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BIOTRANSFORMATION OF QUERCETIN BY *ASPERGILLUS NIGER* FROM *ALLIUM CEPA*

Supriya Chatla *, Saisrilakshmi Gasi, Shaik Abdul Rahaman, Mounika Yaradla and Shruthi Mareedu

Department of Biotechnology, Nirmala College of Pharmacy, Atmakuru, Mangalagiri, Guntur - 522503, Andhra Pradesh, India.

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Correspondence to Author:
Dr. Supriya Chatla

Associate Professor,
Department of Biotechnology,
Nirmala College of Pharmacy,
Atmakuru, Mangalagiri, Guntur - 522503, Andhra Pradesh, India.

E-mail: supriya.chatla@gmail.com

ABSTRACT: Biotransformation is a biological process in which the microbes are converting one form of compound to the another form. In the present research work, we studied about the conversion of Quercetinto kaempferol, which is having more therapeutic potential. Flavonoids are diverse group of secondary metabolites found especially in all plants. Flavonoids have Anti-carcinogenic, Anti-inflammatory, antioxidant, and Anti-allergic properties. Our study Quercetinis transforming into Kaempferol by *Aspergillus niger* (*Allium cepa*) through fermentation process at 37 ºC, PH 6 under aerobic conditions. The Transformed product (150 mg flavones / 200 ml medium) was extracted and analyzed by the spectral analysis (UV, IR, H-NMR, C-NMR) and mass spectrometry analysis process. The trans-formed product has more therapeutic potential in the treatment of wide range of cancer like breast, ovarian, gastric, pancreatic, lung cancer.

INTRODUCTION: Microbial transformation is known to be a useful tool to obtain more high or less toxic compounds and to attain selective conversion of compounds to use derivatives which are often difficult to produce synthetically. In this recent year's microbial transformation of organic compounds has been an important research pursuit as it is well known for no high stereo-selectivity and environment-friendly nature. Bio-trans-formation of flavonoids, steroids [1], glycosides, rutin, and phloridzin has been reported. Flavonoids are the major red, blue and purple pigment in the plants, which have considerable medical importance.

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Previous studies have reported the biosynthesis [2] of flavonols like quercetin, kaempferol, and naringenin by different strains like *Escherichia coli*, *Bacillus subtilis*, and *Saccharomyces cerevisiae*. There are some other studies that have reported the biotransformation of naringenin to Quercetin and also its metabolites.

In the present study, microbial transformation, produced by microorganism *Aspergillus niger* which is isolated from *Allium cepa* is considered as an essential step in this work. Quercetin is a plant flavanol from the flavonoids group of poly phenols. It is mainly found in fruits, vegetables, leaves, and grains. Generally, kaempferol can be produced by using the precursors; when Naringenin [3] was used for feeding the fungal spores, the production obtained was about 0.8 mg/L. Kaempferol has a wide range of pharmacological activities like anti-oxidant, anti-inflammatory, antimicrobial, anti-cancer [4], cardioprotective, neuroprotective anti-

diabetic, antiosteoporotic, estrogenic / anti-estrogenic, anxiolytic, analgesic, and antiallergic activities. By means of microbial transformation, Quercetin can be easily converted to kaempferol [5] in the presence of *Aspergillus niger* at specific conditions by fermentation process. Quercetin is a hydroxylated form of kaempferol. The metabolites obtained is separated by means of a thin-layer chromatography technique to estimate their affinity variations. By means of Quercetin-rich medium, the amount of kaempferol production varied from that of the precursor-rich medium. The whole process undergoes several optimization factors like temperature, pH, concentration, and time. The obtained metabolite after transformation is analyzed by means of several spectral analyses like UV, IR, c-NMR, and H-NMR spectroscopy.

MATERIALS AND METHODS:

1. Quercetin was purchased from sigma Aldrich Company, Bangalore.

2. Test culture – *Aspergillus niger* (16404) was collected from *Allium cepa* from NCPA, Atmakuru.

3. **Pure Cultures:** *Bacillus subtilis* (2063) and *Staphylococcus aureus* (6571) were collected from NCIM (National collection of industrial micro-organisms) PUNE.

Screening of Selected Strains for Quercetin Utilization: Initially, both Potato dextrose medium (fungal spores) and Agar medium (bacteria) were prepared and processed. One consisted of normal media components, and the other consists of minimal concentration Quercetin. Finally, the selected organisms are inoculated and incubated according to their incubation periods *i.e., Aspergillus niger* [6] was incubated for 3-6 days shown in **Fig. 1**. *Bacillus subtilis* and *Staphylococcus aureus* are incubated for 24 – 48 h.

