Biotransformation of Curcumin to Calebin-A: A Pharmacologically Important Novel Curcuminoid from Curcuma Species

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Abstract

Calebin-A, a novel natural product of turmeric (Curcuma longa) rhizome has been reported to possess various pharmacological activities. The minimal natural occurrence has been the major constraint for exploiting its pharmacological use. Thus, this study focuses to investigate the production of calebin-A through biotransformation approach using curcumin as the substrate. Four bacterial strains (Acinetobacter calcoaceticus delfa L360, Pseudomonas putida TGPNP13, P.putida NCIMB 10007 and A.johnsonii NCIMB 9871) and three different media compositions (MTSB, modified tryptic soy broth; TSB, tryptic soy broth; NB, nutrient broth) were used to investigate the conversion of curcumin to calebin-A. Biotransformation studies were carried out by supplementing curcumin (50 mg) into media (1000 ml). After 24 h of interval, samples were withdrawn and ethyl acetate extract was obtained as per partition method followed by determination of calebin-A presence using HP-TLC and HPLC techniques. P. putida NCIMB 10007 and A. johnsonii NCIMB 9871 were found to convert curcumin to calebin-A in a time dependent manner with optimum conversion at 48 h and after 96 h respectively. The highest growth of P. putida NCIMB 10007 and A. johnsonii NCIMB 9871 and the production of calebin-A were observed when grown in MTSB media. Baeyer-Villiger monooxygenase enzyme was produced by P. putida NCIMB 10007 (3.30±0.09 U/ml) and A. johnsonii NCIMB 9871 (4.10±0.10 U/ml), suggesting its possible role in the conversion of curcumin to calebin-A. The findings of this study clearly suggested that P. putida NCIMB 10007 and A. johnsonii could be potential candidates for biotechnological production of calebin-A from curcumin.

Keywords: Calebin-A, biotransformation, Baeyer-Villiger monooxygenase, Acinetobacter calcoaceticus, Pseudomonas putida.
INTRODUCTION

*Curcuma longa* L. (turmeric) has been known as a color food additive, spice and also used as remedy to treat and manage various human ailments in traditional Asian medicine for centuries (Praditya et al., 2019). Turmeric rhizomes containing active curcumin, has been reported to exhibit several pharmacological properties such as antibacterial, anticoagulation, anti-inflammation, anticancer activities and also help improve blood circulation, lipid metabolism, hormonal disorders and indigestion (Amalraj et al., 2017). Curcumin from turmeric has already been proven non-toxic to human and also found to be clinically effective for the treatment of human cancer and other ailments in various human clinical trials worldwide (Kunnunakkara et al., 2016). Regardless of its various biological activities, curcumin is reported to have low bioavailability, low solubility in water and also strong color intensity which limits its uses in pharmacology (Liu et al., 2016). Thus, various researchers have made efforts to explore new metabolites and curcumin derivatives from turmeric rhizomes which have been reported to show better efficacy and also to have improved other characteristics. The study conducted by Younis et al., (2016) suggested that microbial transformation of curcumin can yield more biologically active metabolites which showed highest anticancer, antimicrobial and anti-oxidant activity than that of curcumin (Amalraj et al., 2017). Furthermore, efforts have been made to increase the bioavailability of curcumin using biotransformation approach through glycosylation reaction which resulted in the production of the compound i.e. curcumin (Purpura et al., 2018). However, limited success has been achieved through all the various approaches to overcome the low bioavailability, low solubility in water and strong color intensity. Thus, there is a need existing to search for an alternative curcuminoid compound with improved efficacy and other characteristics. Calebin-A (3E)-4-(4-Hydroxy-3-methoxyphenyl) 2-oxo-3-buten-1-yl (2E)-3-(4-hydroxy-3 methoxy-phenyl) acrylate) is a curcuminoid reported to be present in the *Curcuma* species along with other curcuminoids (curcumin) which has a highly similar structural feature as curcumin (Dosoky and Setzer 2018). Calebin-A was first isolated and reported by the Kim and Kim (2001) from *Curcuma longa* rhizome with the yield of 0.001% (w/w) of the total turmeric extract. There are reports suggesting that the calebin-A has promising activity to prevent β- amyloid (βA) insult to neuronal cells in Alzheimer’s disease (Yan et al. 1996; Park and Kim 2002). Among several pharmacological activities, calebin-A has also been reported to show anticancer effects and to improve hepatic steatosis, suppress adipocyte differentiation and prevented high fat diet-induced obesity (Lai et al., 2015). By considering the pharmacological activities exhibited by the calebin-A till date, there is scope that the calebin-A could be a potential agent in the treatment and management of various human ailments including Alzheimer’s disease, cancer and obesity. However, the trace quantity reported in turmeric rhizome is a limiting factor for the development of calebin-A. There are reports suggesting the chemical synthesis of calebin-A (Majeed et al. 2017), but obtaining calebin-A naturally is still a challenge to the researchers.

