OPTIMIZATION OF MEDIA COMPONENTS FOR HESPERIDINE METABOLISM BY *CUNNINGHAMELLA ELEGANS*

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Summary

Drug metabolism is essential to know the safety and efficacy of a drug as well as in the development of new drug entities. Traditionally several small animal models and *invitro* models like tissue culture, microsomal preparations etc. are being developed of which the microbial model became a very popular. In the present study eight microorganisms were screened for their ability to metabolize hesperidine in a manner comparable to humans to an active metabolite hesperitine. Among the different microorganisms screened, *Cunninghamella elegans* metabolized hesperidine as an extra metabolite peak appeared at retention time of 3.39min. compared to its controls. The influence of the type and concentration of carbon and nitrogen sources in the fungal media on the metabolite production was studied. Maximum biotransformation to produce hesperidine metabolite was found in the presence of dextrose(2%) and yeast extract(0.01%) which might be due to increased mycelia growth or induction of the enzymes required for metabolism.

Key words: Hesperidine, metabolism, *Cunninghamella elegans*, carbon and nitrogen sources

Introduction

Metabolism is a major determinant of the pharmacokinetic properties of most xenobiotics and is often behind bioavailability problems, drug-drug interactions, and metabolic idiosyncrasies [1]. The approval and usage of drugs require extensive studies to establish safety and efficacy, which is an important factor that can be known by drug metabolism. The

metabolic/biotransformation reactions introduce chemical functions into inaccessible sites of molecules to produce rare structures that are highly regio- and stereo selective in nature [2]. Drug metabolism leads to chemical alteration and structural modification of drug that is, conversion of drug to more polar and hydrophilic metabolites, for easy excretion of drug from body compared to lipophillic substances [3]. Different animal models are used traditionally like small animal models(rat, dog etc.), tissue culture, microsomal preparations or perfused organ systems associated with some disadvantages like ethical concerns, bacterial contamination etc..

Microorganism is an excellent source of enzyme, better than plants or animals due to their broad chemical diversity, feasibility of mass culture and ease of genetic manipulation [4,5]. All these enzymes influences the growth of an organism and are essential elements for the synthesis of macromolecules DNA, RNA and proteins [6]. The enzyme synthesis and activation depends on the biosynthesis of nucleic acids and proteins in microbes to maintain the physical and chemical integrity of cell. These may vary with environmental conditions and media composition like effect of carbon and nitrogen sources etc [7]. Rapid enzyme production can be achieved by improvements in media composition and physical parameters. Optimization is defined as highest achievable performance under the given constraints, by maximizing desired factors and minimizing undesired ones. Therefore, by optimizing the biotransformation conditions, the increased metabolite production occurs, which can be evaluated for further pharmacological and toxicological studies [8,9,2].

Flavonoids are a diverse group of secondary metabolites found ubiquitously in the plant kingdom [10]. These occur as aglycons, glycosides and methylated derivatives [11]. Many of these have low toxicity and have a variety of biological effects on various mammalian cell systems, *in vitro* as well as *in vivo* [12]. The two major sites of flavonoids' metabolism are liver and colonic flora. The selected flavonoid, in present study is hesperidine which undergoes hydrolysis by colonic microflora and result in occurrence of intestinal intracellular hesperitin aglycon [13]. Phase II metabolism by UDP-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs) is the predominant metabolic pathway during the first pass metabolism of hesperitin. [14,15,16]. Similarly SULTs catalyse and produce hesperitin-7-O-sulfate and hesperitin-3'-O-sulfate. The extensive metabolism of hesperidine by these cells suggested strongly that the oral bioavailability of hesperidine in humans may be low [13].

Materials and Methods

Microorganisms

Aspergillus flavus(MTCC 1783), Aspergillus ochraceus(NCIM 1140), Cunninghamella elegans(NCIM 689),Cunninghamella blakesleeana (MTCC 3729), Cunninghamella echinulata (MTCC 4279), Rhizopus stolonifer(NCIM 880), Gliocadium roseum (NCIM 1064), Saccharomyces cerevesiae(NCIM 3090). The microbial cultures were obtained from National Chemical Laboratory (NCL), Pune and Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India.

