

## Solid state fermentation of lipase production from *Aspergillus niger* using tannery solid waste

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### ABSTRACT

A novel bi-substrate fermentation (BSF) process was developed for the production of lipase from *Aspergillus niger* using agro-industrial residues, wheat bran (WB), and Beef fleshing of tannery solid waste. The study was carried out to utilization of solid waste substance from the tannery industry for the production of extracellular lipase enzyme from *A. niger* species. The high level lipase activity was  $404.8 \pm 17$  U/g dry substrate (U/gds) at 30 °C and 96 h and growth studies indicated that addition of WB significantly augmented the biomass and lipase production. The fungal lipase was produced from a slaughterhouse waste, and also agro-industrial residue as substrate. The high level lipase activity was at 30 °C and 96h and the growth study indicate that addition of tannery waste substance and agro-industrial residue influence over the production of lipase enzyme. Mixed substrate fermentation was done for single substrate fermentation (SSF), bi-substrate fermentation (BSF), tri-substrate fermentation (TSF), among three fermentation systems the BSF was selected as high enzyme yields one. The optimization of different parameters like substrate concentration, inoculums range, and moisture content was done. The maximum activity was obtained using equal ratio of tannery waste and agro-substance (1:1), when fermented substrate was extracted in phosphate buffer (pH 7). The inducers plays important role in increasing the production of lipase enzyme like oily solid substrate, instead of using oily substance tannery waste substance was used. Thus, the extra cellular enzyme has potential industrial applications and furthermore, the direct application of fermented substrate for beef fleshing hydrolysis makes the process economical for industrial production of biofuel.

Keywords: Beef fleshing, solid state fermentation, bisubstrate fermentation, beef hydrolysis.

## INTRODUCTION

The enzymes are the focal point of biotechnological process since they are involved in all the aspects of biochemical conversion from the simple fermentation into conversion to the complex techniques in genetic engineering and molecular biology.[1-5] They are used as cost- effective and eco-friendly substitutes for chemical process in several industries. Despite the fact that more than 3000 different enzymes have been identified and many of them have found their way into biotechnological and industrial applications, the present enzyme toolbox is not sufficient to meet most of the industrial demands. In view of these limitations, researchers have diverted their attention for isolation and characterization of enzymes from different environments. Whenever required, due attention is also paid towards development of recombinant enzymes with desired characteristics [6-11]and for specific applications (Mala *et al.*, 2007).

Among microorganisms, molds are known to be more potent lipase producers, because they produce lipase both by solid substrate and by submerged fermentation. Hence, it is worthwhile to optimize the fermentation medium, which affects the product yield and volumetric productivity of these enzymes (Mala *et al.*, 2007).

Several reports are available on lipase production by SSF using solid substrates including rice bran (Rao *et al.*, 1993) coconut oil cake (Benjamin and Pandey, 1997), gingelly oil cake (Kamini *et al.*, 1998) and soybean meal (Han, 2003). However, reports on lipase production by SSF using mixed solid substrates (Mala *et al.*, 2007) are very much limited and no report is available on the utilization of fleshing in different substrate combinations for the production of lipase from *Aspergillus niger*. [12-19] It is, therefore, imperative to develop a suitable process for enhanced lipase production.

Lipases are ubiquitous enzymes of considerable physiological significance and industrial potential. Interest in the production of microbial lipases has increased in recent decades, because they find promising applications in the production of pharmaceuticals, detergents, cosmetics, leather, and foods and in other organic syntheses (Hasan *et al.*, 2006).

Furthermore, the fermented solid material was directly evaluated for the hydrolysis [20-25] of fleshing, a low cost feed stock, which is economically and industrially important for the production of fatty acid esters.

## MATERIALS AND METHODS

### Material

All the chemicals used in the present study were of AR grade and purchased from Hi-Media Limited and S.D. Fine Chemicals Limited, Mumbai, India. Wheat bran (WB), coconut oil cake (COC), [26-29] wheat rawa (WR) and beef fleshing was purchased from local market, Chennai, India.

