.0), 2.5 ml of deionised water and 3 ml of olive oil. The reaction mixture was mixed well and incubated at 37 °C for 30 minutes. Both test and blank were performed. Immediately after starting the incubation, 1 ml of the culture supernatant was pipetted into a 50 ml Erlenmeyer flask marked blank and stored at 4 ^oC. After 30 minutes the test solution was transferred to a 50 ml Erlenmeyer flask. 3 ml of 95% ethanol was added to stop the reaction. Liberated fatty acid was titrated against 0.1M NaOH using thymolphthalein blue as indicator. A unit lipase is defined as the amount of enzyme, which releases one micromole fatty acid per minute under specified assay conditions. Enzyme activity was expressed as units per gram of dry substrate.

Protein assay:
Protein content of the supernatant was quantified by the method of Lowry et al. [27] with bovine serum albumin as standard and was expressed as mg/ml.

Optimization studies:
Using different agro-industrial waste materials (wheat bran, rice bran, cottonseed oil cake, coconut oil cake groundnut oil cake, sugar cane bagasses and), lipase production was studied at different pH (7 - 11), temperature (30 – 70 °C), moisture content (10-50%) and incubation period (3-9 days).

Different carbon sources (lactose, maltose, mannitol, starch and sucrose), nitrogen sources (peptone, yeast extract, casein, urea and albumin) and metal ions (magnesium sulphate, calcium chloride, sodium chloride, ferrous sulphate and zinc chloride) were supplemented separately to a final concentration of 1% (w/v) in solid media. After fermentation, the lipase activity was estimated.

RESULTS & DISCUSSION

Screening of fungi for alkaline lipase production:

All the 40 isolates collected from KMCCC were subjected to rapid screening by using Rhodamine B method (**Table 1**). Out of these isolates, 28 showed a zone of fluorescence at 350 nm. Among the active isolates, one isolate (*Penicillim chrysogenum*.) found to produce maximum growth zone of fluorescence (**Figure. 1**). Those 28 strains which showed growth in Rhodamine B agar were further screened for alkaline lipase production using the selective media. Out of 28 microbial sp., *Penicillium chrysogenum* showed the maximum hydrolytic halo around the colony that indicates it produces the highest production of alkaline lipase (**Figure. 2**).

Table: 1 Rapid screening of fungi for their lipase production using Rhodamine B agar medium

S.No	Fungal genera	Growth
1	*Acremonium furcatum*	-
2	*Acremonium murorum*	-
3	*Aspergillus alutaceus*	-
4	*Aspergillus erythrocephalus*	-
5	*Aspergillus flavipes*	+
6	*Aspergillus flavus*	+
7	*Aspergillus fumigatus*	+ +
8	*Aspergillus glaucus*	+ +
9	*Aspergillus japonicus*	+
10	*Aspergillus melleus*	-
11	*Aspergillus nidulans*	+ +
12	*Aspergillus niger*	+ +
13	*Aspergillus ornatus*	+
14	*Aspergillus oryzae*	+ +
15	*Aspergillus sclerotorum*	+ +
16	*Aspergillus sydowii*	+ +
17	*Aspergillus terrus*	+
18	*Aspergillus ustus*	+
19	*Aspergillus versicolor*	+
20	*Aspergillus wentii*	+ +
21	*Fusarium culmorum*	-
22	*Fusarium dimerum*	+
23	*Fusarium merismoides*	+
24	*Humicola insolens*	-
25	*Mucor circinelloides*	-
26	*Penicillium brevicompactum*	+ +
27	*Penicillium chrysogenum*	+ +
28	*Penicillium fellutanum*	-
29	*Penicillium frequentans*	+
30	*Penicillium livindum*	-
31	*Penicillium nigricans*	+ +
32	*Penicillium restrictum*	+ +
33	*Penicillium sacculum*	+
34	*Penicillium thomii*	+
35	*Penicillium oryzae*	+ +
36	*Penicillium canescens*	-
37	*Penicillium citrinum*	+
38	*Penicillium claviforme*	+
39	*Trichoderma koningii*	-
40	*Rhizopus stolonifer*	+ +

