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Production of lipases by *Alternaria* sp. (mbl 2810) through optimization of environmental conditions using submerged fermentation technique

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Abstract

The present investigation was conducted for the biosynthesis of extracellular and intracellular lipases by *Alternaria* sp. (MBL 2810) through submerged fermentation technique. To increase the lipases production various parameters were optimized such as nitrogen, carbon sources and additional additives besides the basic cultural conditions *e.g.,* rate of fermentation, volume of fermentation medium, inoculum size and type and initial pH of fermentation medium. The maximum extracellular and intracellular lipases production was achieved, when fermentation was optimized at room temperature for 24 h at the agitation speed of 100 rpm. Other cultural conditions such as inoculum type and inoculum size (4.0 mL) was also optimized, glycine as an additional carbon source and urea as an additional nitrogen source gave the maximum extracellular lipases activity. Maximum production of extracellular lipases (9.04 ± 0.05[a] U/mL) was achieved when volume of fermentation was optimized at 25mL and maximized intracellular lipases (10.04 ± 0.13[a] U/mL) production was achieved when the medium pH was adjusted at 5.

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Introduction

Enzymes play important role in number of biotechnological processes and products that were commonly found in the form of food, pharmaceuticals, beverages etc. An excess of important enzymes along with other secondary metabolites are secreted by yeast and filamentous fungi in growth medium. Most of these enzymes are hydrolytic in nature and used in various food processing industries. These are also used to refine the fodder quality. Only 2% microorganisms of the world have been tested as enzyme sources (Ghorai *et al.,* 2009). Filamentous fungi are considered as an important source of industrial enzymes (Li *et al*., 2006).

The introduction of enzymology represents an imperative advancement in the biotechnology industry (Kirk *et al*., 2002). The hydrolytic enzymes (proteases, amylases, amidases, esterases and lipases) have main contribution to the industrial enzyme market. Lipases have appeared as most important enzymes in the developing field of biotechnology (Pandey *et al.,* 1999). Lipases belong to the one of the most significant class of industrial enzymes (Babu and Rao, 2007).

Lipase-producing microorganisms have been found in various habitats for example industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, oil seeds, and decaying food (Sztajer *et al.,* 1988). Compost heaps, coal tips, and hot springs (Wang *et al*., 1995). They can be produced easily as compared to the lipases obtained from other natural sources and thus are considered commercially important (Jaeger and Eggert, 2002). Microbial lipases are commercially essential. At commercial level, fermentation of different microorganisms like bacteria, fungi, yeast and actinomycetes can produce lipases (Sharma *et al*., 2001). Microorganisms can produce both extracellular and intracellular lipases (Haq *et al*., 1988).

Fungal lipases are used in various industries such as detergents, pharmaceuticals, beverages, dairy etc (Hiol *et al*., 2000). Lipases are used in specific organic synthesis, hydrolysis of fats and oils, flavor enhancement in food processing, resolution of racemic mixture, fat modification and chemical industry (Sharma *et al*., 2001). Lipases are considered unique for a wide range of biotransformation because they are responsible for the breakdown and mobilization of lipids inside cells and transfer of lipids from one organism to the other one (Martinelle *et al.,* 1995).

Submerged and solid state fermentation techniques can be used for the production of lipases but submerged fermentation is found to be more attractive because all parameters can be controlled easily (Hiol *et al*., 2000). Complex culture media consisting of yeast extract, peptone, soymeal and corn steep liquor are used for fungal lipase production (Chahinian *et al*., 2000). *Alternaria* species is a ubiquitous organism associated with various kinds of organic materials in damp places. The genus *Alternaria* is composed of many saprophytic and endophytic species. Most of them are disreputable critical plant pathogens and show necrotrophic life style (Lawrence *et al.,* 2008). Lipases which are obtained from microbes have been widely studied because of their promising applications but endophytic fungi have obtained comparatively little attention for production of lipases. The two most important features of *Alternaria* sp. are melanin production, particularly in spores and production of host-specific toxins in the case of pathogenic species (Thomma, 2003). The objective of this study was to evaluate the extracellular and intracellular lipases production by *Alternaria* sp. (MBL 2810) through submerged fermentation technique.

Materials and methods

Microorganism

An identified culture of *Alternaria* sp. (MBL 2810) was obtained from the Laboratory of Mycology and Biotechnology, Government College University, Faisalabad. Fungal culture was revived after every 15 days on potato dextrose agar slants (4%).