### Inoculum and fermentation conditions

A fungal strain of *A. niger* MTCC 2594 screened and isolated in our laboratory was used in the present research and was maintained on Czapek Dox agar slants at 4°C. The spore suspension for inoculation was prepared by adding 2 ml of sterile distilled water to the culture slant and the spores were dislodged using an inoculation needle under aseptic conditions. A spore suspension containing  $4.3 \times 10^8$  spores/ml was used as an inoculum. Lipase production was carried out in 250 ml Erlenmeyer flasks, each containing 10 g of different solid substrates either alone or in mixed combinations with bi-substrate fermentation (BSF) or tri-substrate fermentation (TSF), [30-35] respectively, with initial moisture content adjusted to 55%, 60% respectively with distilled water. The flasks were inoculated with 2.5 ml of spore suspension and the contents were mixed and incubated at 30°C for 5 days. Samples were taken at 24 h intervals and extraction of the enzyme was carried out according to Kamini et al. (1998). All the experiments were carried out in triplicates and the results were expressed as the mean  $\pm$  SD of triplicates.

### Lipase Production by Single Substrate Fermentation

Fermentation studies were investigated by the initial moisture content was 55% and inoculum concentration 2.5% ( $4.3 \times 10^8$  spores/g substrate). Using optimized parameters, time course studies on lipase production were carried out for single substrate fermentation.

Substrates are wheat bran, wheat raw, coconut oil cake and beef fleshing

### Mixed Substrate Fermentation

#### Lipase Production by BSF

Consecutive optimization studies were investigated by the initial moisture content was 55% and inoculum concentration was about 2.5% ( $4.3 \times 10^8$  spores/g substrate). Using optimized parameters, time course studies on lipase production were carried out for BSF.[36-39]

**Table 1 Lipase Production by BSF**

| S.No | Substrate (%)   |
|------|-----------------|
| 1    | WB(7.5)+FL(2.5) |
| 2    | WB(5)+FL(5)     |
| 3    | WB(2.5)+FL(7.5) |

#### Lipase Production by TSF

TSF studies were investigated by varying the initial moisture content 60% and inoculum concentration 2.5% ( $4.3 \times 10^8$  spores/g substrate). Using optimized parameters, time course studies on lipase production were carried out for TSF.

**Table 2 Lipase Production by TSF**

| S.No | Substrate (%)          |
|------|------------------------|
| 1    | WB(5)+WR(2.5)+COC(2.5) |
| 2    | WB(5)+WR(2.5)+FL(2.5)  |
| 3    | WB(5)+FL(2.5)+COC(2.5) |
| 4    | FL(5)+WR(2.5)+COC(2.5) |

### Optimization of BSF Parameters

#### Effect of Inoculums Size on Lipase Production for BSF

Various concentrations of inoculums were used for BSF medium. The percentage ranges from 1 % to 10% of inoculums. [29-34]The production of enzyme various with inoculums percentage. The spore suspension for inoculation was prepared by adding 2 ml of

sterile distilled water and the spores were dislodged using an inoculation needle under aseptic conditions. A spore suspension containing  $4.3 \times 10^8$  spores/ml was used as inoculums.

### **Effect of Moisture Content on Lipase Production by BSF**

The moisture content was optimized ranges from 30 % to 60 %. The production of lipase varies with moisture percentage.

#### **Calculation Formula**

$$\text{Moisture (\%)} = \frac{\text{Vol. of dis. H}_2\text{O}}{\text{Vol. of dis. H}_2\text{O} + \text{Weight of the sample}} \times 100 \quad (3.2)$$

### **Effect of Extraction of the Enzyme**

Extraction of the enzyme from BSF was carried out with tap water, distilled water, buffer, salts (sodium chloride - 1%) and ammonium sulphate - 1%) and surfactants (Triton X-100 – 0.5%).[33-39]

### **Scale up of Lipase Production**

Scale up of lipase production from 10 g flask level to 40 g were carried out. Substrate inoculums grown for 72 h were inoculated into 40g of mixed substrate (moisture content adjusted to 55%) in series of sterilized flasks 96 h and assayed for lipase activity at 24 h intervals. [18-24]The variation of temperature and humidity during the process was monitored using a portable humid meter.

### **Enzyme Assay**

At the end of fermentation period, 1 g of the fermented substrate was homogenized with 10 ml of 0.05 M phosphate buffer (pH 7.0) and the supernatant was squeezed through a double layered muslin cloth and centrifuged at 10,000 rpm for 5 min. The supernatant obtained was used as the enzyme source. Lipase activity was determined according to Yamada et al. (1962) using olive oil as substrate.[25-30] One unit of activity was defined as the amount of enzyme releasing 1  $\mu\text{mol}$  of free fatty acid in 1 min at pH 7.0 and 37 °C and the lipase activity was expressed as U/g of dry substrate (U/gds). Moisture content was determined after total drying of the sample in a hot air oven at 100°C for 6 h.

