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Process Engineered Production of Betulinic Acid As A Prominent Anticancer Molecule

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ABSTRACT

Betulin (B) and Betulinic acid (BA) are naturally occurring lupane-type pentacyclic triterpenes with significant pharmacological relevance like anti-tumor and anti-HIV activities. AIDS is a mixture of conditions that gradually reduces the immunity of the host making them more susceptible to even common infections like influenza. The cure of HIV is generally based on a blend of 3 or more type of medication which is named as HAART (Highly active anti Retroviral Therapy). This blending of medication supports in the reduction of chances for virus replication by masking their mutations. Even though there are more than 25 known drugs with diverse mechanism of action, available in the market but still there is need of alternative mechanism of action with better cure as well lower side effect. Now Betulin and Betulinic acid (BA) with its derivatives represents as significant group of anti-HIV agents with novel mechanisms. Previously Betulin and Betulinic acid derivatives were chemically synthesized by altering different groups specially at C3, C20 and C28 positions but here our aim is through microbial transformation. Range of betulin 0.25 to 4.0 mg/l were tried and 1.4 mg/l was found best for maximum growth at 250 ml shake flask. Highest growth 58.51mg/ml and yield of betulinic acid 22.32 mg/l (statistically optimization). Biotransformation was confirmed by RP-HPLC analysis and further characterization of transformed product as betulinic acid was done by ESI-MS.

Keywords:

Betulin, Microbial Transformation, *Bacillus megaterium*, HPLC, Anti-cancer, Betulinic acid

Introduction

Betulin, Betulinic Acid and its derivatives attracted more attention because it has wonderful pharmacological activities, (Alakurtti and Makela 2006). Betulinic acid (BA) Fig 1(A), a naturally occurring pentacyclic lupane type triterpenoid is distributed among a wide range of plant kingdom including *Betula alba* (Betulaceae), *Ziziphus jujube* (Rhamnaceae) (Yogeeswari, and Sriram, 2005). Researchers indicates that betulinic acid as a derivative of betulin, is significantly cytotoxic against non-melanoma human tumors Because of its selective cytotoxicity against tumor cells and favorable therapeutic mode of action, betulinic acid is a very promising newer chemotherapeutic agent for the treatment of cancer and HIV infections (Fulda 1997, Quan et al. 2013). For commercial use, through chemical synthesis, betulin is used as precursor to betulinic acid but due to the specific reaction conditions, environmental safety and pollution issues; it is not advisable (Csuk et al. 2006). While on the other hand biotransformation is more beneficial and appropriate key factor for obtaining

bioactive molecules such as betulinic acid, betulone and other derivatives of betulin that's why biotransformation is advisable (Saxena 2006; Bastos 2007; Mao et al. 2012). All the biocatalytic reactions can be carried out under certain safer, healthy, environment friendly and economic conditions. The target of our work was to be found out suitable bacteria for the bioconversion of betulin to its derivatives either betulinic acid (BA) or else by suitable betulin utilizing bacteria. As most of the reported betulin transformations were done by fungi as biocatalyst where betulinic acid is one of them vital molecules of interest (Bastos 2007; Liu et al. 2010; Mao et al. 2012; Feng et al. 2013). Betulin, 3 β -lup-20(29)-ene-3, 28-diol) fig 1(B) was one of the first natural products isolated in 1788 from the bark of the white birch, *Betula alba*. Betulin from birch bark is a triterpene that can easily be extracted by different methods viz. by organic solvents, thermal sublimation or supercritical carbon dioxide up to the yield of 30 % of total dry weight. A small amount of betulinic acid is often present in extraction of betulin.

Fig 1 Showing the Chemical structure of (A) betulinic acid (B) botulin

BA protects the lungs against inflammation and could be a prospective modulator of inflammation in sepsis-induced acute lung damage (Lingaraju et al., 2015). Betulinic acid induces HeLa cell apoptosis by triggering both the endoplasmic reticulum pathway and the reactive oxygen species (ROS)-mediated mitochondrial pathway (Xu et al., 2014). Betulinic acid mediates the anti-estrogenic effects of *Proteus vulgaris*; which suggests its potential use as a therapeutic agent in estrogen-dependent tumors also (Kim et al., 2014). Betulinic acid dose-dependently inhibits proliferation and induces apoptosis in neuroblastoma and melanoma cell line apoptosis (Tiwari et al, 2014). BA reduces lactate dehydrogenase (LDH) and creatine kinase (CK) release, prevents cardiomyocyte apoptosis, and alleviates the extent of myocardial ischemia /reperfusion injury (Xia et al., 2014). The betulin derivative BT06 and betulinic acid derivative AB13 may be promising alternatives for leishmaniasis therapies, particularly in combination with miltefosine (Gheorgheosu et al., 2014). Betulinic acid derived from *Vitis amurensis* root plays a novel role in melanogenesis. This finding has advanced our understanding of cosmetic therapy to reduce skin hyper pigmentation (Jin et al., 2014). Spray drying of betulinic acid is a superior alternative formulation that significantly increases betulinic acid oral bioavailability and enhances anticancer efficacy (Godugu et al., 2014). Betulinic acid may prevent bone loss in patients with bone metastases and cancer treatment-induced estrogen deficiency (Park et al., 2014). Betulinic acid exerts hepatoprotective effects by increasing antioxidant capacity, through the improvement of the tissue redox system, maintenance of the antioxidant system, and decreased lipid