The term biotransformation refers to the organic reactions involving biological catalysts which can either be whole cells or enzymes, in order to chemically modify a chemical compound (Lin and Tao, 2017). Of late, biotransformation approaches have gained a lot of popularity among industries as these processes are environment friendly and products obtained through these processes can also be considered natural if the substrate is from natural origin, according to the FDA and European legislation (Gallage and Miller, 2015). Due to the natural origin of curcumin and also its simple and similar structure as calebin-A, curcumin could be an ideal substrate to obtain calebin-A through biotransformation approach. To the best of our knowledge, there is no report suggesting the bacterial biotransformation of natural curcumin to calebin-A. Thus, the current study aimed to investigate the potential of *Acinetobacter johnsonii* NCIMB 9871 and *Pseudomonas putida* NCIMB 10007 for the bioconversion of curcumin to calebin-A.

MATERIALS AND METHODS

Tryptone soy broth (TSB) and nutrient broth (NB) were purchased from HiMedia Laboratories Pvt. Ltd (Mumbai, India). tetrahydrofuran, mercuric chloride, sodium hypo-
chlorate, methanol, dimethyl sulfoxide (DMSO), β-nicotinamide adenine dinucleotide 22-phosphate, reduced tetradsodium salt hydrate (NADPH) and cyclohexanone were procured from Sigma-Aldrich (St. Louis, MO, USA). TLC Silica Gel 60 F254 was procured from Merck KGaA (Darmstadt, Germany). Curcumin and calebin-A were obtained from Sabinsa Corporation (Piscataway, NJ, USA) and were used as standard throughout the study.

**Bacterial strains**

A. calcoaceticus delfa L360 and P. putida TGPNP13 were procured from National Centre for Cell Science (NCCS), Pune, India. P. putida NCIMB 10007 and A. johnsonii NCIMB 9871 were obtained from NCIMB Ltd, Aberdeen, Scotland.

**Screening of microbes for the growth and biotransformation ability**

The screening of bacteria for the potential to biotransformation of curcumin to calebin A was performed. Different bacterial strains i.e. A. calcoaceticus delfa L360, P. putida, TGPNP13 P. putida NCIMB 10007 and A. johnsonii NCIMB 9871 were screened by growing in different media i.e. MTSB (pancreatic digest of casein 17 g/L, papain digest of soybean meal 3 g/L, sodium chloride 5 g/L, dextrose 5 g/L, dibasic potassium phosphate 2.5 g/L, manganese(II) sulfate 0.1 g/L, magnesium sulfate g/L, calcium chloride 0.1 g/L, potassium dihydrogen phosphate 1 g/L; pH 7.0±2), TSB (pancreatic digest of casein 17 g/L, papain digest of soybean meal 3 g/L, sodium chloride 5 g/L, dextrose 2.5 g/L, dibasic potassium phosphate 2.5 g/L, pH 7.3±2), NB (peptone 5 g/L, sodium chloride 5 g/L, HM peptone 1.5 g/L, yeast extract 1.5 g/L; pH 7.3±2). Overnight grown cultures of P. putida NCIMB 10007 and A. johnsonii NCIMB 9871 were inoculated in to MTSB, TSB and NB media. After every 24 h of time intervals, samples were collected and analyzed for total reducing sugars (TRS) as described earlier and optical density (OD) was recorded by using a UV-VIS spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

**Biotransformation of Curcumin into Calebin-A**

Overnight grown culture of P. putida NCIMB 10007 and A. johnsonii NCIMB 9871 were inoculated to MTSB media (1000 ml) supplemented with curcumin (50 mg dissolved in 20 ml of DMSO) and then flasks were incubated at 37°C for 144 h in shaker incubator (Scigenics Biotech, Chennai, India) with 140 rpm. At every 24 h interval, samples were withdrawn (100 ml) and extracted using ethyl acetate (300 ml) in a separating funnel (Borosil, Mumbai, India). The organic layer was collected and concentrated to dryness using rotary evaporator (Heidolph, Schwabach, Germany). Samples were further dissolved in 20 ml of methanol and this sample was used to identify and for quantitative and qualitative analysis of curcumin and calebin-A.