### Direct Application of Fermented Substrate for Fleshing Hydrolysis

The fermented substrate was directly evaluated for the hydrolysis of fleshing. The hydrolytic reactions were carried out in series of 100 ml screw capped Erlenmeyer flasks containing 10 g of sliced fleshing, 25 ml of 0.1 M phosphate buffer, pH 7.0 and hydrolysis was carried out with fermented substrate (lipolytic activity of 25 U/g of fleshing) for 96 h with shaking at 150 rpm and 30 °C. Samples were taken at 24 h intervals and reaction was stopped by addition of 20 ml of acetone and the free fatty acids liberated were titrated with 0.1 N KOH using phenolphthalein as indicator. A control was carried out similarly, except the fermented substrate was added after the addition of acetone.[31-39] The control value was subtracted from the experimental value and the acid value was calculated.

Hydrolysis ratio (%) = acid value/saponification value  $\times 100$

The effects of lipase concentration, substrate: buffer ratio, temperature and additives to the reaction mixture were also determined to achieve maximum hydrolysis of Beef fleshing.

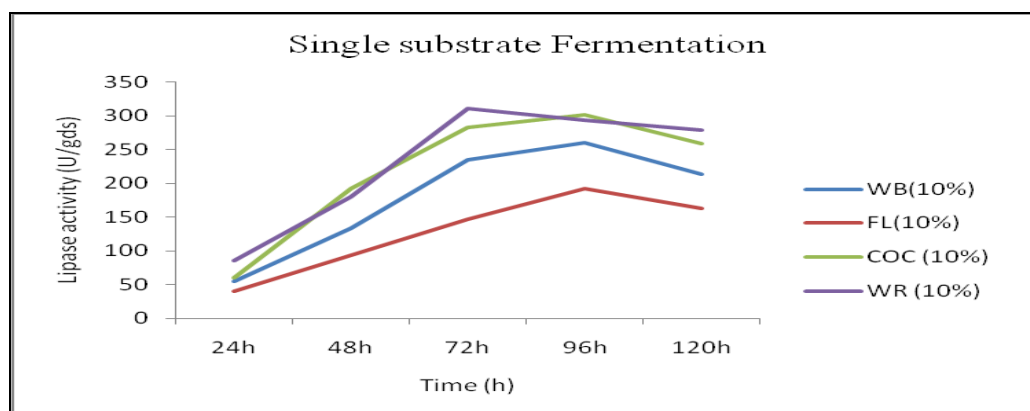
## RESULTS AND DISCUSSION

The effect of different substrates such as oil cakes, wheat bran, wheat raw, cotton seed oil cake and spent barley on lipase production by *A. niger* was reported earlier by Kamini *et al.* (1998). Mixed solid substrates are viable substrates for the growth of microorganisms on SSF, since they act as support matrix,[18-27] nutrient source and as inducers for the production of enzymes. Accordingly, to enhance the growth and production of lipase from *A. niger*, a BSF was developed using the substrates WB and beef fleshing.

### Mixed Substrate Fermentation

#### Single Substrate Fermentation

Lipase production from *A. niger* was carried out with the substrates WB, COC, WR and beef fleshing and the results are shown in Table 4.2. The lipase activities were 260, 192 and 301 U/gds with WB, FL and COC, respectively, at 96 h, while a higher lipase activity of 310 U/gds was obtained with WR at 72h.

**Fig 1 Lipase Production by Single Substrate Fermentation****Table 1 Lipase Production by Single Substrate Fermentation**

| Substrate | 24h       | 48h        | 72h        | 96h        | 120h       |
|-----------|-----------|------------|------------|------------|------------|
| WB(10%)   | 55.3 ± 12 | 134 ± 19   | 235.8 ± 15 | 260.5 ± 12 | 214.1 ± 11 |
| FL(10%)   | 40.5 ± 17 | 93.8 ± 13  | 147.4 ± 18 | 192.9 ± 17 | 163 ± 14   |
| COC (10%) | 60.1 ± 19 | 192.3 ± 18 | 281.7 ± 11 | 301.4 ± 18 | 258.6 ± 18 |
| WR (10%)  | 85.5 ± 12 | 179 ± 17   | 310.1 ± 16 | 293.1 ± 18 | 278.5 ± 16 |

**Bi-substrate Fermentation**

Accordingly, BSF was carried out by addition of fleshing to WB in different ratio (Table 4.3). Maximal lipase activities of 404 and 345 U/gds were obtained [16-20] with combinations of FL: WB (1:1) and WB: FL (1:3), respectively, at 96 h. The enzyme yields obtained with other combinations were also higher than individual substrates.