Fermentation Medium

In the present studies fifty milliliter of fermentation medium was used as per composition reported earlier (Gupta *et al.*, 2007) [% (w/v), Glucose 0.1; Olive oil 3.0; NH_4Cl 0.5, Yeast extract 0.36, K_2HPO_4 0.1, $MgCl_2$ 0.01, $CaCl_2$ 0.4]. All the experiments were carried out in triplicate.

Extraction of the Enzyme

Extracellular lipase Assay

After 24 hours the content of the flasks were filtered out and supernatant was assayed for extracellular lipase activity.

Intracellular lipase Assay

Pre-weighed mycelial mass was then grinded with sand in 10 mL of phosphate buffer pH 7. Then filter the contents and filtrate was assayed for intracellular lipase activity.

Enzyme assay estimation

Lipase estimation was carried out by adding assay substrate (10mL of 10% olive oil into 10% gum acacia, 2mL of 0.6% $CaCl_2$, and 5mL of 1M PO^{-4} buffers) to the flask containing 1mL of the filtrate and then shaking was done for 1h at 150 rpm. Then, 20 mL of alcohol: acetone (1:1) mixture was added to the reaction flask to stop the ongoing reaction between assay substrate and enzyme. Lipase activity was determined titrimetrically. Liberated fatty acids were titrated with 0.1N NaOH using phenolphthalein as an indicator. The end point was light pink color. One unit of enzyme is defined as "the amount of enzyme which releases one micromole fatty acid per minute under specified assay conditions" (Kundu *et al.*, 1970).

Statistical analysis

Experiments were performed in triplicate and the results were statistically analyzed using computer software Costat.

Results and discussion

Effect of additional Nitrogen Sources

Enzyme synthesis is also affected by different organic and inorganic nitrogen sources. In the present investigation 1% (w/v) peptone, NH_4NO_3, casein, $NaNO_3$ and urea were used. Figure 1 illustrates the influence of different nitrogen sources on lipolytic activity by *Alternaria* sp (MBL 2810). Among the nitrogen sources tested the highest extracellular lipase activity (5.05 ± 0.03[a] U/mL) was obtained when urea was added to the fermentation medium. The maximum intracellular lipase activity (5.62 ± 0.02[a] U/g) was achieved by the addition of casein to the fermentation medium. Nitrogen sources referee lipase production is well recognized in submerged fermentation (Carzo and Revah, 1999).

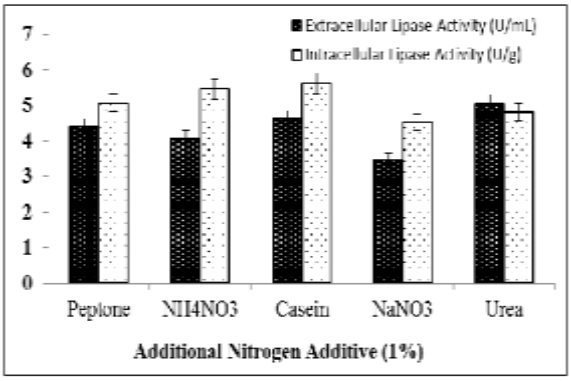

Fig. 1. Effect of additional nitrogen sources on extracellular and intracellular lipases production by *Alternaria* sp. (MBL 2810) through submerged fermentation.

Concentration and type of nitrogen sources affects the lipase production (Sharma *et al.*, 2001). Among all nitrogen sources, urea gave the highest extracellular (5.05 ± 0.03[a] U/mL) lipase production by *Alternaria* sp (MBL 2810). This result was confirmed by Babu and Rao, (2007) who observed the maximum lipase activity by using urea as nitrogen source by *Yarrowia lipolytica* NCIM 3589. Urea is also reported as best nitrogen source for enhanced lipolytic activity by six different yeast cultures (Nahara *et al.*, 1982). Casein gave the maximum intracellular (5.62 ± 0.02[a] U/g) lipolytic activity. It was also found that the casein works as the best nitrogen source for intracellular lipase production by using *Rhizopus oligosporus* DGM 31 (Iftikhar *et al.*, 2008).