+ + = Very good, + = Average

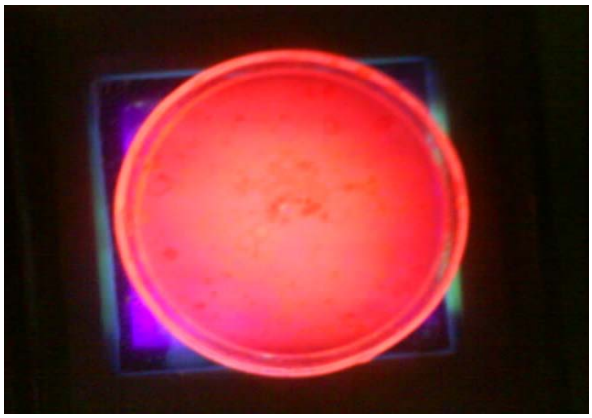

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Figure 1: Growth of *Penicillim chrysogenum* showing fluorescent halo on Rhodamine B agar medium

a

Figure 2 *Penicillim chrysogenum* showed the maximum hydrolytic halo around the colony

Optimization of SSF system for lipase production:

Effect of different solid substrates on lipase production

Among all the substrates, the maximum lipase activity of 420 U/ gds was observed with wheat bran (**Figure: 3**). Different substrates occupied surface area according to their size was an important parameter in solid state fermentation.

Ten grams of substrate yield maximum production of lipase. Due to its easy penetration, the microbial mass showed high growth rate with wheat bran [28]. Wheat bran found to be widespread suitability, presence of sufficient nutrients and its ability to remain loose even in moist condition, thereby providing a large surface area [10]. According to Mala et al [29], the maximum lipase activity of 271.6 U/ gds was obtained with wheat bran as substrate.