**Fig 2 Lipase Production by BSF**

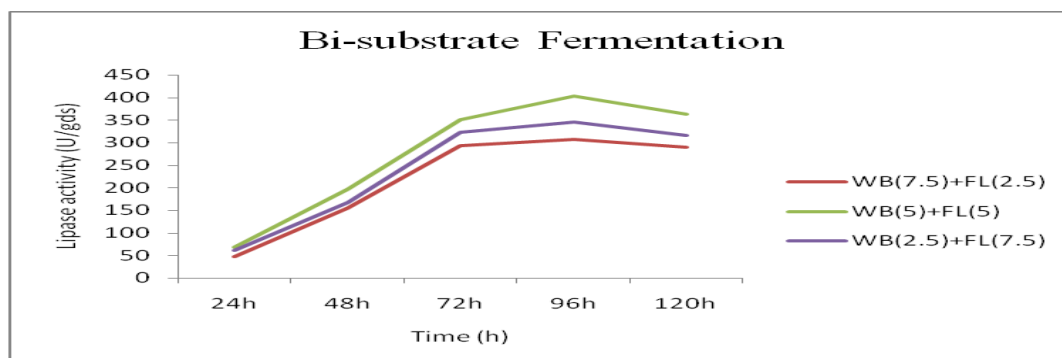


**Table 2 Lipase Production by BSF**

| Substrate Combination | 24h       | 48h        | 72h        | 96h        | 120h       |
|-----------------------|-----------|------------|------------|------------|------------|
| WB(7.5)+FL(2.5)       | 48.2 ± 12 | 156.5 ± 16 | 294.8 ± 12 | 308.7 ± 18 | 291.8 ± 12 |
| WB(5)+FL(5)           | 68.1 ± 15 | 198.7 ± 10 | 352.3 ± 18 | 404.8 ± 17 | 363.5 ± 11 |
| WB(2.5)+FL(7.5)       | 62.4 ± 19 | 168.6 ± 18 | 323.5 ± 15 | 345.3 ± 15 | 315.2 ± 18 |

Accordingly, BSF was carried out by addition of COC and WR to WB independently. Maximal lipase activities of  $459.1 \pm 16$  and  $362.2 \pm 18$  U/gds were obtained with combinations of COC: WB (1:3) and WR: WB (1:1), respectively, at 96 h. The enzyme yields obtained with other combinations [37-39] were also higher than individual substrates, since the nutrients and trace elements present in the BSF enables the organisms to yield more protein favourably by influencing its biochemical pathways for lipase production (Cordova *et al.*, 1998).

The effect of different substrates such as gingelly oil cake, cottonseed oil cake, groundnut oil cake, rice bran, wheat bran, gingelly seed, castor bean seed, groundnut seed, sugarcane baggasse, groundnut kernel, coffee husk and spent barley on lipase production by *A. niger* was reported earlier by Kamini *et al.*, (1998). Among the substrates, gingelly oil cake was found to be the best substrate with a lipase activity of 363.6 U/dgs at 72 h. Later, Mala *et al.* (2007) reported a 36.0% [24-29] increase in lipase activity by adding GOC to WB in a mixed solid substrate fermentation (GOC: WB, 1:3, w/w), which was comparatively higher than the activity obtained with WB alone.

### Tri-substrate Fermentation

In order to enhance the lipase production, TSF experiments were carried out using FL, COC, WB and WR (Table 4.4), since maximum lipase activity was obtained with a combination of 5 g of FL and 5 g of WB in BSF (Table 4.3). In TSF, an optimal lipase activity of 422 U/gds [16-21] was obtained using a mixture of WB, 5 g; COC, 2.5 g and WR, 2.5 g (Table 4.4), which was comparatively higher than other combinations of TSF.