Effect of additional Carbon sources

Five different carbon sources (sucrose, Galactose,

Starch, Glucose and Glycine) were used to study the enhanced lipolytic activity by *Alternaria* sp. (MBL 2810) Figure 2. The extra cellular lipase production ranged from 2.08 ± 0.07^{e} U/mL to 5.14 ± 0.05^{a} U/mL. The intracellular lipase activity ranged from 4.08 ± 0.1^{e} U/g to 4.95 ± 0.04^{a} U/g. The maximum extracellular (5.14 ± 0.05^{a} U/mL) and intracellular (4.95 ± 0.04^{a} U/g) lipase activity was observed when glycine was added to the fermentation medium as an additional carbon source. The minimum extracellular (2.08 ± 0.07^{e} U/mL) and intracellular (4.08 ± 0.1^{e} U/g) lipase yield was observed by the addition of starch and galactose respectively. Carbon is the major component of cells and other substances such as fats, fatty acids, plant oils, triglycerides, and ester-based detergents were the excellent inducers of lipases by microorganisms (Benjamin and Pandey, 1997). The maximum extracellular (5.14 ± 0.05^{a} U/mL) and intracellular (4.95 ± 0.036^{a} U/g) lipase activity was observed by *Alternaria* sp. (MBL 2810) when glycine was added to the fermentation medium as an additional carbon source. Contrary to this finding, starch was also reported as good inducer for maximized intracellular lipase production *Rhizopus oligosporus* DGM 31 (Iftikhar *et al.*, 2008).

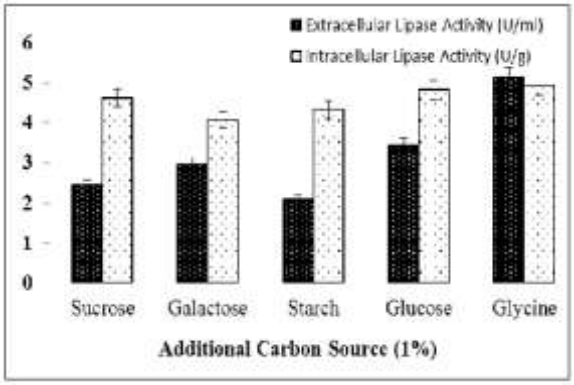

Fig. 2. Effect of different carbon sources on extracellular and intracellular lipases production by *Alternaria* sp. (MBL 2810) through submerged fermentation.

Effect of additional oils

In this study, the effect of inducers on the production of extracellular and intracellular lipase by *Alternaria* sp. (MBL 2810) was investigated Figure 3. For this purpose 1% (v/v) almond oil, coconut oil, brassica oil, sunflower oil and caster oil were used. The yield of extracellular lipases ranged from 2.13 ± 0.14^{e} U/mL to 3.11 ± 0.06^{a} U/mL. The intracellular lipase production ranged from 3.58 ± 0.065^{e} U/g to 5.07 ± 0.09^{a} U/g. The highest extracellular lipase activity (3.11 ±0.06^{a} U/mL) was obtained when Sunflower oil was added to the fermentation medium as an inducer. The maximum intracellular lipase activity (5.07 ±0.09^{a} U/g) was achieved when brassica oil was added to the fermentation medium as an inducer for achieving enhanced lipase production. The additions of oils to the fermentation medium enhance the lipolytic activity (Wang *et al.*, 1995). The imperative role of different oils on lipase production by *Alternaria* sp. (MBL 2810) was explained by incorporating the selected oils 1% (v/v) to the fermentation medium. The highest intracellular lipase production (5.07 ±0.09^{a} U/g) was observed with brassica oil. The maximum extracellular lipase activity (3.11 ±0.06^{a} U/mL) was observed with sunflower oil. This finding matches with the earlier reported results in which sunflower oil was used as the best inducer for lipase production by *Yarrowia lipolytica* NCIM 3589 (Babu and Rao, (2007). The results are also in accordance with those reported by (Moataza *et al.*, 2004) who observed the maximum lipolytic activity by *Fusarium oxysporum*.

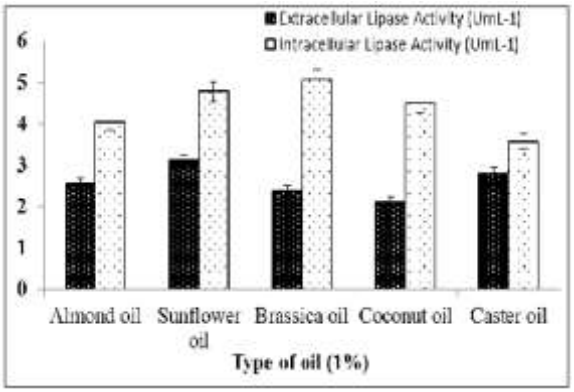

Fig. 3. Effect of additional oils on extracellular and intracellular lipase production by *Alternaria* sp. (MBL 2810) through submerged fermentation.