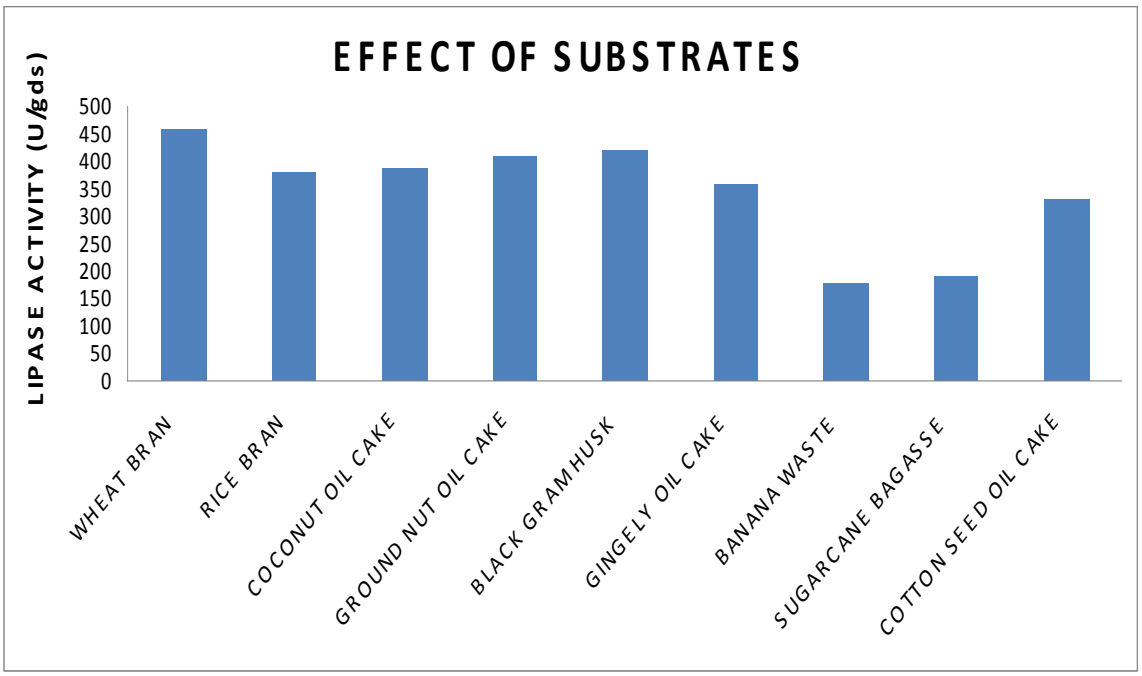
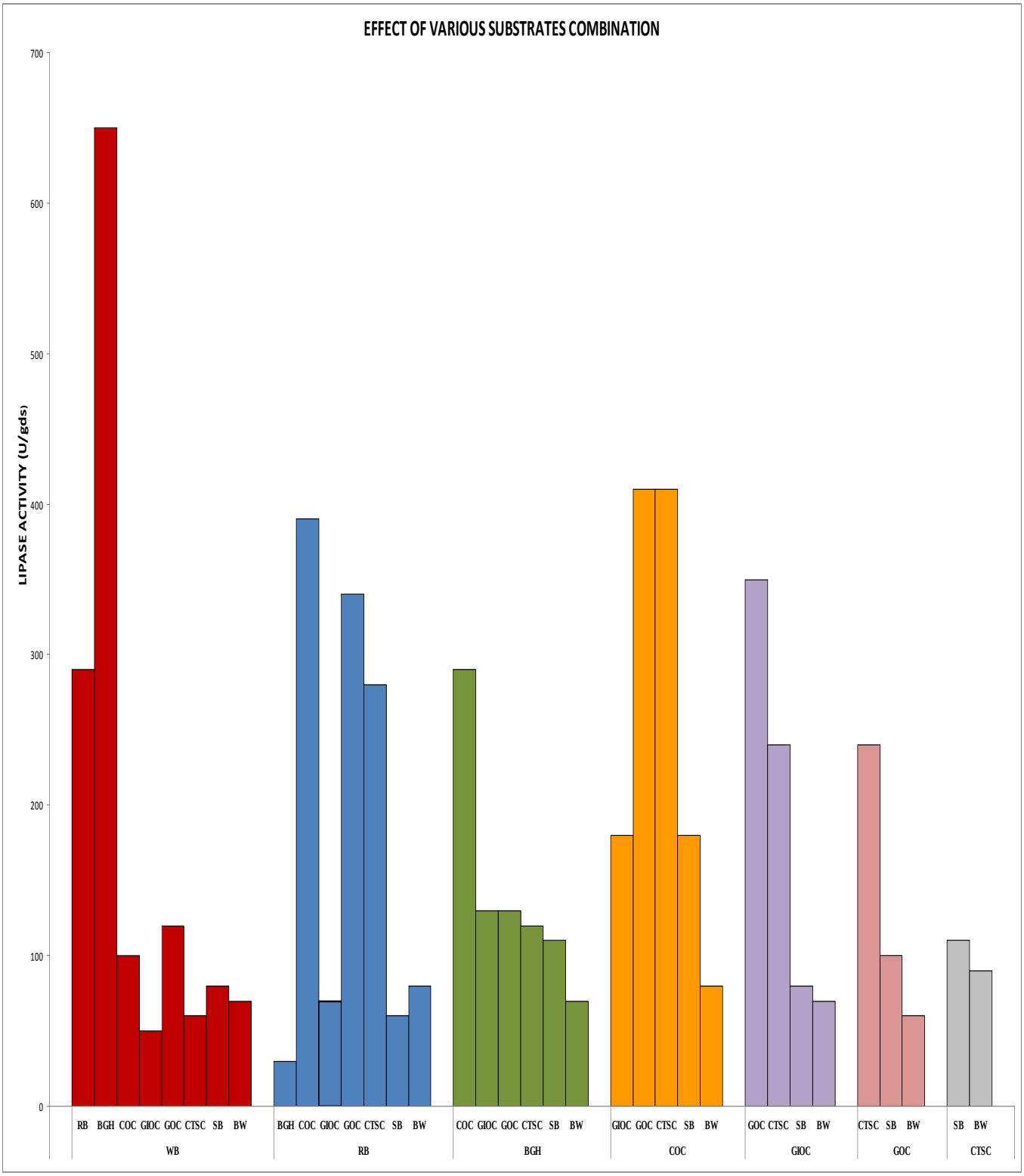