After their incubation period, it was observed that among the selected micro-organisms, *Aspergillus niger* has grown more rapidly in Quercetin-rich media when compared to normal media. *Bacillus subtilis* and *Staphylococcus aureus* growth is more in normal nutrient agar media compared with the Quercetin-rich media. Hence, we concluded that *Aspergillus niger* has the greater potential to utilize and to transform the Quercetin

TABLE 1: COMPOSITION OF POTATO DEXTROSE MEDIUM

Name of the ingredients	composition
Dextrose	20 g
Potatoes (sliced washed)	4g (from 200g infused potato)
Agar	20 g
water	1000 ml
pH	5.6±0.2
Temperature	25 ℃

TABLE 2: NUTRIENT AGAR MEDIUM

Name of the Ingredients	Quantity/1000ml
Meat Extract	3g
Peptone	5g
Sodium chloride	5g
Agar	15g
Distilled water	1000 ml
pH of the medium	7.4

Optimization of Quercetin Concentration for Maximum Utilization by *Aspergillus niger*: After selection of the organism, the optimum Concentration of Quercetin was tested for the maximum trans-formation. Potato dextrose medium was prepared, and transfer into six Petri-dishes, in which five Petri-dishes consist of different concentrations of Quercetin (0.03, 0.05, 0.07, 0.09, 0.10 grams/10 ml) and remaining one Petri-dish is considered as a standard without Quercetin. Finally, after solidification *Aspergillus niger* was inoculated by simple streaking under aseptic conditions and incubated for 3 -6 days. It was observed that the Petri-dish, which contains 0.05gm/10ml concentration of Quercetin, has more grown culture than the other concentrations. The same process was repeated was three times to obtain accurate concentration, and finally, 0.05 gm/10 ml of Quercetin was set as an optimal concentration shown in **Fig. 2**.

Effect of Agitation over the Growth of the Selected Strain: 100 ml of potato dextrose medium was prepared and transferred into two 150 ml conical flasks, each containing 50 ml of PDA **Table 1**. Later the culture was inoculated into the conical flasks under aseptic conditions. After inoculation, one conical flask was kept in a normal incubator, and the other was kept in an orbital shaker at about 150 rpm at 27 ºC. Both the conical flasks were allowed to stand for one week of the incubation period. After the incubation period, it was observed that the conical flask, which was incubated in an orbital shaker, has more growth of culture when compared to the one which is

incubated normally. Hence orbital shaker is used in the further process for biotransformation.

Biotransformation Phase:

Step-1: Growth Phase: The biotransformation of Quercetin was carried out by using submerged Fermentation method [7]. The liquid fermentation cultures were inoculating by about 5ml of selected fungal strain *Asparagillus niger* (two-week old slants) into 50 ml sterile liquid medium **Table 2** contained in 250 ml conical flasks placed on orbital shaker operating at about 150 rpm at 27ºC for 48 h.

Composition of Fermentation Medium: The fermentation liquid medium was composed of 10 g glucose, 5 g peptone, 5 g yeast extract, 5 g sodium chloride, 5 g K_2HPO_4, 10 ml glycerol per one liter, and the pH was adjusted to 6 before sterilizing it in an autoclave.

Step 2: Transformation Phase: Transfer about 5 ml of the above Step-1 culture to 50 ml of the sterile fermentation broth. Cultures were allowed to incubate for 24 h before adding the substrate. Now dissolve the transforming substrate Quercetinin N, N-dimethyl formamide (DMF) at a concentration of 5 mg/50 ml medium, add it to the broth to incubate for 6 days. Keep the uninoculated broth (without spores [8] of *Aspergillus niger*) as a control. After the 6 days incubation period, filter the broth to separate the cell debris. The obtained clear filtrate should be analyzed to detect the transformed product.

Isolation and Detection of Transformed Product:

Chromatographic Analysis (TLC) of Transformed Product: Sampling of the trans-formed product was done by taking 5ml if the culture suspension and then extracted with 5ml chloroform. Then, chloroform was evaporated, and the residue was dissolved in a few drops of methanol, then 20 µl was spotted into silica gel plate [9]. The applied spots were eluted out using methanol-water-aceticacetic 955:45:0.5V/V/V). The separated bands on the thin layer of silica gel were first dried and then sprayed with sulphuric acid or exposed to vapors of ammonia [10].

UV- analysis: The selected flavonoid Quercetin and the transformed product Kaempferol were analyzed by UV-analysis (Perken Elmer lambda 4B UV spectrophotometer) at 326 nm wavelength.

IR-analysis: The selected flavonoid Quercetin and the transformed product Kaempferol were analyzed by 1340 ratio recording infrared spectrophotometer.

Microbiological Assay of Transformed Flavonoid: Microbiological assay of Flavonoids was carried out by disc diffusion method [11] against three different test cultures -Gram-positive [12] *Staphylococcus aureus*; Gram-negative- *E. coli* and fungi- *Candida albicans*.