Chemicals

Hesperidine was obtained from Sigma, Mumbai, India. Solvents used were Methanol, Water (HPLC grade) and glacial acetic acid obtained from Merck, Mumbai, India. Culture media components, carbon and nitrogen sources obtained from Merck, Qualigens, SD fine chemicals, Loba, Mumbai, India.

Maintenance of Cultures

All the cultures (fungi and yeast) were maintained on the respective agar slants at 4^0C and transferred for every 6 months to maintain viability. For fungi**,** Potato dextrose broth (Potato chips, 20 gm / 100 ml (steamed for 30min); dextrose, 2 gm ; yeast extract ,10 mg ; distilled water up to 100 ml; final pH 5.6), for *C.elegans, A.terreus, A.ochraceus, A.flavus, Gliocadium roseum and Rhizopus stolonifer*; For yeast MGYP broth consisted of malt extract 0.3gm, glucose 1gm, yeast extract 0.3gm, peptone 0.5gm, distilled water 100ml, pH 6.4-6.8. The respective media were prepared and autoclaved in individual Erlenmeyer flasks at 121^0C at 15 lb/sq. in for 30min before incubation.

Media for studying the influence of carbon and nitrogen sources

The influence of carbon and nitrogen sources in the production of hesperidine metabolite in fungal media was studied by substituting dextrose with different carbon sources like starch, D-fructose, D-sorbitol, D-maltose, D(+) galactose and substituting yeast extract with different nitrogen sources like ammonium acetate, urea, barium nitrate and calcium nitrate tetrahydrate in same quantities. Further the influence of concentration of dextrose and yeast extract in media was also studied by keeping other components constant.

Fermentation procedure

The fermentation was carried out in 250ml Erlenmeyer flasks containing 50ml medium labelled as drug control, culture control and sample. For each biotransformation study two controls and one sample was used. The study included the drug control to which drug solution was added and incubated without organism. Culture control consisted of the medium inoculated under identical conditions with loop full of respective fungi or yeast culture to study whether hesperidine would be chemically transformed during incubation period or bio transformed. The two controls were run simultaneously with sample flask, that consisted of both drug and culture. All the flasks were incubated on the orbital shaker for 24-48hrs, operated at 120rpm at 37^0C for metabolism study.

Extraction Procedure

The incubated medium was heated on a water bath at 50^0C for 30min and centrifuged at 1000rpm for 10min at 37^0C(R8C: Remi instruments, Mumbai, India). Clear supernatant liquid was collected into boiling tubes, cotton plugged closely and stored in refrigerator. The supernatant liquid is filtered and 25µl. portions were injected into the HPLC for analysis. Controls were also prepared similarly to provide suitable blanks.

Analytical technique:

High Performance Liquid Chromatography

High pressure liquid chromatography(HPLC) analysis was carried out using a HPLC system (Waters, USA) consisted of Waters 515 solvent delivery module and Waters 2489 UV-detector and a Wakosil IIS C-18 rs-100[a], 5µl, 4.6 × 250mm stainless steel column. Sensitivity was set at

0.001 a.u.f.s. Mobile phase consisted of methanol:water(0.07% glacial acetic acid) in the ratio 33:67 [17] (Zhang Hongyan., 2009) was used at a flow rate of 1ml/min. Elution was monitored using UV-detector set at 270nm.

The percentage of metabolism was calculated based on peak areas of drug and metabolite obtained in HPLC analysis and the percentage of metabolite formation in presence of different carbon and nitrogen sources in media was compared.