Figure 3: Effect of different solid substrates on lipase production



Note: WB – Wheat bran, RB – Rice bran, COC – Coconut oil cake, GOC – Ground nut oil cake, BGH – Black gram husk, Gi OC – Gingely oil cake, BW - Banana waste, SCB – Sugarcane bagasse, CTSC – Cotton seed oil cake.

Figure: 4 Lipase production with mixed substrate

Mixed substrates are viable substrate for SSF processes [30]. Accordingly mixed SSF was developed with various substrates combination (**Figure: 4**).Among the various combinations, the mixture of wheat bran (WB) and black gram husk (BGH) showed the maximum lipase activity of 650 U/ gds. This is the first report on lipase production using a mixture of WB and BGH. Though there were report on the production of lipase by mixed SSF using *Aspergillus niger* MTCC 2594 (384.3 U/ gds, wheat bran and gingely oil cake) [29], *Candida rugosa* (118.2 U/ gds, Coconut oil cake and wheat bran) [30], *R. rhizopodiformis* (79.6 U/ gds, olive oil cake and

sugarcane bagasse) [31], and *A.flavus* (7.2 U/ gds rice husk and rubber wood dust) [32], lipase activities were found to be low with all these systems.

Wheat bran and Black gram husk were tested with various ratios (**Table: 2**). Addition of BGH (5 g) to WB increased the lipase activity significantly (650 U/ gds) over using WB alone. Furthermore, there was no appreciable increase in lipase activity upon addition of either increasing or decreasing the amount of BGH to WB.

Table: 2 Effect of Black gram husk (BGH) to Wheat bran (WB) on lipase production

Substrate (g)	Lipase activity (U/g dry substrate)
WB(10)	460
WB(8.75)+BGH(1.25)	520
WB(7.5)+BGH(2.5)	580
WB(5)+BGH(5)	650
WB(2.5)+BGH(7.5)	480
BGH (10)	420

Effect of incubation periods

The lipase enzyme production for *Penicillium chrysogenum* was examined on various incubation days ranging from day 3 to 9. *Penicillium chrysogenum* showed the maximum of 680 U/g of dry substrate enzyme productions on day 7 (**Figure. 5**). Cho et al [33] observed maximum lipase activity (40 U/ml) for *Penicillium chrysogenum* when incubated at 20ºC on the fifth day. Mohawed et al [34] reported peak lipase productivity in *Aspergillus fumigatus* after 5 days of incubation. *Aspergillus fumigatus* and *Aspergillus nidulans* exhibit a peak lipase production after 10 days of incubation [35].

