Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 5, Suppl 4, 2013

Research Article

EFFECT OF DIFFERENT PARAMETERS DURING FERMENTATION ON BIOTRANSFORMATION OF FENOFIBRATE BY *ASPERGILLUS TERREUS*

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Received: 18 May 2013, Revised and Accepted: 15 Sep 2013

ABSTRACT

Objective: The objective of present research is to study the effect of different parameters like media components such as carbon and nitrogen sources used in media, incubation period and agitation speeds on metabolite production, also on their respective cell dry weight.

Method: The fermentation was carried out using Potato dextrose broth media. Broth medium was substituted with different carbon sources like D-fructose, D-sorbitol, D [+] galactose, D-maltose, and starch and nitrogen sources like ammonium acetate, urea, barium nitrate and calcium nitrate. At 2,12,14,16,18,20,22,24,36,42,48,52,60 hrs of incubation period and 60,80,100,120,140,160 rpm of agitation speed fermentation was conducted to find the suitable conditions for maximum metabolite production and cell dry weight

Results: Maximum metabolite formation and cell dry weight was observed with dextrose [2%], yeast [0.01%] when used as different carbon, nitrogen sources. Maximum biotransformation and cell dry weight was observed at 48th hour of incubation period compared to other time intervals and when agitation intensity was kept at 120 rpm.

Conclusion: Based upon the results obtained it can be concluded that there is an effect of media components, incubation period and agitation speeds on biotransformation of fenofibrate to its active metabolite fenofibric acid.

Keywords: Fenofibrate, Biotransformation, *Aspergillus terreus*, Carbon, Nitrogen sources, Fenofibric acid.

INTRODUCTION

Drug metabolism leads to chemical alteration of the drug to more polar and hydrophilic metabolites, which are easily excreted from the body[1]. The ability of humans to metabolize and clear the drugs is a natural process that involves the same enzymatic pathways and transport systems that are utilized for normal metabolism of dietary constituents. Human come into contact with crores of foreign chemicals or xenobiotics through exposure to environmental contaminants as well as drugs. All the molecules [endogenous and exogenous] undergo biotransformation interaction with biological systems [enzymes, enzymatic systems, cells, tissues, cells, organs]. Hence understanding of drug metabolism plays an important role in the development of new drug entities[2]. Hence the approval and usage of drug in human subjects require extensive studies to establish its safety and efficacy[3]. Hence a better understanding of the metabolism of drug is essential to know about the drug action, toxicity, distribution, excretion and storage in the body. The knowledge of drug metabolism is also important for studying induction or inhibition of several drugs[4]. Microbial models have a number of advantages over studies with animals and humans, due to reduced animal use, ease of setup and manipulation, higher yield and diversity of metabolite production, and lower cost of production. In some cases microbial models can provide sufficient amounts of putative metabolites for complete structural elucidation. Metabolite production is especially important for metabolites that are not easily synthesized by organic chemical methods[5].

Fenofibrate is used in treatment of type II a, type II b, type III, type IV and type V hyperlipoproteinaemias[6]. It is metabolised into active metabolite fenofibric acid[7].

In present research work different microorganisms were screened for metabolism of fenofibrate to its active metabolite fenofibric acid and effect of different parameters on this biotransformation were performed using different carbon, nitrogen sources at various concentrations, incubation period and agitation speeds.

MATERIALS AND METHODS

Microorganisms

Aspergillus niger [NCIM 545], *Aspergillus terreus* [NCIM 657], *Aspergillus ochraceus* [NCIM 1140], *Aspergillus flavus* [MTCC 1783], *Cunninghamella elegans* [NCIM 689], *Cunninghamella echinulata* [NCIM 691], *Cunninghamella blakesleeana* [MTCC 3729], *Rhizopus stolonifer* [NCIM 880], *Gliocladium roseum* [NCIM 1064] were procured from National Chemical Laboratory [NCL] Pune, India and microbial type culture collection and Gene bank [MTCC], Chandigarh, India.

Chemicals

Fenofibrate was obtained as gift sample from Therdose Private Limited, Hyderabad, India. All the solvents used for analysis were HPLC grade. The remaining chemicals and culture media components were purchased from Qualigens & S.D. fine chemicals, Mumbai, India.