Results

The results of HPLC analysis of hesperidine and its metabolite in different extracts are given in Table 1. The peak at retention time of 2.7min. represented the solvent peak and peaks at 2.3, 2.5min. represented various culture content peaks where as peak at 12.8min. corresponds to hesperidine. An extra peak at 3.39min. observed in sample of *Cunninghamella elegans* represented the formation of metabolite as shown in Fig.1. 30.32% of drug was bio transformed into the metabolite in the potato dextrose medium by *Cunninghamella elegans.*

Effect of carbon and nitrogen sources

The results obtained in the present investigation revealed that the type and concentration of carbon and nitrogen sources have effect on the biotransformation of hesperidine. In the presence of dextrose and yeast extract, produced maximum metabolite i.e. 30.32% compared to other carbon and nitrogen sources as shown in Fig.2 and 3. Similarly, dextrose (2%) and yeast extract (0.01%) produced 30.32% compared to other concentrations of carbon and nitrogen sources as shown in Fig 4 and 5.

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

Name of the organism	Retention time (min.)			
	Blank I	Blank II	Pure hesperidine	Sample
Aspergillus flavus	2.7 12.8	2.7 -	2.7 12.8	2.7 12.8
Aspergillus ochraceus	- 2.7 12.8	2.3 2.7 -	- 2.7 12.8	2.3 2.7 12.8
Cunninghamella elegans	2.7 - 12.8	2.7 - -	2.7 - 12.8	2.7 **3.39*** 12.8
Cunninghamella echinulata	2.7 12.8	2.7 -	2.7 12.8	2.7 12.8
Cunninghamella blakesleeana	2.7 12.8	2.7	2.7 12.8	2.7 12.8
Gliocadiuum roseum	2.7 12.8	2.7 -	2.7 12.8	2.7 12.8
Rhizopus stolonifer	- 2.7 12.8	2.5 2.7 -	- 2.7 12.8	2.5 2.7 12.8
Saccharomyces cerevesiae	2.7 12.8	2.7 -	2.7 12.8	2.7 12.8

"*" – Metabolite

Fig. 1: HPLC chromatogram of hesperidine from culture extracts of *Cunninghamella elegans*

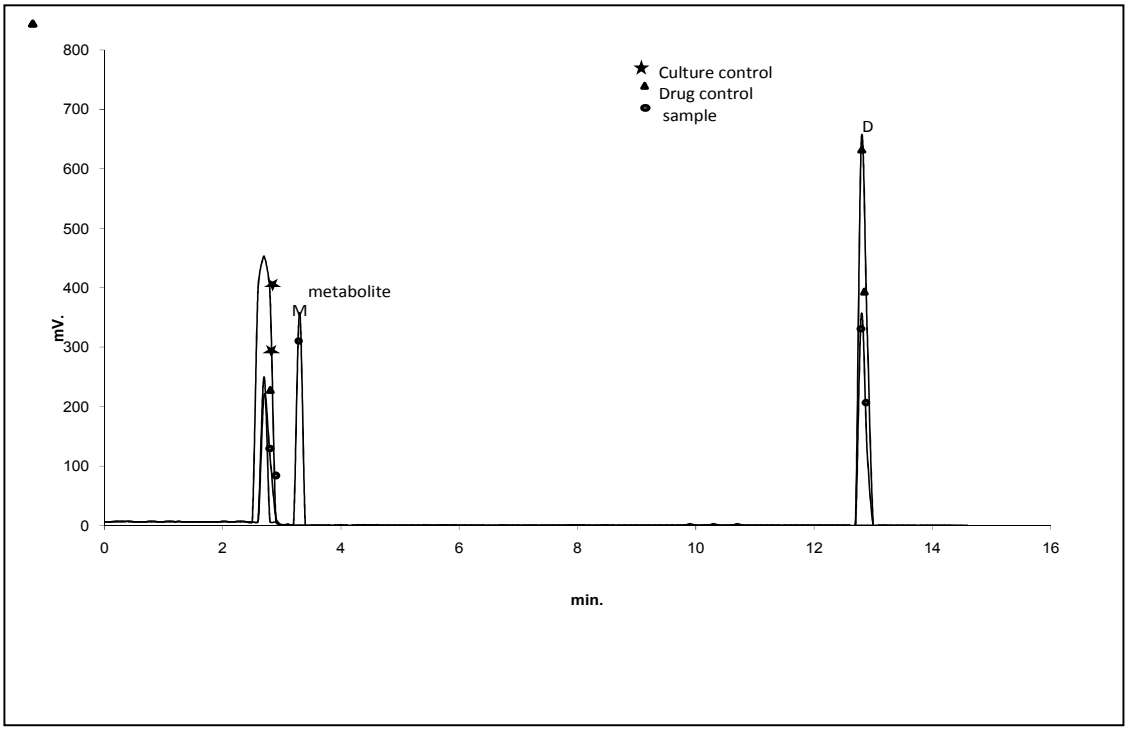


Fig. 2: Histogram representation of effect of different carbon sources on metabolite production