**Fig 3. Lipase Production by TSF**

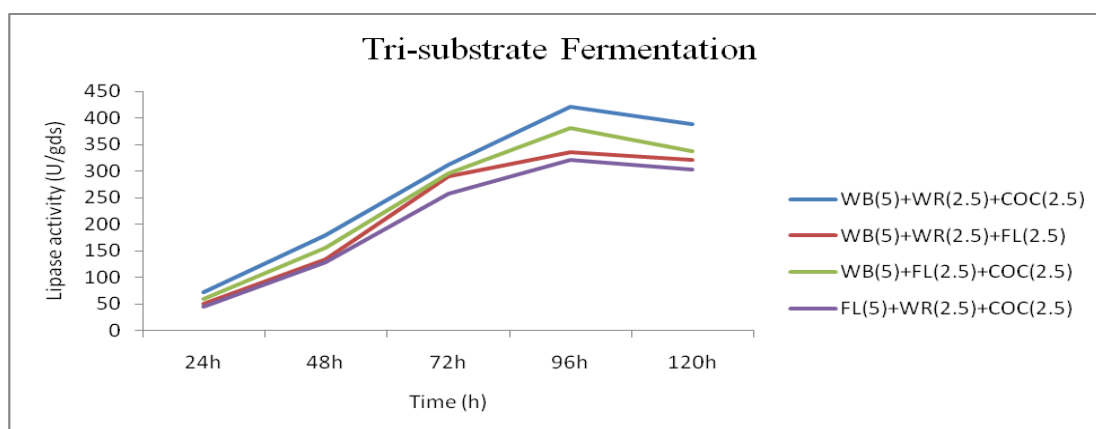
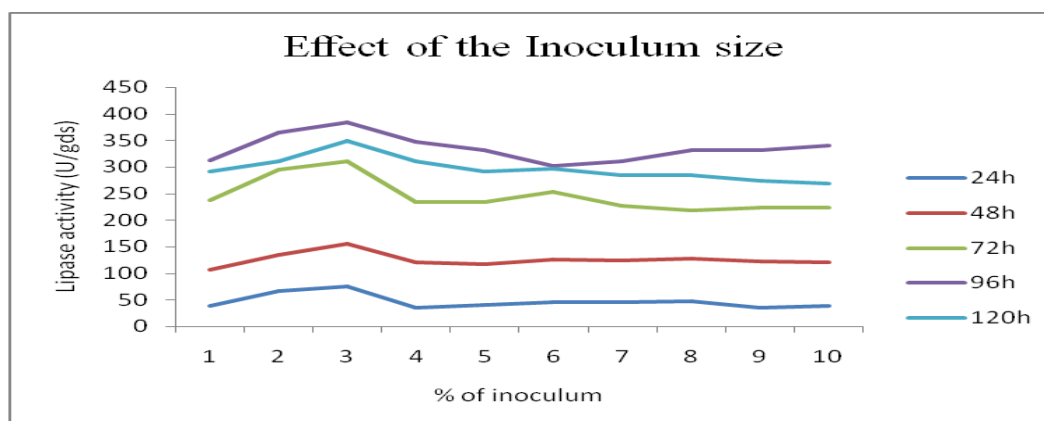


**Table 3 Lipase Production by TSF**

| Substrate Combination  | 24h      | 48h       | 72h       | 96h       | 120h      |
|------------------------|----------|-----------|-----------|-----------|-----------|
| WB(5)+WR(2.5)+COC(2.5) | 72.1 ±11 | 180.8 ±16 | 312.2 ±14 | 422.7 ±11 | 389.4 ±18 |
| WB(5)+WR(2.5)+FL(2.5)  | 51.3 ±15 | 135.1 ±13 | 289.7 ±13 | 335.6 ±17 | 320.2 ±11 |
| WB(5)+FL(2.5)+COC(2.5) | 60.8 ±10 | 156.7 ±12 | 295.3 ±11 | 381.4 ±15 | 338.1 ±15 |
| FL(5)+WR(2.5)+COC(2.5) | 44.5 ±18 | 128.6±12  | 258.4 ±17 | 321.9 ±13 | 302.1 ±19 |