Maintenance of pure cultures

The pure cultures were regularly transferred into respective fresh agar slants for every 6 months and were stored at 4°C to maintain viability. Potato dextrose broth consisted of Potato chips [20gm/100ml, steamed for 30 min], dextrose 2gm, yeast extract 10mg, distilled water upto 100ml [final pH 5.6] used for *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus ochraceus, Aspergillus flavus, Cunninghamella elegans, Rhizopus stolonifer , Gliocladium roseum.* For Potato dextrose broth consisted of Potato chips, 20 gm / 100 ml [steamed for 30min]; dextrose, 2 gm; distilled water up to 100 ml; [final pH 5.6] used for *Cunninghamella echinulata.* Oat meal flakes, 3gm/100ml [steamed for 30min] used for *Cunninghamella blakesleeana*.

Fermentation procedure

The fermentation was carried out in 250ml Erlenmeyer flask containing 50ml of broth media labelled as drug control, culture control and sample. For each two controls and one sample was used. Study consisted of one drug control which had drug being incubated without organism, culture control consisted of broth medium inoculated with a loopful of respective fungi. Sample flask consisted of both drug and culture. Two controls and sample flasks were incubated on orbital shaker under identical conditions to obtain the prominent growth of microorganisms for biotransformation study.

Extraction procedure

The incubated flasks were taken out from shaker incubator and heated on water bath at 50°C for 30 min for inactivation of grown microbes. Then, these were transferred into centrifuge tubes and

centrifuged at 3000 rpm for 10 min [R8C: Remi instruments, Mumbai, India]. The supernatant obtained was collected in separate boiling tubes. The supernatant of drug and its metabolites were extracted by using dichloromethane. Then organic layer was collected and air dried. The dried extract was reconstituted with mobile phase for HPLC analysis.

Effect of influence of carbon and nitrogen sources on biotransformation

To study the influence of carbon and nitrogen sources on metabolite production, the carbon source i.e. dextrose in fungal medium was substituted with other carbon sources like D-fructose, D-sorbitol, D-[+] galactose, D-maltose, and starch in same quantity. Similarly the nitrogen source i.e. yeast in fungal medium was substituted with other nitrogen sources like ammonium acetate, urea, barium nitrate and calcium nitrate.

Further to study the influence of concentration of carbon source on metabolite production, 0%, 1%, 2%, 3%, 4% and 5% of dextrose was used in the fungal medium. To study the influence of nitrogen source on metabolite production, 0.005%, 0.01%, 0.02% of yeast extract was used in the fungal medium, by keeping other components constant.

Effect of incubation time on biotransformation

The effect of incubation period on transformation of fenofibrate was studied by analyzing the culture broth at the end of 0,2,12,14,16,18,20,22,24,36,42,48,52,60 hrs of incubation.

Effect of different agitation speeds on biotransformation

The general method is repeated at different shaking speeds of shaker incubator such as 60, 80, 100, 120, 140, 160 rpm and flasks are taken out after 48 hrs of incubation [optimal time] for analysis of the amount of metabolite formed.

Determination of biomass production [cell dry weight]

The biomass production at different conditions was determined as it was correlated with the quantity of metabolite produced under same conditions by the following procedure. An empty eppendroff tube is weighed [w_1]. Fixed amount of filtered suspension cell material is added into the eppendroff tube. The eppendroff tube [cell material] is weighed again [w_2]. The eppendroff tube with the cell material is placed into an oven at 60 °C until consecutive readings are obtained. The eppendroff tube [including dried cell material] is weighed again [w_3], cell dry weight was calculated from the three weights with the following formula.

Dry weight percentage = [w_3-w_1] / [w_2-w_1] x 100 is calculated.

Analytical Methods

High pressure liquid chromatography

Fenofibrate and its metabolite in the extracted samples were estimated by High Performance Liquid Chromatography [HPLC] method[8]. The HPLC system [Waters, USA] consisted of Waters 515 solvent delivery module and Waters 2489 UV-visible spectrophotometric detector. The mobile phase consisted of Acetonitrile : Water [70:30] with a flow rate of 1ml/min. The column used was C-18 [stainless steel column of 25 cm length and 4.6 mm internal diameter packed with porous silica spheres of 5 µ diameter, 100 Å pore diameter – II 5C-18 rs – 100a, 5 µm, 4.6 x 250 mm]. The eluent was monitored at 286 nm. sensitivity was set at 0.001 a.u.f.s.

The percentage of drug and metabolite was calculated based on the area of peak obtained during HPLC analysis and percentage of metabolite formed under different optimized conditions was compared.