Standard Stock solutions of both Quercetin and transformed product were prepared at 50 µg/ml for disc impregnation.

Disc Preparation: A solution of 50 µg/ml of Quercetin and transformed product impregnated discs were prepared for antimicrobial assay..

Development of Inoculum: Sterilized Nutrient Agar media and Sabouraud's media were inoculated by *Staphylococcus aureus*, *E.* coli in cubated for 24 h at 37 ºC, and *Candida albicans* (Fungi) for 96 h at 28 ºC for antifungal activity [13].

Assaying Procedure: The above-developed inoculums were inoculated into PDA (Potato Dextrose Agar) media and nutrient agar media and allow to solidify. After solidification, the impregnated discs of the transformed product (test compound) and the quercetin, standard Streptomycin discs (30 µg/ml), and clotrimazole discs (10 mg/ml) were placed at the surface of the agar plates. Then the plates were incubated for 24 h at 28 ºC (for yeast), and at 37 ºC (for bacteria) to know the potency of the transformed product. The antimicrobial activity of flavone and transformed product against the tested microorganisms were detected by taking the growth inhibition zone diameter [14].

RESULTS AND DISCUSSION:

Rate of Quercetin Utilization by Selected Strains: Quercetin utilization by selected strains like *Bacillus subtilis*, *Stapylococcus aureus*, *Eschericia coli* 15, and *Aspergillus niger* were studied. .In the present study, we found that the selected fungal strain, *Aspergillus niger* has the more Quercetin utilization capacity shown in **Fig. 4**. Based on nutritional characteristics as the fungi need more carbohydrates and less nitrogen source; hence, these fungal spores are highly replicated in

the quercetin-rich media at 37 ºC. The growth rate is measured in U.V Spectrophotometer at 520 nm,

Optimization of Quercetin for Maximum Utilization by *A. niger*: The optimum concentration of quercetin required for the maximum conversion of Quercetin into Kaempferol by *Aspergillus niger* is estimated. The optimum concentration of Quercetin is studied by taking the quercetin at different concentrations like 0.03, 0.05, 0.07, 0.09, 0.10 mg, and a control (potato dextrose medium, Quercetin without test culture).

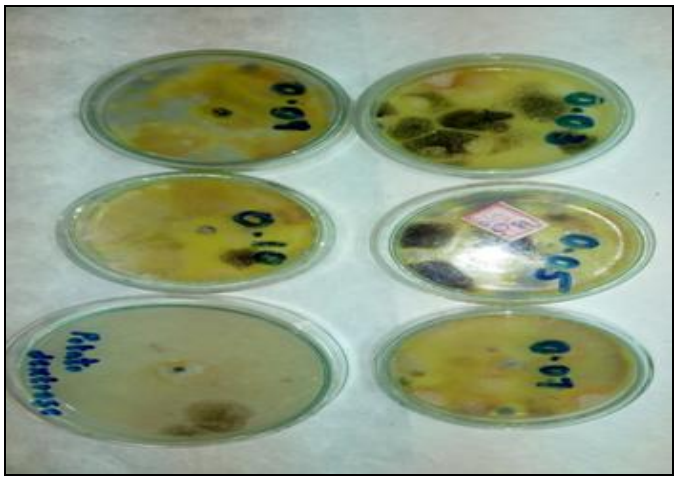

FIG. 1: TWO DAYS OLD CULTURE (*ASPERGILLUS NIGER*) IN DIFFERENT CONCENTRATIONS OF QUERCETIN MEDIUM

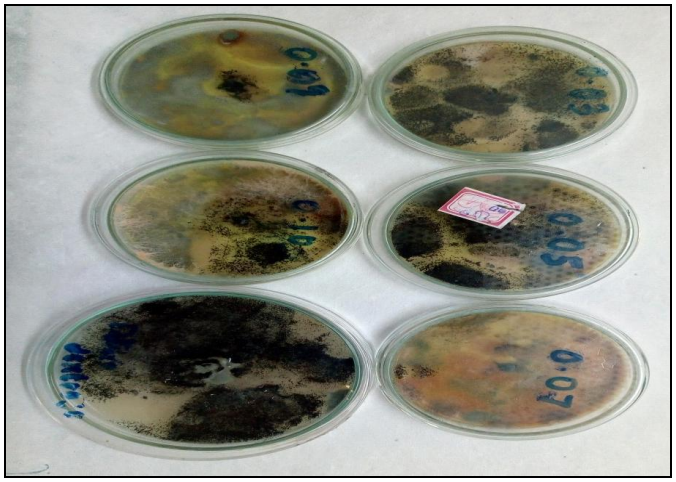

FIG. 2: ONE WEEK OLD CULTURE IN QUERCETIN RICH MEDIUM

Where 5 mg of Quercetin is used in 50 ml of media is used as a standard preparation according to the literature survey. Estimation of the fungal spores present in the different dose variant media in petridish has shown a difference in the live cultural spores. While in 0.05 mg/10 ml Quercetin rich media has shown more fungal growth when compared to remaining dose levels media. Whereat 0.03 and 0.05 mg quercetin-containing media has shown freshly developed spores when compared to other media shown in **Fig. 1** and **2**.