Effect of rate of agitation

The data of Figure 4 shows the effect of shaking conditions on lipase production by *Alternaria* sp (MBL 2810). The extra cellular lipase activity ranged from 3.5 ± 0.02^{e} U/mL to 5.33 ± 0.05^{a} U/mL. The intracellular lipase activity ranged from 1.413 ± 0.44^{d}

U/g to 5.57 ± 0.03[a] U/g. The maximum extra cellular lipase activity (5.33 ± 0.05[a] U/mL) was detected at 100 rpm. The highest intracellular lipase activity (5.57 ± 0.03[a] U/g) was observed at 200 rpm. The lipolytic activity is also affected by the shaking speed (Riffat *et al.*, 2010). It is clear from the results shaking speed affects the lipase production. The maximum extracellular lipolytic activity (5.33 ± 0.05[a] U/mL) was obtained at 100 rpm by *Alternaria* sp (MBL 2810). This result is in accordance with the lipase production by *Geotrichum Candidum*-M2 (Mladenoska and Dimitrovski, 2002). Contrary to this finding highest lipase activity was obtained at 150 rpm by *Fusarium oxysporum* (Riffat *et al.*, 2010). The highest intracellular lipase production (5.57 ± 0.03[a] U/g) was obtained at 200 rpm. Contrary to this finding, the investigations showed the highest intracellular lipase production at 300-400 rpm by *Rhizopus oligosporus* DGM 3 (Iftikhar *et al.*, 2008). It was stated that increase in agitation rate did not enhance the lipase production (Cihangir and Sarikaya, 2004). Maximum lipase production was observed at increased agitation rate this might be due to increased oxygen transfer rate, improved dispersibility of carbon sources or enhanced surface area of contact with the components of medium.

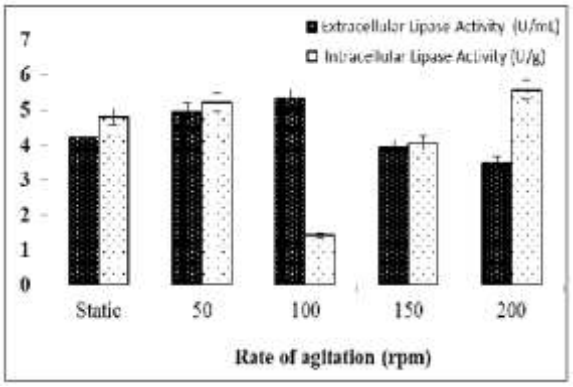

Fig. 4. Effect of rate of agitation on extracellular and intracellular lipases production by *Alternaria* sp. (MBL 2810) through submerged fermentation.

Effect of Rate of Fermentation

In the present study the effect of incubation period on the extracellular and intracellular lipase production by *Alternaria* sp. (MBL 2810) was studied Figure 5. The strain was incubated at time intervals of 24, 48, 96, 72, 120 hours. The extracellular lipase activity ranged from 1.13 ± 0.09[e] to 5.33 ± 0.35[a] U/mL. The intracellular lipase activity ranged from 3.16 ± 0.14[e] to 6.2 ± 0.26[a] U/g. The maximum extracellular (5.33 ± 0.35[a] U/mL) and intracellular (6.2 ± 0.26[a] U/g) lipase activity was achieved after 24 hours. The lipase production decreased after more than 24 hours. The growth rate and lipase production by microorganism is very important in understanding their fermentation pattern (Sztajar and Maliszewska, 1988).

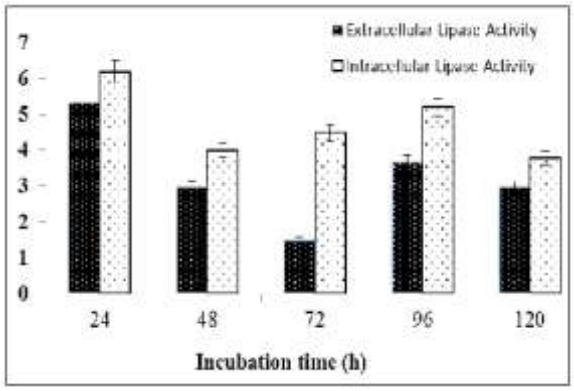

Fig. 5. Effect of rate of fermentation on extracellular and intracellular lipases production by *Alternaria* sp. (MBL 2810) through submerged fermentation.