RESULTS

The results of HPLC analysis of fenofibrate and its metabolite in different culture extracts are shown in the Table 1. The peak at retention time of 1.8min represented solvent peak, peaks at 1.4min and 2.2min represented various culture contents and peak at retention time of 10.8min indicated fenofibrate. Interestingly, the sample of *Aspergillus terreus* shown an extra metabolite peak at 2.64 min compared to its controls as shown in Figure 1.

Effect of different conditions on biotransformation of fenofibrate:

The effect of different parameters on biotransformation of fenofibrate by *Aspergillus terreus* was studied to optimize the conditions for maximum production of fenofibrate metabolite using the fungal biotransformation method.

Effect of Carbon sources

The effect of selected carbon sources and cell dry weight on metabolite production by *Aspergillus terreus* in its growth medium was studied and results are given in Figure 2. The percentage of metabolite was in the range of 1.1% - 62% at different carbon sources and cell dry weight was 0.5%-2.37%.

Table 1: HPLC data of fenofibrate and its metabolite from microbial culture extracts

Name of the organism	Retention time[min]			
	Blank I [Drug control]	Blank II [Culture control]	Control [fenofibrate]	Sample
Gliocladium roseum	1.8	1.8	-	1.8
[NCIM 1064]	10.8	-	10.8	10.8
Aspergillus flavus	1.8	1.8	-	1.8
[MTCC 1783]	2.2	-	-	2.2
	10.8	-	10.8	10.8
Cunninghamella elegans	-	1.4	-	1.4
[NCIM 689]	1.8	1.8	-	1.8
	10.8	-	10.8	10.8
Cunninghamella echinulata	-	1.4	-	1.4
[NCIM 4279]	1.8	1.8	-	1.8
	10.8	-	10.8	10.8
Cunninghamellablakesleeeana	-	1.4	-	1.4
[MTCC 3729]	1.8	1.8	-	1.8
	10.8	-	10.8	10.8
Aspergillus terreus	1.8	1.8	-	1.8
[NCIM 657]	-	-	10.8	2.64*
	10.8	-	-	10.8
Rhizopus stolonifer	1.8	1.8	-	1.8
[NCIM 880]	10.8	-	10.8	10.8
Aspergillus ochraceous	1.8	1.8	-	1.8
	2.2	-	-	2.2
[NCIM 1140]	10.8	-	10.8	10.8

"*"- Metabolite

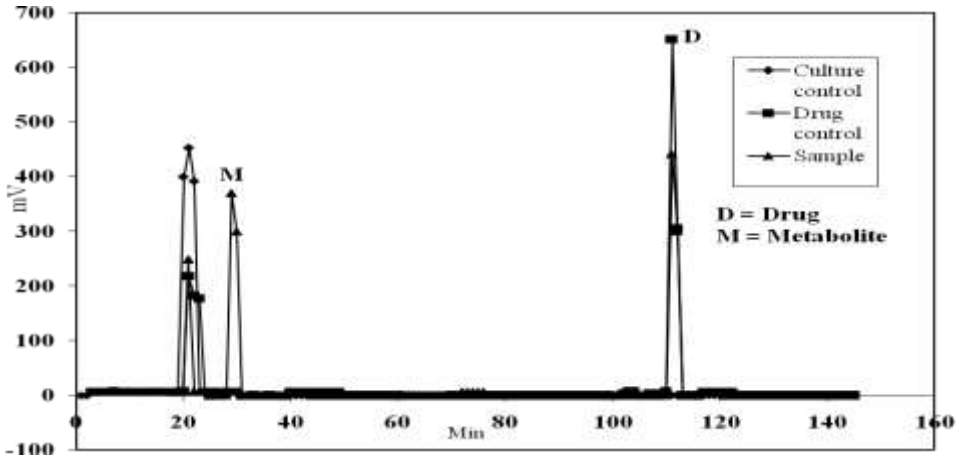

Fig. 1: HPLC chromatogram of fenofibrate from culture extracts of *Aspergillus Terreus*