**High Performance Thin Layer Chromatography (HPTLC)**

Qualitative identification of curcumin and calebin-A was performed by using HPTLC system (Camag, Muttenz, Switzerland) using silica gel plates 60 F₂₅₄, 20x10 cm (Merck Millipore) developed by the solvent system chloroform:

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**Table 1.** The utilization of reducing sugar (g/L) by the A. johnsonii NCIMB 9871 and P. putida NCIMB 10007 in three different media compositions determined by following DNSA method

| S.N. | Time (h) | A. johnsonii NCIMB 9871 | P. putida NCIMB 10007 |
|------|----------|------------------------|----------------------|
|      | MTSB     | TSB                    | NB                   | MTSB       | TSB          | NB            |
| 1    | 0        | 3.51±0.09              | 2.81±0.09            | 0.72±0.02  | 3.53±0.09    | 2.82±0.08     | 0.74±0.08    |
| 2    | 24       | 1.91±0.07              | 1.34±0.08            | 0.43±0.01  | 0.60±0.05    | 1.45±0.06     | 0.45±0.07    |
| 3    | 48       | 0.51±0.04              | 0.67±0.06            | 0.39±0.01  | 0.52±0.04    | 0.51±0.05     | 0.42±0.05    |
| 4    | 72       | 0.48±0.02              | 0.47±0.06            | 0.38±0.01  | 0.47±0.03    | 0.43±0.04     | 0.43±0.04    |
| 5    | 96       | 0.45±0.02              | 0.45±0.05            | 0.39±0.01  | 0.45±0.02    | 0.44±0.02     | 0.42±0.02    |
| 6    | 120      | 0.43±0.02              | 0.41±0.04            | 0.37±0.01  | 0.43±0.01    | 0.43±0.01     | 0.41±0.01    |
| 7    | 144      | 0.41±0.01              | 0.39±0.01            | 0.38±0.01  | 0.42±0.01    | 0.41±0.01     | 0.39±0.01    |

MTSB, modified tryptic soy broth; TSB, tryptic soy broth; NB, Nutrient Broth.
Data are expressed as mean ± standard deviation (SD) of two independent experiments performed in triplicate.
methanol (98:2). Each sample (2µl) was loaded with Camag Linomate V semi-automatic sample applicator, Linomat syringe (100µl), with 10 mm band width. The plates scanned at 280 nm with deuterium illumination using scanner 3 (Camag). The images were captured on Camag reprostar 3 with win CATS software (ver. 1.4.3.6336) and compared the Rf value with the standard of calebin-A.

**High Performance Liquid Chromatography (HPLC)**

The quantification of curcumin and calebin-A was performed by using HPLC (Shimadzu Class Vp series) which was coupled to an auto sampler and DAD–UV detector (SPD-M10A Vp), C18 column (250 x 4.6 mm) and binary gradient pump (LC20 AD). The injection volume was 20µl. The mobile phase used was tetra-hydrofuran 0.6% citric acid in water (40:60) at a flow rate of 1.0 ml/min. Data acquisition and analysis was carried out using Shimadzu LC Solution version 1.25. Samples were taken in triplicates for the analysis.

**Baeyer Villiger Monooxygenases (BVMO) enzyme activity**

The overnight grown culture of *P. putida* NCIMB 10007 and *A. johnsonii* NCIMB 9871 was inoculated into the MTSB, TSB and NB media containing 0.05 mg/ml of curcumin (dissolved in 2.5 mg/ml of DMSO) and incubated for 144 h at
37 ± 0.5°C at 140 rpm. After every 24 h, culture was collected and centrifuged (10,000 x g for 10 min) to remove cells and then supernatant was collected separately. The cell pellet was washed three times with sterile phosphate buffer (0.1 M PBS, pH 7.5). The washed cells were re-suspended in 10 ml phosphate buffer (50 mM; pH 7.5), and then were sonicated by (30 seconds each cycle, with gap of 3 min after each cycle) using ultrasonic homogenizer (Sartorius AG, Gottingen, Germany). The cell homogenate was centrifuged (10,000 x g) at 4°C and supernatant used as crude enzyme for the BVMOs activity. The BVMOs assay were performed in tris-HCl buffer (50 mM, pH 8.5) containing 10 mM cyclohexanone, 0.8 mM NADPH, by adjusting absorbance between 0.9 to 1.0 at 340 nm using UV-VIS spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Enzyme activity was measured by monitoring the NADPH consumption at 340 nm for 180 seconds. One unit (U) of the enzyme activity was defined as the amount of enzyme to oxidize 1µmol of NADPH for

![Graph A](image1)

**A. johnsonii NCIMB 9871**

![Graph B](image2)

**P. putida NCIMB 10007**

Fig. 2. Effect of different media composition on the production of calebin-A by the *A. johnsonii* NCIMB 9871 (a) and *P. putida* NCIMB 10007 (b) as determined by the HPLC method. Each value represents the mean ±SD (n= 3).
1 min under the reaction conditions. Estimation of protein was determined as per the Bradford’s method as described earlier (Bradford, 1976).