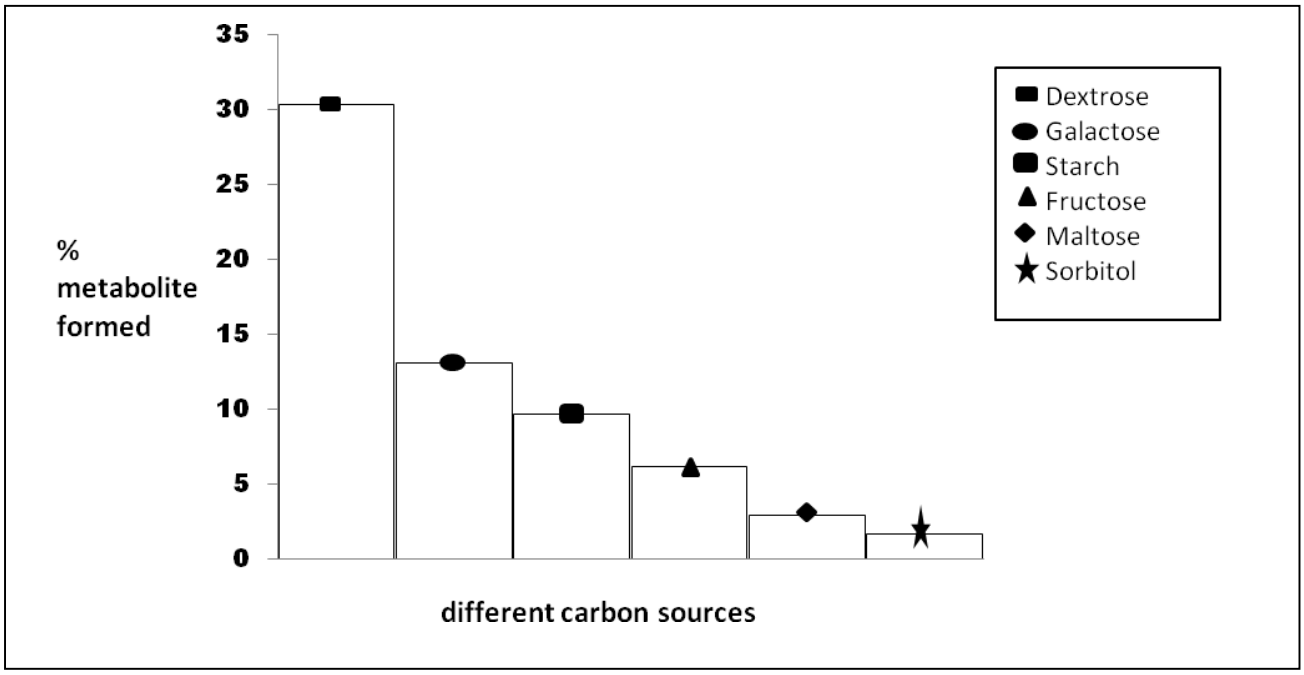


Fig. 3: Histogram representation of effect of different nitrogen sources on metabolite production