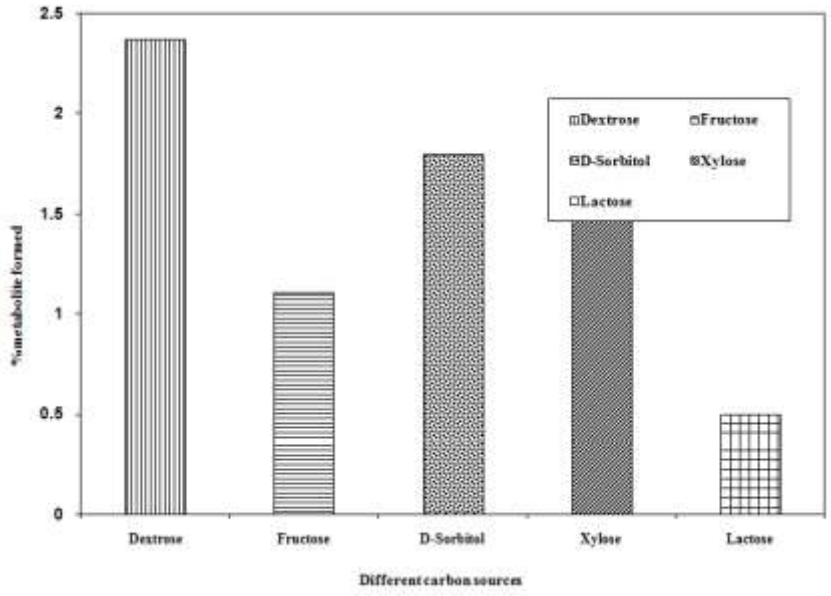

Fig. 2: Histogram representation of effect of different carbon sources on metabolite production

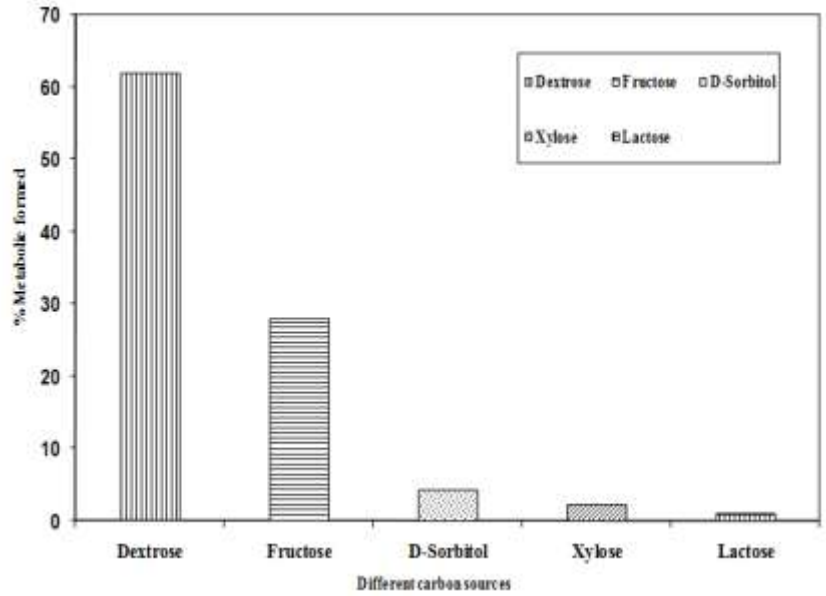

Fig. 3: Histogram representation of effect of different nitrogen sources on metabolite production

Effect of Nitrogen sources

Similarly the effect of selected nitrogen sources and respective cell dry weight on metabolite production by *Aspergillus terreus* in its medium was studied and results are given in Figure 3. The percentage of metabolite formation at different nitrogen sources was in the range of 5.2% - 62% and and cell dry weight 0.4% - 3.3%.

Based on percentage metabolite formed in the above results, dextrose and yeast extract were selected as the best carbon and nitrogen sources. Then the effect of their concentration on metabolite production so as to optimize the conditions that favours the maximum growth of *Aspergillus terreus* to collect the fenofibric acid in large amounts was studied and results are shown in Table 2 and Table 3.

Table 2: Effect of concentration of dextrose on metabolite production and its percent cell dry weight at different concentrations of dextrose.

% of dextrose	Peak area of the metabolite in HPLC analysis	Average % of metabolite	% cell dry weight
0	236821	2.7	0.23
1	874682	9.9	0.89
2	5437879	**62***	**2.37***
3	3912645	44.6	2.06
4	2164147	24.6	0.85
5	1984295	22.6	0.82

"*" – Maximum % of metabolite and cell dry weight formed.

Table 3: Effect of concentration of yeast extract on metabolite production and its percent cell dry weight at different concentrations of yeast extract.