**RESULTS**

**Growth profile of bacterial strains**

The growth profile of bacterial strains was determined using different media composition (without the supplementation of curcumin). Bacterial strains *P. putida* NCIMB 10007 and *A. johnsonii* NCIMB 9871 achieved maximum growth at 48 h in the MTSB media with optical density of 0.75 and 0.72 (1:10 dilution) at 600nm respectively (Fig. 1a & b) which suggested that growth of these bacterial strains was higher in MTSB media compared to other media compositions (TSB and NB) (Fig. 1a & b). The total reducing sugar (TRS) was also analyzed to understand the growth of bacteria. We found that there was decrease in TRS with the increase in growth irrespective of the media used for the growth of *P. putida* NCIMB 10007 and *A. johnsonii* NCIMB 9871 (Table 1). However, the reduction of reducing sugar by the *P. putida* NCIMB 10007 and *A. johnsonii* NCIMB 9871 was highest in MTSB media composition compared to TSB and NB.

![HPTLC Chromatograms](image1)

**Fig. 3.** HPTLC Chromatograms of ethyl acetate extract of (a) *A. johnsonii* NCIMB 9871 and (b) *P. putida* NCIMB 10007 at different time intervals of incubation. Lane 1 and 2 represent the curcumin and calebin-A standards in each chromatogram. Lanes 3 to 9 represent the ethyl acetate extract of (a) *A. johnsonii* NCIMB 9871 and (b) *P. putida* NCIMB 10007 after 0, 24, 48, 72, 96, 120 and 144 h of incubation.

![HPLC Chromatogram](image2)

**Fig. 4.** HPLC chromatogram of *A. johnsonii* NCIMB 9871 at different time intervals of growth and extracted with ethyl acetate (A) Calebin-A standard; (B), 24 hours of growth; (C), 48 hours of growth; (D), 72 hours of growth; (E), 96 hours of growth; (F), 120 hours of growth. The chromatogram shows the presence of calebin-A from the 24 hours of growth after adding curcumin in the broth.
Screening of microbes for the bioconversion of curcumin to calebin-A

Four different bacterial strains were screened for the bioconversion of curcumin to calebin-A using three different media composition, aiming to investigate the suitable media and strain for the conversion of curcumin to calebin-A. Data of the experiments revealed that the bioconversion of curcumin to calebin-A was achieved by *P. putida* NCIMB 10007 and *A. johnsonii* NCIMB 9871 in all three media composition (MTSB, TSB and NB). *P. putida* NCIMB 10007 and *A. johnsonii* NCIMB 9871 were fermented in MTSB, TSB and NB media supplemented with curcumin. The ethyl acetate extract was analyzed for the presence of calebin-A by following HPTLC, HPLC techniques. The HP-TLC results showed the similar band to the calebin-A standard with the same Rf value. This indicated that the *P. putida* NCIMB 10007 and *A. johnsonii* NCIMB 9871 were able to convert the curcumin into calebin-A in the suitable growth time and conditions provided (Fig. 3). Furthermore, quantitative analysis of bio-conversion of curcumin to calebin-A was confirmed by HPLC technique.

The HPLC chromatogram confirmed the presence of calebin-A in samples as it showed similar retention time as standard of calebin-A (Fig. 4 & 5). The HPLC chromatogram showed that the primary metabolite and calebin-A appeared after incubation time of 24 h with retention time (RT) of 7.1 in the *A. johnsonii* NCIMB 9871 and *P. putida* NCIMB 10007 group (Fig. 4 & 5). The maximum conversion of calebin-A was observed at 96 h and 48 h in the *A. johnsonii* NCIMB 9871 and *P. putida* NCIMB 10007 when grown in MTSB media respectively (Fig. 2a & b). However, the production of calebin-A by the *A. johnsonii* NCIMB 9871 was significantly higher compared to *P. putida* NCIMB 10007 (Fig. 2a & b), thereby suggesting better efficiency of bioconversion of curcumin to calebin-A.