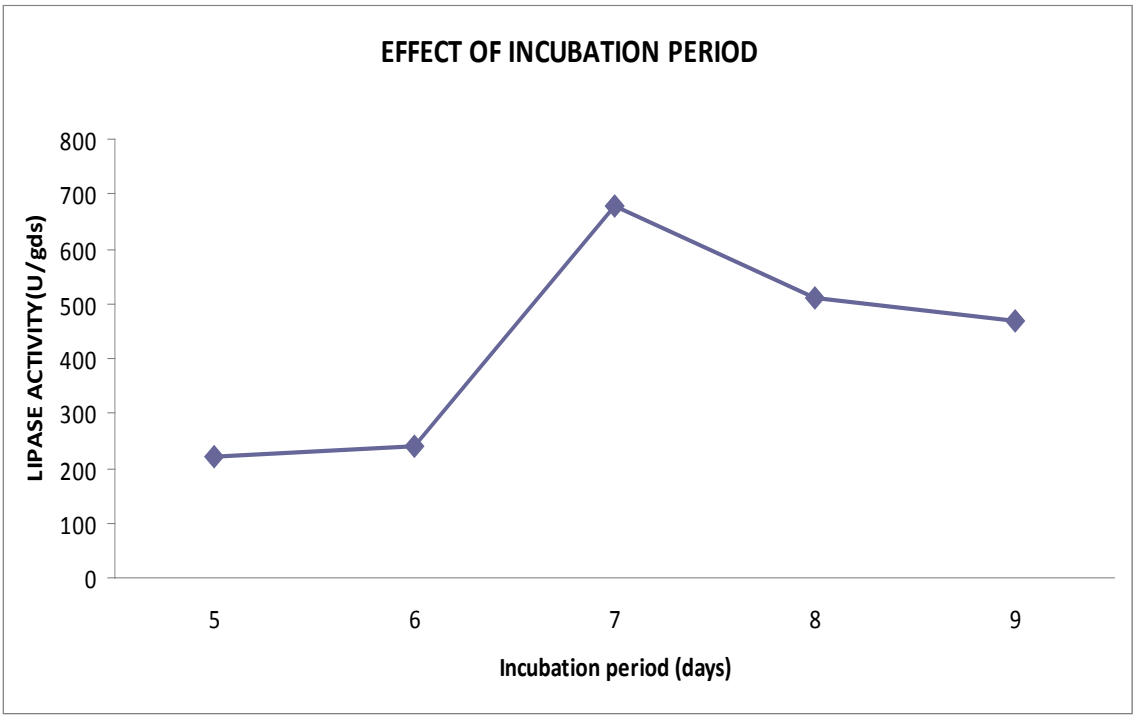


Figure: 5 Effect of various incubation period

Effect of initial moisture content of substrate:

Variation in initial moisture content of substrate showed that the enzyme synthesis was related to the availability of moisture. Substrate moisture is a crucial factor in SSF and its importance for enzyme production has been well established. With the initial moisture content of 10%, lipase yield was 320 U g/ds, which considerably increased with increase in moisture content. The maximum yield of 660 U g/ds was found at 40% (**Figure 6**). Higher moisture would lead to decrease porosity, promotes development of stickiness and increases the chances of contamination [36]. The critical importance of the moisture level in SSF media and its influence on the biosynthesis and secretion of enzyme can be attributed to the interference of moisture level was believed to reduce the porosity of the substrates [37].