### Effect of Inoculums Size and Moisture Content on Lipase Production

**Fig 4 Effect of Inoculums on Lipase Production**

**Table 4 Effect of Inoculum on Lipase Production**

| Percentage of Inoculum | 24h       | 48h        | 72h        | 96h        | 120h       |
|------------------------|-----------|------------|------------|------------|------------|
| 1                      | 38.1 ± 11 | 106.7 ± 16 | 238.8 ± 13 | 313.2 ± 17 | 292.1 ± 13 |
| 2                      | 67.2 ± 16 | 134.1 ± 18 | 295.9 ± 16 | 365.3 ± 12 | 310.2 ± 13 |
| 3                      | 75.7 ± 18 | 156.5 ± 16 | 310.4 ± 17 | 384.5 ± 14 | 350.6 ± 12 |
| 4                      | 36.7 ± 19 | 121.8 ± 12 | 234.2 ± 18 | 347.6 ± 13 | 310.9 ± 17 |
| 5                      | 41.2 ± 10 | 118.1 ± 19 | 234.4 ± 13 | 332.5 ± 11 | 292.8 ± 18 |
| 6                      | 46.5 ± 14 | 126.6 ± 12 | 253.8 ± 15 | 302.1 ± 12 | 296.6 ± 18 |
| 7                      | 46.7 ± 19 | 125.6 ± 16 | 227.5 ± 18 | 310.2 ± 14 | 284.5 ± 15 |
| 8                      | 47.1 ± 15 | 128.6 ± 16 | 219.1 ± 10 | 332.5 ± 19 | 284.4 ± 12 |
| 9                      | 36.7 ± 18 | 122.4 ± 17 | 223.6 ± 14 | 332.6 ± 15 | 275.6 ± 11 |
| 10                     | 38.3 ± 16 | 121.5 ± 18 | 223.3 ± 16 | 341.9 ± 16 | 269.8 ± 17 |

The initial moisture content and inoculum concentration were found to be the critical factors for lipase production. Accordingly, [21-29] a maximum yield of 422 U/gds was obtained at 96 h with an initial moisture content of 60% and an inoculum concentration of 2.5%.

**Fig 5 Effect of Moisture Content on Lipase Production**

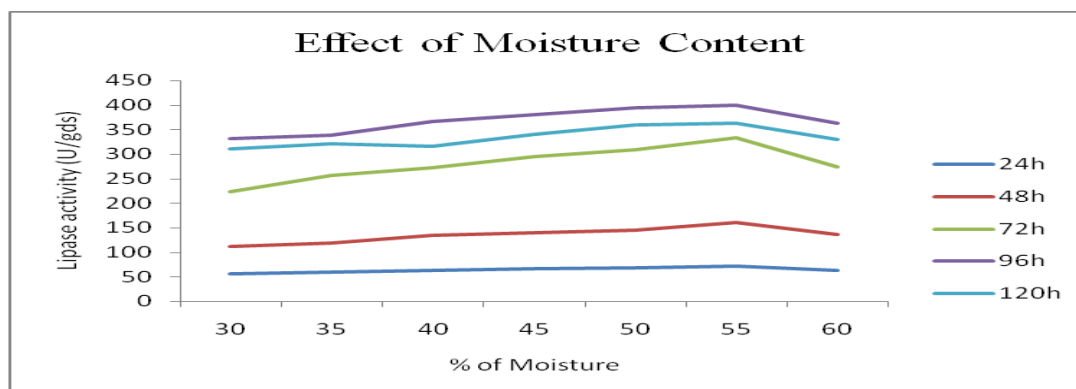
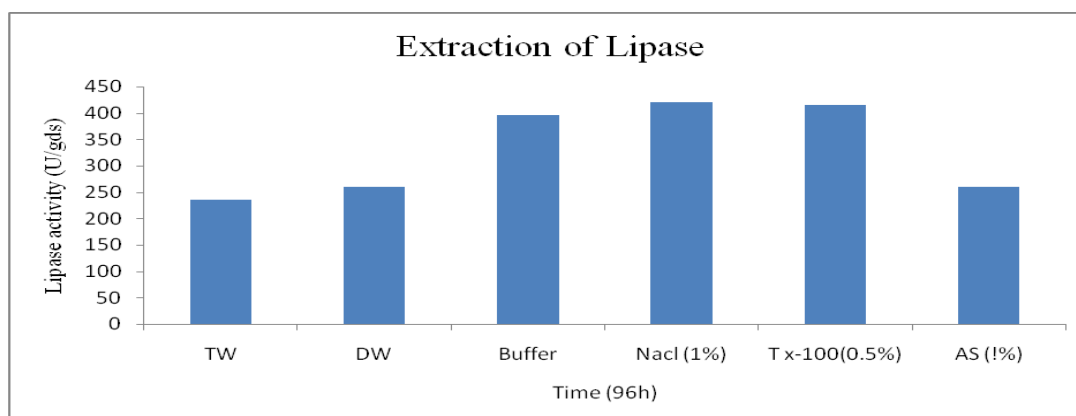