Biotransformation of Quercetin to Kaempferol: The optimized quercetin dose (0.03-0.05 mg) is inoculated into the medium and allow to incubate for 24 h to be transformed into kaempferol. The Quercetin is transformed into Kaempferol by hydroxylation, dehydroxylation, methylation, hydrogenation reaction. Biotransformation reactions are based on microbial enzymatic activity such as oxidase, hydroxylase, methylases, *etc*.

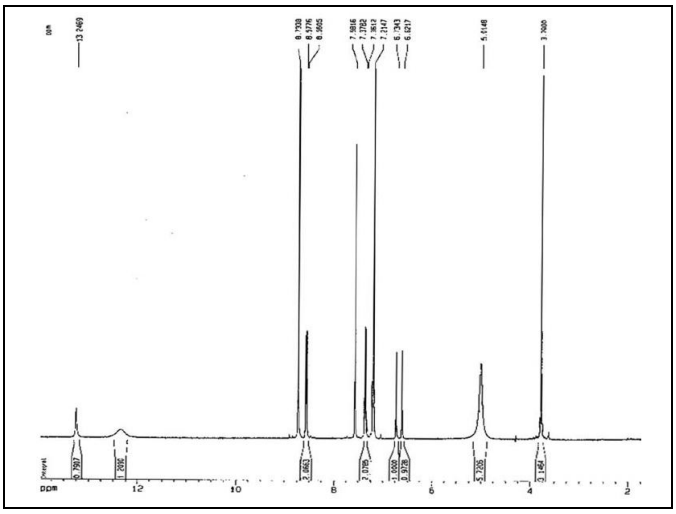

FIG. 3: H-NMR SPECTRUM FOR THE TRANSFORMED PRODUCT (KAEMPFEROL)

Spectrophotometric Analysis of the Transformed Product: The structural elucidation of Transformed product done by UV spectroscopy. UV spectrum lambda maximum values of the obtained metabolite are 265 nm, 293 nm, 320 nm, 342 nm, and 377 nm. The purity of the obtained metabolite can also be estimated with that of standard (Kaempferol). Where the standard Kaempferol lambda max is about 365 nm and its purity is about 98.56%, but the obtained metabolite has a purity of 57.54%. The IR spectroscopy of the metabolite was done with potassium bromide by means of Fourier transform infrared spectroscopy. The molecular weight of the standard Kaempferol is 286.236 Daltons.

The transformed product (Kaempferol) with Dimethyl sulfoxide (DMSO) substance is used in the H-NMR spectroscopy and has peaked at certain

ppm like -3.1464,-4.684, 9.527, 1.023, 2.086, 2.075, 1.243, 0.987 ppm shown in **Fig. 3**.

Microbiological Assay of Transformed Product: The obtained metabolite is studied for its antimicrobial activity. Antifungal and Antibacterial activity was studied for the Quercetin and for a transformed product after biotransformation [16], which is an unidentified product.

The antimicrobial activity of the transformed product (Kaempferol), standard compound Kaempferol is determined against *Staphylococcus aureus*, *E. coli*, *Candida albicans* by disc diffusion method. The activity is measured in terms of growth inhibition zones. The growth inhibition zone diameter of the Transformed product (Kaempferol) is more at a concentration of 0.5 mg/ml than the Quercetin and it is equal to the standard Kaemferol shown in **Fig. 4**.

FIG. 4: ANTIMICROBIAL ACTIVITY FOR THE OBTAINED METABOLITE (KAEMPFEROL)

CONCLUSION: Biotransformation is an inexpensive and less time taking process to convert cost-effective drugs or compounds into another form. In the present study, we selected Quercetin flavonoid to convert into Kaempferol. The selected fungal strain *Aspergillus niger* has the great potential to transform Quercetin into Kaempferol by dehydrogenation. The transformed product was analyzed by different spectrophotometric analytical techniques like NMR, UV, and IR. We concluded that most of the biotransformation reactions are through hydroxylation, dehydroxylation, methylation, hydrogenation reaction. In the present study, the TLC method was used for the separation and determination of transformed flavonoids. The transformed product showed better therapeutic potential in treating several disorders and good antimicrobial activity.

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CONFLICTS OF INTEREST: Nil

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