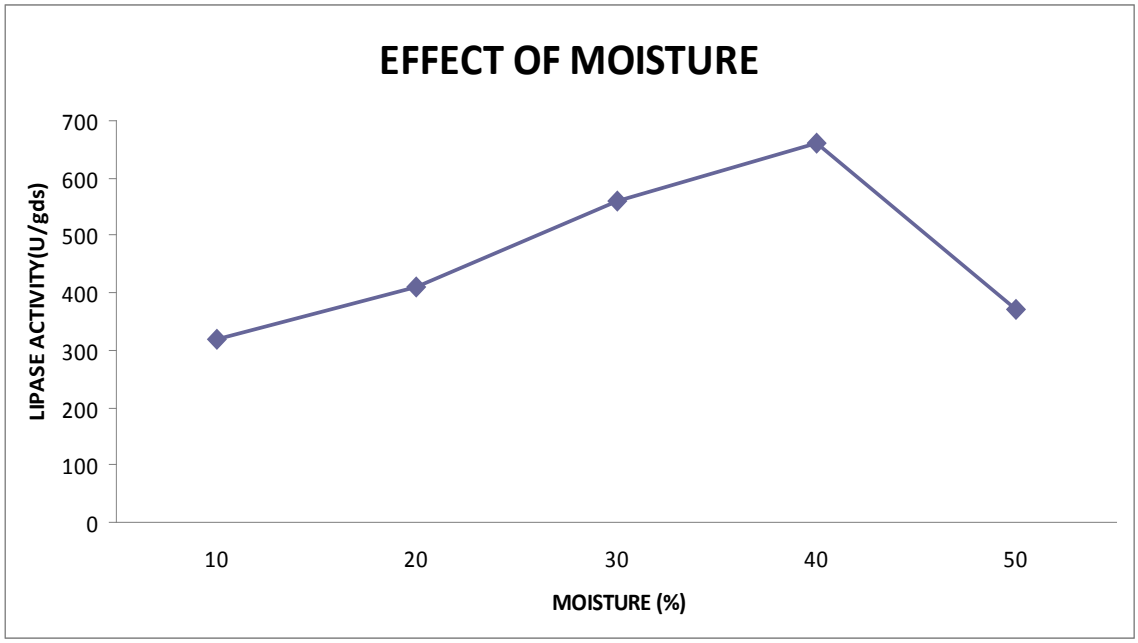


Figure: 6 Effect of various moisture content

Influence of temperature and pH on enzyme production

Lipase production at different temperature (30 - 70 °C) was examined for 7 days keeping the other fermentation conditions constant. Lipase production increased with increase in temperature from 30 to 50 °C. Maximum production of lipase (652 U/g of dry substrate) was obtained at 40 °C and production declined at 50 °C (**Figure 7**). Pau et al [32] reported that *A. flavus* USM A10 showed maximum lipase activity of 5.9±0.03g $^{-1}$ substrate within the temperature range of 28 to 30 °C. Higher temperature resulted lower lipase activity which may be related to the low enzyme stability at higher temperature. Although many other enzymes produced by bacteria and yeast show maximum activities at high temperatures, such as *Pseudomonas aeruginosa* (70 ºC) [38] and yeast *Kurtzmanomyces* sp. (75 ºC) [39], just few fungal lipases reported in literature present such thermophilic behavior [40].

Lipase production by *Penicillium chrysogenum* was observed on different pH range from 7-11. Growth and lipase production ceased at pH 10. Maximum lipase production of 680 U/g of dry substrate was observed at pH 9 (**Figure 8**). Optimum pH for the production of lipase by *Aspergillus carneus* is 8.0 [41], 7.0 *for candida deformans* [42] and 8 for *Fusarium oxysporum* [43].