**BVMO enzyme assay**

BVMOs are widely known for oxidization of heteroatom-containing molecules and also to catalyze the insertion of an oxygen atom in ketones and aldehydes. In this study, we have evaluated the intracellular enzyme activity of *P. putida* NCIMB 10007 and *A. johnsonii* NCIMB 9871

![Fig. 5. HPLC chromatogram of *P. putida* NCIMB 10007 at different time intervals of growth and extracted with ethyl acetate (A) Calebin-A standard; (B), 24 hours of growth; (C), 48 hours of growth; (D), 72 hours of growth; (E), 96 hours of growth; (F), 120 hours of growth. The chromatogram shows the presence of calebin-A from the 24 hours of growth after adding curcumin in the broth.](image-url)
during the bioconversion of curcumin to calebin-A, in order to investigate the possible role of BVMOs for this conversion. *P. putida* NCIMB 10007 and *A. johnsonii* NCIMB 9871 produced significant BVMOs enzyme with maximum activity of 3.30±0.09 U/ml and 4.10±0.10 U/ml respectively (Fig. 6). Data of the study indicated that the pattern of the specific activity was exactly similar with the bioconversion of curcumin to calebin-A by *P. putida* NCIMB 10007 and *A. johnsonii* NCIMB 9871, thereby indicating BVMOs role in this bioconversion.

**DISCUSSION**

This study reported for the first time the bioconversion of curcumin to calebin-A aiming to obtain calebin-A naturally apart from its natural occurrence which was first time reported by Kim and Kim (2001) in *C. longa* rhizome. Previous studies suggested that the calebin-A was found to be a pharmacologically active compound to protect cells from beta-amyloid insult and also effective in the treatment of multidrug resistance cancer and human gastric (Kim and Kim, 2001; Li et al., 2008). Furthermore, calebin-A also reported for higher ability to inhibit the proliferation of HepG2 cells and anti-obesity potential (Chen et al. 2009). In view of these studies, to achieve maximum growth in order to get maximum bioconversion of curcumin to calebin-A conditions for the growth of culture are important. The conditions for the growth of the culture, initial pH, temperature and media play a pivotal role in the bioconversion (Xiao-kui Ma and Daugulis, 2014). Moreover, previous report on curcumin glucoxidation by *Catharanthus roseus* cells in liquid culture suggested that the glucoxidation efficiency was dependent on the culture growth and sucrose concentration (Kaminaga et al., 2003). Moreover, literature suggested that the biotransformation process have strong association with the growth
of microbes and enzyme production during the fermentation which depend on the availability of nutrients to the microbes (Singh et al., 2017). In the present study, MTSB media composition was the most suited media to achieve the maximum growth of P. putida NCIMB 10007 and A. johnsonii NCIMB 9871 and to attain maximum bioconversion of curcumin to calebin-A (Fig. 1 & 2). These results indicated that MTSB media fulfilled all requirement to grow and produce enzymes which was required for the bio-conversion of curcumin to calebin-A. Interestingly, out of four strains two strains successfully transformed curcumin to calebin-A while growing in MTSB media, suggesting that the failure of biotransformation was not only dependent on the media but it also involved type of culture used in the study.

To obtain pharmacologically active and novel structurally analog compounds, microbial biotransformation has emerged as an important tool (Bianchini et al., 2015). Events in the biotransformation reaction are challenging to get stable, pharmacologic active and non toxic compound, although it has become quite promising due to the availability of large diversity of microbes (Fura, 2006). Pseudomonas putida NCIMB 10007 and A. johnsonii NCIMB 9871 were widely studied bacteria for the camphor and cyclohexanone metabolism (Donoghue 1976; Grogan et al., 1993). Thus, the current study investigated the role of P. putida NCIMB 10007 and A. johnsonii NCIMB 9871 in the production of calebin-A using curcumin as a substrate. The production of calebin-A was also confirmed by the HP-TLC and HPLC techniques. The pattern of change in composition of the curcumin and calebin-A in the HPLC chromatogram revealed that the P. putida NCIMB 10007 and A. johnsonii NCIMB 9871 were able to convert the curcumin into calebin-A with the highest conversion after 48 and 96 h of incubation period respectively (Fig. 2). Although this study is preliminary and required further optimization and scale-up in order to develop a commercially economical process to produce calebin-A naturally, this study has provided lab scale evidence to produce calebin-A which will be very essential information for the future studies.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS’ CONTRIBUTION

All authors have made substantial, direct and intellectual contribution to the work and approved it for publication.
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None.

DATA AVAILABILITY
All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT
This article does not contain any studies with human participants or animals performed by any of the authors.

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