The maximum extracellular lipolytic activity (5.33 ± 0.35[a] U/mL) and highest intracellular lipase yield (6.2 ± 0.26[a] U/g) was observed after 24 hrs of cultivation by *Alternaria* sp (MBL 2810). This result is in accordance with the lipase production by *Geotrichum Candidum*-M2 (Mladenoska and Dimitrovski, 2001). Beyond 24 h, gradual decrease in lipolytic activity was observed. The increase in incubation decreases the lipase production (Sangeetha *et al.*, 2008). This may be because of reduction in nutrients, accretion of noxious end products and change in the pH of medium. The incubation period of 24 hrs was optimized for further studies.

Effect of inoculum type

Two types of inoculum (spore and vegetative) were used to check their effect on production of enzyme (Figure 6) by *Alternaria* sp (MBL 2810). The highest intracellular (5.91 ±0.1[a] U/g) and extracellular (4.75 ±0.07[a] U/mL) lipase production was achieved when spore inoculum was used. The role of inoculum in enzyme production is very important, particularly for fungi (Papagiani, 2014). In submerged fermentation,

the density of inoculum acts as a significant variable (Iftikhar *et al.*, 2008). The highest extracellular (6.06 ± 0.02[a] U/mL) and intracellular lipolytic activity was observed by the use of 4ml of inoculum. The highest lipase activity was not obtained beyond this level. In contrast to this finding, earlier reports in literature observed maximum intracellular lipase production at 5% inoculum size (Iftikhar *et al.*, 2008). It was studied that the maximum extracellular lipolytic activity at inoculum of 5% (v/v) was obtained (Mladenoska and Dimitrovski, 2001). So, spore inoculum was optimized for further investigations.

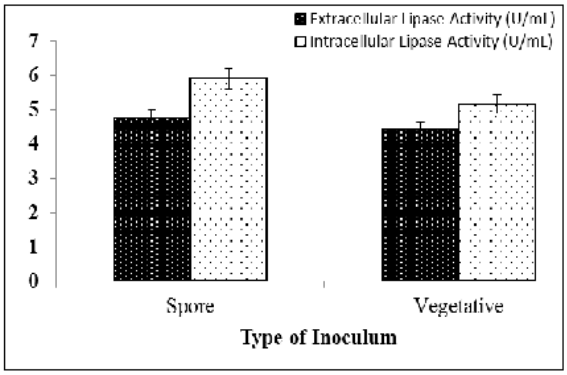

Fig. 6. Effect of type of inoculum on extracellular and intracellular lipases production by *Alternaria* sp. (MBL 2810) through submerged fermentation.

Effect of inoculum size

In the present study effect of inoculum size on production of lipase by *Alternaria* sp. (MBL 2810) was also investigated (Figure 7). Different inoculum levels (1mL, 2mL, 3mL, 4mL & 5mL) were used for study. The extracellular lipase activity ranged from 4.16 ± 0.025[e] U/ml to 6.06 ± 0.02[a] U/mL. The intracellular lipase activity ranged from 4.15 ± 0.026[e] U/g to 6.53 ± 0.06[a] U/g. The highest extracellular lipase production (6.06 ± 0.02[a] U/mL) was achieved at 4mL of inoculum. The maximum intracellular lipase production (6.53 ± 0.06[a] U/g) was obtained at 1 mL of inoculum. The number of fungal spores in inoculum also affects the lipase production (Papagiauni *et al.*, 2001). In the present investigations, spore and vegetative inoculum were tested with the aim to get enhanced lipase production by *Alternaria* sp (MBL 2810). An increased lipase production (4.75 ±0.07[a] U/mL) was observed when spore inoculum was used. This might be due to the reason that more concentration of spores is present in spore inoculum[7].

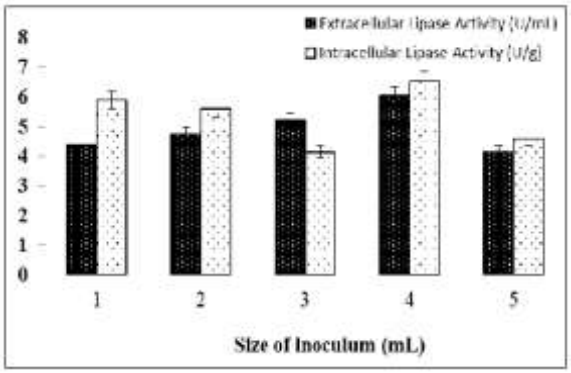

Fig. 7. Effect of inoculum size on extracellular and intracellular lipases production by *Alternaria* sp. (MBL 2810) through submerged fermentation.