% of the yeast extract	Peak area of the metabolite in HPLC analysis	Average % of metabolite	% of cell dry weight formed
0.005	3246421	37	1.18
0.01	5437879	**62***	**2.37***
0.02	3946472	45	2.09

"*" – Maximum % of metabolite and cell dry weight formed.

Incubation time

Effect of incubation time [hrs] on amount of metabolite formed was studied at different time periods such as 0, 2, 12, 14, 16, 18, 20, 22, 24, 36, 42, 48, 52, 60 hrs of incubation. The percentage of metabolite and percentage cell dry weight formed at different time periods is given in Figure 4.

Agitation speed

Effect of agitation speed on amount of metabolite formed was studied at different rpm such as 60, 80,100, 120, 140, 160 rpm and the percentage of metabolite formed at different agitation speeds is shown in Figure 5.

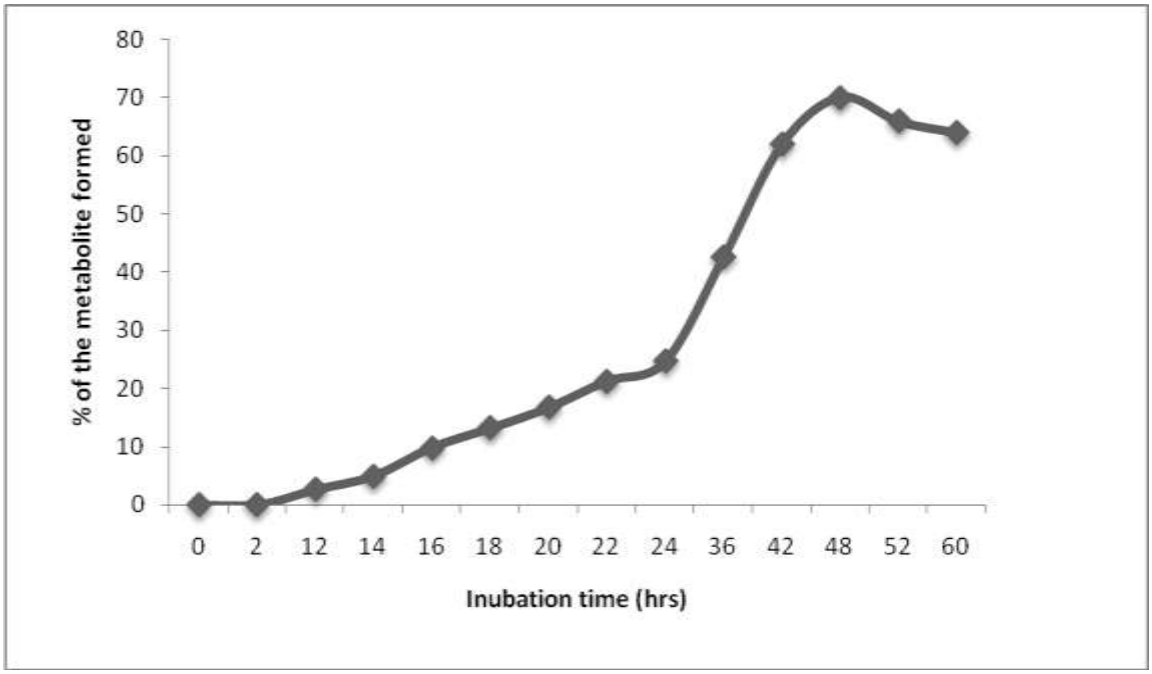

Fig. 4: Graphical representation of effect of incubation time on metabolite production