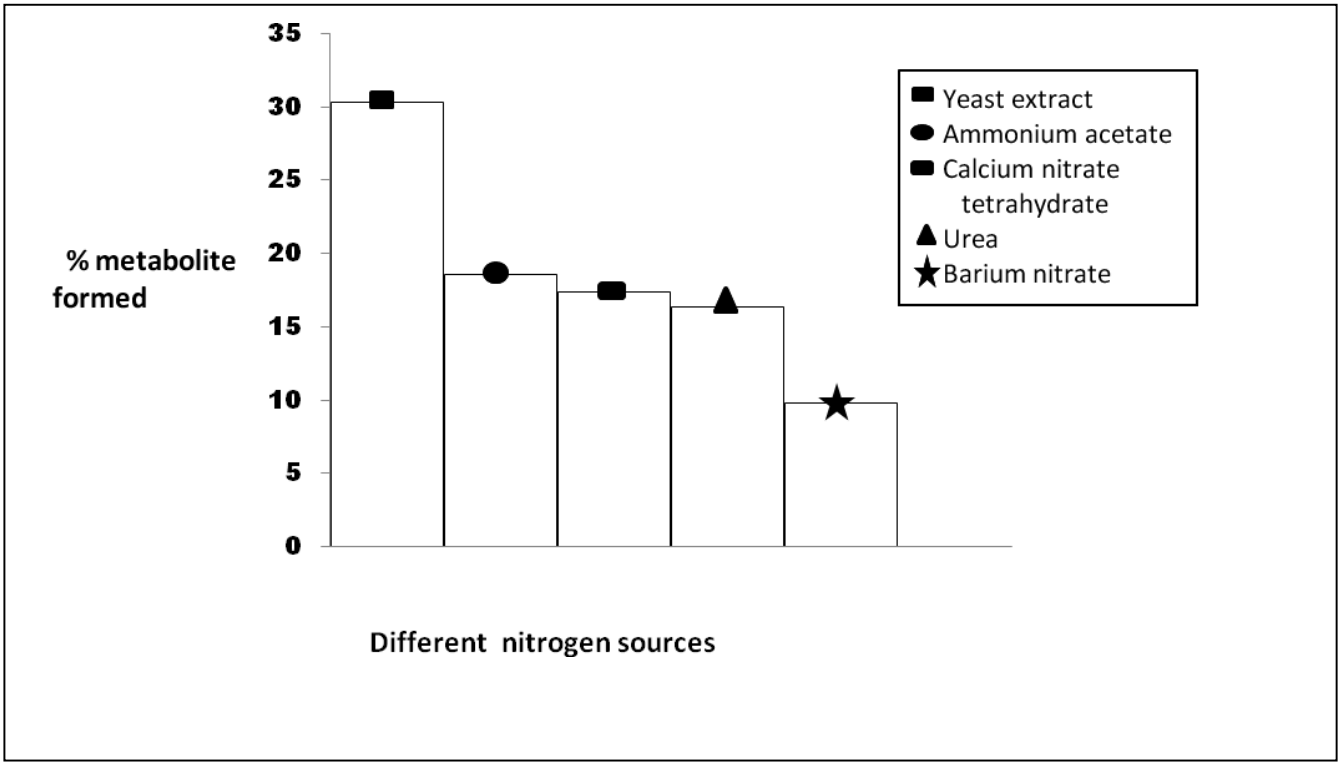


Fig. 4: Effect of concentration of dextrose on metabolite production

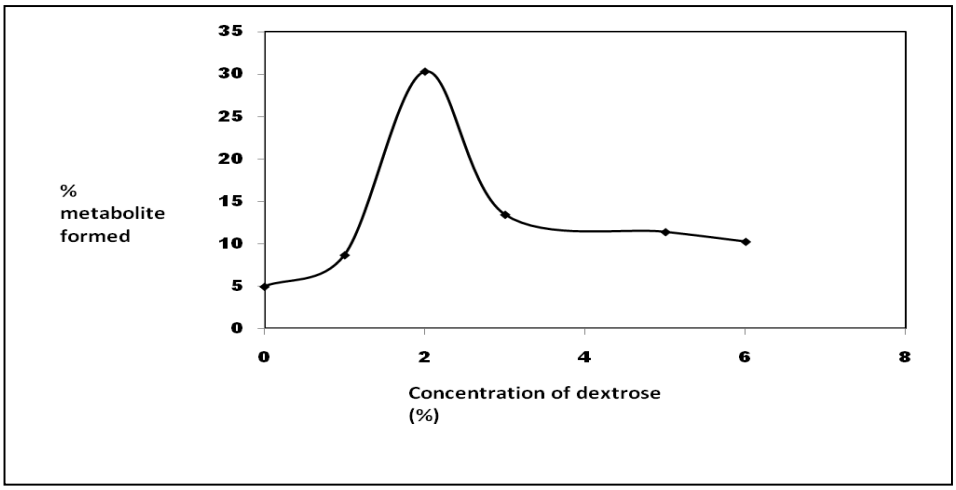
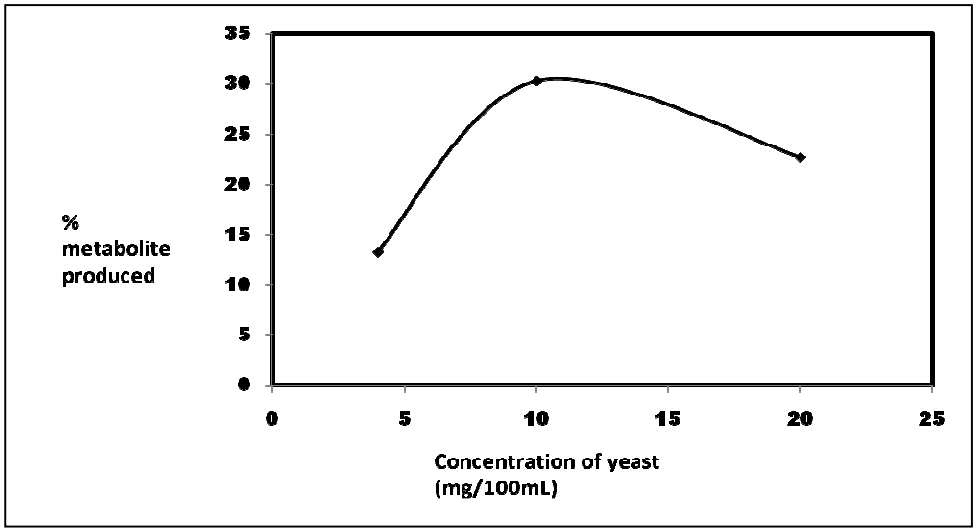


Fig. 5: Effect of concentration of yeast extract on metabolite production



Discussion

The HPLC chromatogram of sample of *Cunninghamella elegans* showed an extra peak at retention time of 3.39min. compared to its controls as given in table 1 and Fig.1. The extra peak represented the formation of hesperidine metabolite by *Cunninghamella elegans*. Other microbes showed identical peaks in sample and controls which indicated that, organisms tested could not metabolize the drug.

The change in type and concentration of carbon and nitrogen sources in media did not show difference in the type of the metabolite formation but shown difference in the quantity of the metabolite production. The maximum percentage of metabolism was observed in the presence of dextrose and yeast extract i.e., 30.32% by *Cunninghamella elegans* and significant decreased production of metabolite was observed with D-sorbitol and barium nitrate i.e., 1.7% and 9.72% as carbon and nitrogen sources respectively as shown in Fig 2 and 3.

It can be concluded that the 2% dextrose and 0.01% yeast extract is the most suitable percentage of carbon and nitrogen sources for the activity of enzyme as it is generally suited for maximum growth of fungus, similar to the reports of Shyam Prasad et al 2009 [18] achieved maximum transformation of meloxicam with 2% glucose by *Cunnninghamella blakesleeana.* The present results revealed that the maximum production of hesperidine metabolite was found when D-glucose was used as carbon source similar to findings of Hossein V et al., 2006 [19] , Sun et al., 2004 [20] and Kreiner et al., 1996 [21] by *Cunninghamella blakesleeana,* Jin and Li 2002 [22] by *Aspergillus niger*.

Further the highest yield of hesperidine metabolite was favoured by yeast extract as nitrogen source. Maximum biotransformation in the presence of dextrose(2%) and yeast extract(0.01%) might be due to increased mycelia growth or induction of the enzymes required for metabolism [23]

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