**Table 5. Effect of Moisture Content on Lipase Production**

| Percentage of Moisture | 24h       | 48h        | 72h        | 96h        | 120h       |
|------------------------|-----------|------------|------------|------------|------------|
| 30                     | 56.1 ± 17 | 112.6 ± 14 | 224.2 ± 12 | 332.6 ± 12 | 310.4 ± 19 |
| 35                     | 58.8 ± 13 | 120.4 ± 15 | 257.3 ± 16 | 340.6 ± 14 | 321.2 ± 11 |
| 40                     | 62.3 ± 18 | 135.1 ± 18 | 273.6 ± 13 | 367.1 ± 12 | 315.1 ± 12 |
| 45                     | 66.4 ± 17 | 140.2 ± 18 | 296.8 ± 13 | 380.4 ± 18 | 340.4 ± 12 |
| 50                     | 69.6 ± 18 | 146.3 ± 19 | 309.3 ± 15 | 394.6 ± 19 | 360.3 ± 17 |
| 55                     | 72.7 ± 16 | 161.4 ± 16 | 334.5 ± 14 | 399.4 ± 11 | 363.2 ± 14 |
| 60                     | 63.9 ± 16 | 136.6 ± 17 | 275.1 ± 12 | 363.2 ± 18 | 330.2 ± 11 |

There was a decline in enzyme production an increase or decrease in moisture content from 60% to 30%, respectively, at 96 h (Table 4.6). This could be due to the suboptimal growth of the fungus and lower degree of substrate swelling at lower moisture levels as reported by Zadrazil and Brunnert (1981), while at increased moisture levels, the porosity of the substrate was decreased and thereby, limiting the gas exchange and increasing the chances of contamination (Mahadik *et al.*, 2002; Adinarayana *et al.*, 2003b). There was no significant increase in [30-34] lipase activity upon increasing the inoculum concentration up to  $4.3 \times 10^8$  spores/g substrate. Supplementation of additives such as carbon and nitrogen sources and inducers to the TSF did not show any effect on production of lipase (data not shown). Moreover, these additives could possibly lead to contamination problems and also increase the cost of production as reported by Mala *et al.* (2007). Hence, the control system without any additives was used for further studies.

### Extraction of the Enzyme

**Fig 6 Effect of Salts and Surfactants on Lipase Extraction****Table 6 Effect of Salts and Surfactants on Lipase Extraction**

| Salts and Surfactants  | Activity (U/gds) |
|------------------------|------------------|
| Tap Water              | 235.5 ± 12       |
| Distilled Water        | 260.2 ± 18       |
| Buffer                 | 396.1 ± 15       |
| Nacl (1%)              | 420.2 ± 15       |
| Triton x-100(0.5%)     | 415.6 ± 14       |
| Ammonium Sulphate (1%) | 260.7 ± 16       |

Extraction of the enzyme from TSF was carried out with tap water, distilled water, buffer, salts (sodium chloride or ammonium sulphate) and surfactants (Tween 80 or Triton X-100). The recovery of the enzyme was [35-37]optimal with sodium chloride (1%), however, supplementation of sodium chloride with Triton X-100 (0.5%) helped in increasing the recovery of enzyme from 420 and 415 U/gds (Table 4.7). This could be due to the use of surfactant, Triton X-100, which might increase the permeability of cells resulting in higher recovery of enzyme from TSF. Similar results were reported for the extraction of lipase from *A. niger* with sodium chloride (1%) and Triton X-100 (0.5%) (Mahadik *et al.*, 2002),

Extraction of lipase was inhibited by ammonium sulphate (1%) and this could be due to the increased hydrophobic interactions between lipase and solid support, thereby preventing the enzyme release as reported by Rodriguez *et al.* (2006). However, supplementation of sodium chloride with Triton X-100 (0.5%) helped in increasing the recovery of enzyme from  $662.4 \pm 16$  to  $745.7 \pm 11$  U/gds. This could be due to the use of surfactant, Triton X-100,[38-41] which might increase the permeability of cells resulting in higher recovery of enzyme from TSF. Similar results were reported for the extraction of lipase from *A. niger* with sodium chloride (1%) and Triton X-100 (0.5%) (Mahadik *et al.*, 2002), while Balaji and Ebenezer (2008) reported a maximum recovery of lipase from *Colletotrichum gloeosporioides* using Triton X-100 alone. Furthermore, the reported lipase activity ( $745.7 \pm 11$  U/gds) and productivity (7.76 U/g/h) obtained with our strain are comparatively higher than that of Mahadik *et al.* (2002), 4.37 U/g/h, ul-Haq *et al.* (2002), 2.16 U/g/h, Babu and Rao (2007), 0.05 U/g/h, Mahanta *et al.* (2008), 5.21 U/g/h and Godoy *et al.* (2009), 0.62 U/g/h.