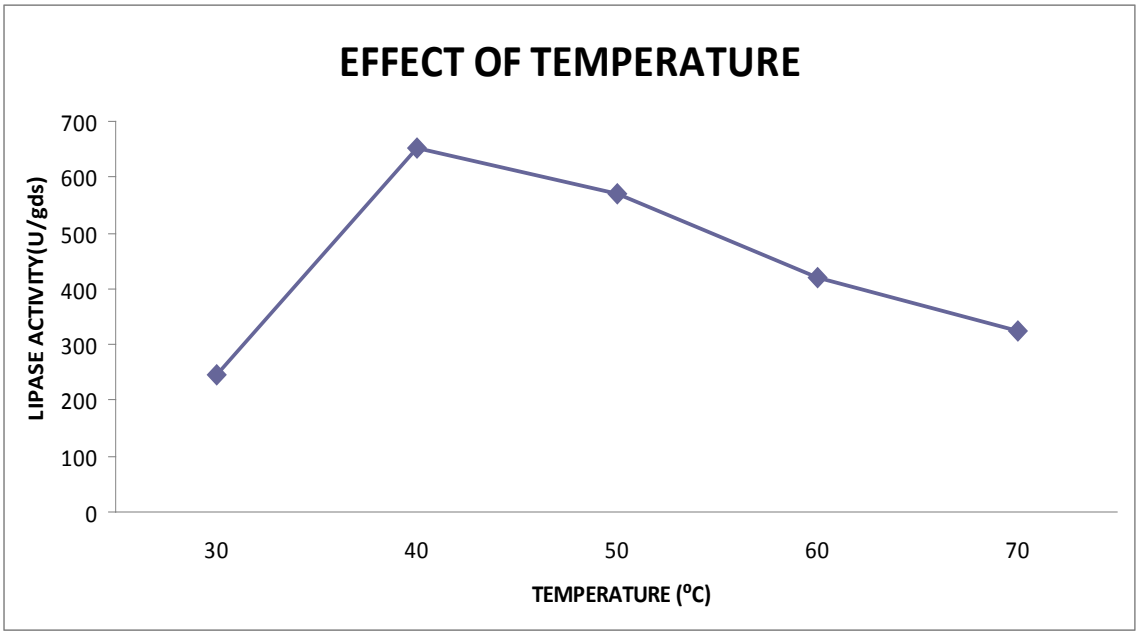


Figure: 7 Effect of various temperature

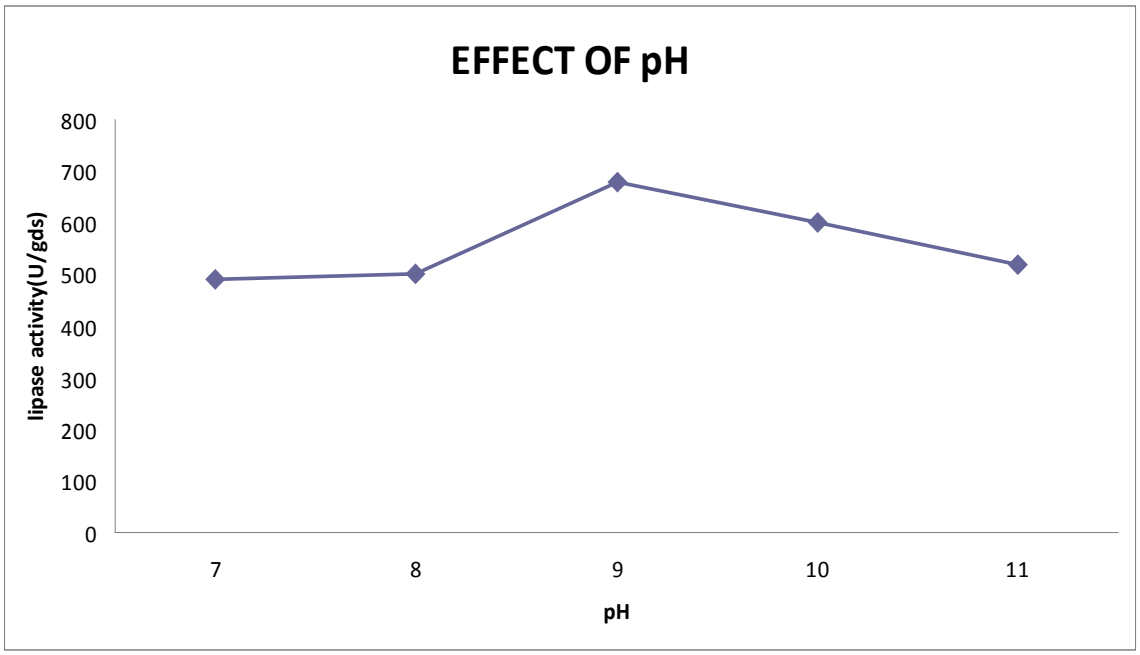


Figure 8: Effect of various pHs

Influence of chemical parameters on lipase production

Supplementation of additives such as carbon sources, nitrogen source and metal ions to the mixed SSF did not influence the lipase production. Similar results were found by Mala et al [29].

CONCLUSION

Lipases that are stable at alkaline pH are highly desirable for various field particularly detergent formulations. In the present study, an alkaline lipase was produced from solid-state fermentation of mixed agro-industrial wastes by lipase producer strain of *Penicillium chrysogenum*. Use of such wastes as substrates instead of commercial synthetic media was done to develop a cost-effective method for lipase production. Various physicochemical parameters were studied to determine the optimum conditions for the lipase production. The obtained results showed that the maximum lipase was produced with the mixed substrate of WB (5 g) and BGH (5 g) at pH 9 for 7 days with moisture content of 40% at 40 ºC.

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