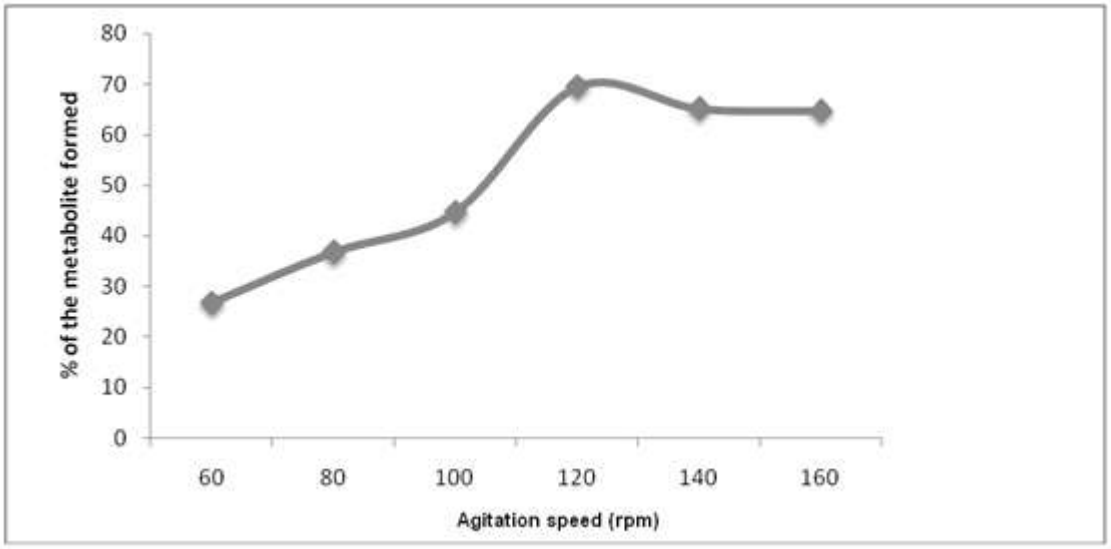

Fig. 5: Graphical representation of effect of agitation speed during incubation on metabolite production

Relationship between cell dry weight and metabolism

The relationship between cell dry weight and metabolite production at different conditions was determined by calculating correlation coefficient.

DISCUSSION

The enzyme synthesis and activation of enzymes also vary with the environmental conditions and media composition like carbon, nitrogen source[9]. It was also reported that the metabolite production by microbes was influenced by the composition of media i.e., type and concentration of carbon and nitrogen sources employed for the study[10,11].

The effect of different carbon and nitrogen sources on the metabolite production and its cell dry weight at different sources were determined as the media components influence the physical and chemical integrity of the cells of an organism. The change in carbon and nitrogen sources and change in concentration of these sources have shown differences in height and area of the peak, indicated the influence on quantity of metabolite formed or on biotransformation of fenofibrate by *Aspergillus terreus*. In the present study, effect of different carbon sources [dextrose, fructose, D-sorbitol, xylose, lactose] on metabolite production and cell dry weight was determined. Maximum production of metabolite i.e. 62% and its cell dry weight 2.37% by *Aspergillus terreus* was found with dextrose compared to other carbon sources used. Similarly among the different nitrogen sources used, yeast extract has shown maximum metabolite production i.e. 62% and cell dry weight 2.37%.

Among the selected concentrations of carbon and nitrogen sources 2% of dextrose and 0.01% of yeast extract shown maximum metabolite production i.e. 62% with cell dry weight of 2.37%.

Effect of incubation time

The microbial biotransformation was carried out for a period of 60 hrs and the samples were taken at different regular intervals. The maximum metabolite production i.e. 66.7% with cell dry weight of 2.96% was found after incubation for 48th hr.

Effect of agitation speed

A proper agitation speed is important for appropriate air supply and proper mixing of media components. The degree of agitation required for a fermentation study will be dependent on the organism and the composition of the fermentation medium[12]. In the present study, 69.5 % of metabolite and cell dry weight of 2.96% was found when agitation intensity was kept at 120 rpm. Decrease in the metabolite production at higher agitation speeds might be due to breakage of the fungal cell at higher agitation speed and at lower agitation speeds less amount of metabolite produced might be due to improper mixing of the medium[13,14]. Different agitation speeds seemed to provide different distribution and transportation of air and nutrients to the cells[15]. Correlation coefficient between cell dry weight and metabolite formed was 0.84, which indicated a positive relationship between cell dry weight and metabolite production.

CONCLUSION

Based on the results obtained it can be concluded that there is an influence of the media components, incubation periods and agitation speeds on biotransformation of fenofibrate. From the present results it was found that the dextrose is the best suitable carbon source compared to other carbon sources at 2% concentration and yeast extract at 0.01% is the best suitable nitrogen source as maximum metabolite i.e. 66.7% was obtained at 48th hr of incubation period and at 120 rpm by *Aspergillus terreus*. It can be revealed that *Aspergillus terreus* can be used as an *in vitro* model for the study of metabolism of fenofibrtae [hydrolysis] and also for the production of fenofibric acid, an active metabolite of fenofibrate in maximum yields using 2% dextrose and 0.01% yeast extract in its medium at 120 rpm speed and 48 hrs of incubation.

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