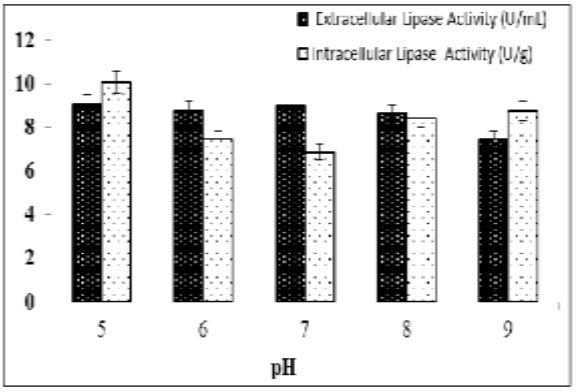

Fig. 8. Effect of initial pH of fermentation medium on extracellular and intracellular lipases production by *Alternaria* sp. (MBL 2810) through submerged fermentation.

Effect of initial pH of fermentation medium

Initial pH of the culture is one of the most significant environmental parameter which affects the lipase production. The data of Figure 8 indicates that the fungus is able to grow in the pH range from 5 to 9. The highest extracellular (9.04 ± 0.05[a] U/mL) and intracellular (26.86 ± 0.13[a] U/g) lipase production was achieved at pH 7. The decrease in lipase production was observed below and above this value which indicates that acidic and basic both conditions are not favorable for enhanced lipase production. The initial pH of medium was optimized for highest lipolytic activity. The highest extracellular lipase production (9.04 ± 0.05[a] U/mL) was observed at pH of 7 by *Alternaria* sp (MBL 2810). This finding matches with the earlier reported results in which

maximum crude lipase activity was obtained by *Penicillium wortmani* at pH 7.0 (Freire *et al.*, 1997). It was also observed the highest lipolytic activity was achieved at pH 7.0 by *C. rugosa* (Morais, 2001). The maximum intracellular lipolytic activity (26.86 ± 0.13[a] U/g) was achieved at pH of 7.0. Similar results were reported in literature by using *Rhizopus oligosporus* DGM 31 (Iftikhar *et al.*, 2008). As the pH increase or decrease from this value there was a gradual decrease in lipase production. Therefore, the neutral pH was selected for further studies.

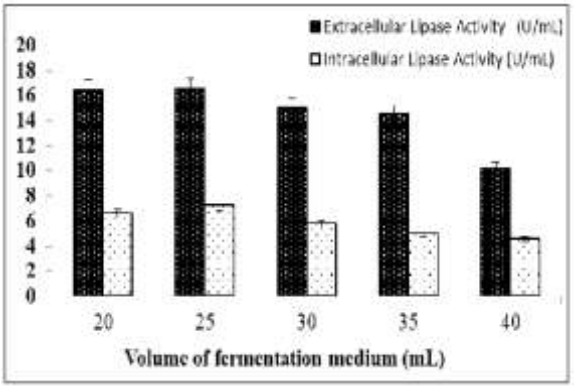

Fig. 9. Effect of volume of fermentation medium on extracellular and intracellular lipases production by *Alternaria* sp. (MBL 2810) through submerged fermentation.

Effect of volume of fermentation medium

The volume of fermentation medium also affects the lipase production Figure 9. The volume of fermentation medium ranged from 20-40 mL with an interval of 5 mL. The highest extracellular (16.58 ± 0.08[a] U/mL) and intracellular (7.23 ± 0.04[a] U/g) lipase activity was achieved at volume of 25 mL in 250 mL flasks. Increase in quantity of medium shows the decrease in the production of lipase by *Alternaria* sp (MBL 2810). Volume of medium has a considerable effect on lipase production (Martinez *et al.*, 1993). The lipase activity was also influenced by variations in volume ratio. The maximum extracellular (16.58 ± 0.08[a] U/mL) and intracellular (7.23 ± 0.04[a] U/g) enzyme yield was achieved at volume of 25 mL in 250 mL flasks by *Alternaria* sp (MBL 2810). Increase in volume shows the decrease in lipase activity. But the investigations performed by (Riffat *et al.*, 2010), found maximum extra cellular lipase activity at volume of 20 mL by using *Fusarium oxysporum*. Hence, 25 mL volume of fermentation medium was optimized for further studies.

Acknowledgements

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