#### **Hydrolysis of Beef Fleshing using Lipase from *Aspergillus niger***

The fleshing was hydrolyzed efficiently to 71.3% at 24 h initially and optimization studies showed an increase in the hydrolytic ratio by 15.6% at 24 h, when the reaction mixture contained 10 g of fleshing, 25 ml of 0.1 M phosphate buffer (pH 7.0), and 250 U of lipase. Accordingly, the fermented substrate could be directly used for the hydrolysis of tallow and in the production of fatty acid esters, since the fuel produced from tallow has the advantage of a higher calorific value and cetane number than the fuels obtained from vegetable oils as reported by Lebedevas *et al.* (2006).[42-44] Moreover, the reported hydrolytic ratio was comparatively higher with our lipase, than the hydrolytic ratios of beef fleshing obtained with lipases from *Rhizomucor miehei* (73%) and *Yarrowia lipolytica* (65%) (Adamczak and Bednarski, 2004), while a higher hydrolytic ratio of 93.86% was reported by Gao *et al.* (2009) using surfactant coated commercial lipase of *C. Rugosa* (Novoenzymes, China). However, the direct application of the fermented substrate for fleshing hydrolysis by *A. niger* makes the process economical and avoids the need for expensive enzyme recuperation and immobilization processes. The potential of this system in transesterification reaction deserves to be explored further.

#### **Fig 7 Hydrolysis of Fleshing by *A. niger* Lipase**

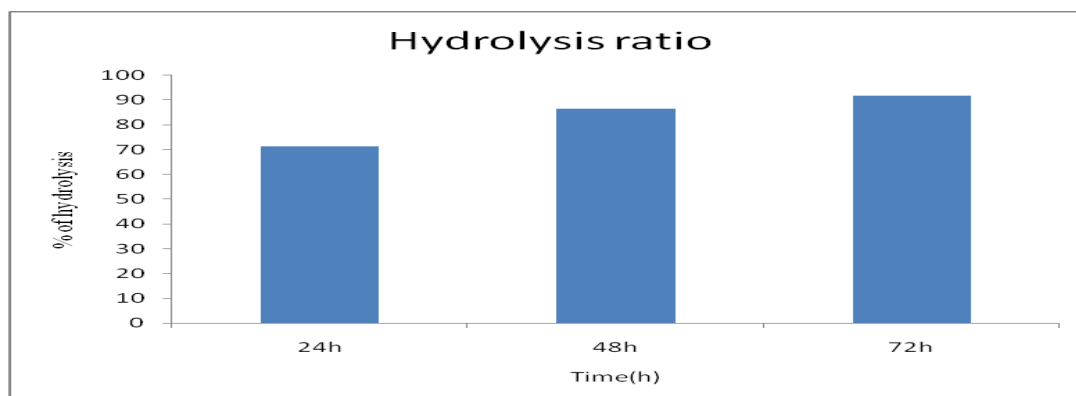


**Table 7 Percentage of Fleshing Hydrolysis by Lipase**

| S.No | Incubation | Hydrolysis Percentage |
|------|------------|-----------------------|
| 1    | 24h        | 71.30                 |
| 2    | 48h        | 86.58                 |
| 3    | 72h        | 91.67                 |

The direct application of fermented substrate on fleshing hydrolysis was evaluated, since the use of fleshing is declined due to the changing feeding habits of human beings and all the excess tallow produced are not used in soap industry (Bhatti *et al.*, 2008). Hence, it is economical to consider fleshing, a low cost feedstock, in oleochemical industries for the production of fatty acids and their corresponding esters using enzymes, because they make the process energy efficient than the conventional thermal fat splitting process, which requires operations at elevated temperature and pressure (Edwinoliver *et al.*, 2010).[44,45]

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