peroxidation in the liver (Yi et al., 2014). Betulinic acid regulates glycemia through classical insulin signaling by stimulating GLUT4 synthesis and translocation. Additionally, it does not cause hypercalcemia, which is highly significant from a drug-discovery perspective (Castro et al., 2014). Betulinic acid has thyroid-enhancing potential by lowering thyroid-stimulating hormone levels and reducing thyroid tissue damage, thereby minimizing the symptoms of hypothyroidism when used anaphylactically in rats (Afzal et al., 2014). Betulinic acid induces erythroptosis in human erythrocytes through Ca^{2+} loading and membrane permeabilization (Gao et al., 2014). A novel mechanism for BA-mediated ATP-binding cassette transporter A1 (ABCA1) expression has been proposed, which may provide new methods to modulate vascular inflammation and atherosclerosis (Zhao et al., 2013). In recent years, derivatisation of betulin has attracted much more attention, because its derivatives such as betulinic acid and betulone have more potential activities than itself (Aiken 2005; Chen 2005; Mao et al. 2012). Betulin has three positions in its structure, namely secondary hydroxy group at position C-3, primary hydroxy group at position C-28 and alkene moiety at position C-20, where chemical modifications can be easily performed to yield derivatives. Studies at the positions C-3 and C-28 demonstrate that chemical modifications of the parent structure of betulin can produce potentially important derivatives, which can act as antitumor and antiviral agents (Aiken and Chen 2005). Due to poor water solubility of betulin and better solubility of betulinic acid it's a potentially lead compound against human melanoma (Dan et al. 2007; Jie et al. 2013). Microbial transformation is the best tool with great potential specially for improving properties of betulin with high specificity like better solubility and less toxicity. Very few reports are available incorporating microbial systems for conversion of betulin, so it is highly significant to get betulinic acid from betulin, through biotransformation.

2. Materials and Methods

2.1 Chemicals and Reagents

Standard betulin (98%) and betulinic acid (90%) was purchased from Sigma Aldrich and dissolved in dichloro-methane (also known as Methylene Chloride) (1mg/ml) as a stock solution for our experiments. Acetonitrile, Methanol, Water HPLC grade was obtained from SRL Limited (Mumbai). Media components and chemicals used in the growth and down stream process were of analytical grades from Himedia, Merck and SRL Mumbai.

2.2 Screening and isolation of suitable biocatalyst for betulin biotransformation

Different soil samples (each 0.5gm) were dissolved separately, in 100 ml sterile water then 1 ml of this suspension was inoculated to 50 ml betulin screening media (BSM) containing 0.005gm betulin with 0.5 ml (50%) triton X-100 as surfactant to increase betulin hydro-solubility, 4.5 gm of dextrose, 1.25 gm yeast extract, 1.25 gm peptone, 1.75 gm of NaCl, 1.75gm of K_2HPO_4 , 0.75 gm of beef extract, in 50ml of distilled water, at 30°C, pH 7.0 for 48 hours. Further from this, 0.1 ml culture broth was inoculated to solid medium and cultivated for next 3 days. The strains were isolated and maintained on self modified LB medium and PDA medium plates for bacteria and fungi respectively for further tests. Stock cultures of microbes were stored on slants of nutrient agar at 4°C.

2.3 Identification of the suitable biocatalyst strain

The morphology of strain was observed under a microscope (Labomed - Luxedo 4D, camera attached microscope). The biochemical tests such as Malonate Test, Voges Prauskaue Test, ONPG, Nitrate reduction and Catalase test were performed for the identification of our isolated strains specially, *Bacillus spp.* The pure cultures were grown

on nutrient agar or potato dextrose medium and transferred to Luria-Bertoni, Mac-conkey agar medium, EMB agar medium, and Mannitol salt agar medium to differentiate and identify bacteria. The plates were incubated inverted at 28-37°C in the incubator and growth were observed on every 24 hrs intervals after inoculation for 05 days.

2.3.1 Phynotypic characterization

Different isolated strains were grown on LB agar (Luria- Bertani Agar) and some other self modified media were examined for their morphological and cultural characteristics, including cell shape, colonial appearance, endospore formation and pigmentation, after incubation at different pH (4.5, 6, 6.5, 7, 8 and 9), and at different temperatures (25, 30, 35, 40 and 45 °C) were tested by using LB agar medium. All tests were carried out by incubating the cultures at different combinations of condition.

2.3.2 Biochemical characterization

Variety of biochemical tests were done for identification of different groups of microbes. Different microbes carry distinguished characterization as per their group, genera and species i.e. based on “Bergey’s Manual Of Determinative Bacteriology”, Voges-Prauskaue (VP), ONPG, Catalase, Citrate, Arginine utilization, Malonate are some of the key factors that we considered in our studies. Different sugars were also tested to get the best carbon source other than glucose but we focused specifically on bacillus strains, and finally by strictly following physiologically and biochemically characterization we selected some bacillus strains for our use.

2.3.3 Phylogenetic identification of strain KD235

The strain was assigned to be the genus *Bacillus*, according to their morphological and biochemical characterization the strain identification was confirmed by carrying its 16S rDNA sequencing and comparing of the reference strains available. The 16S rDNA analysis of reference stains with our strain i.e. KD235 shown 99% similarities to *Bacillus megaterium*. Our results indicated that the isolated stain is identified as *Bacillus megaterium* KD235 (sequence submitted to NCBI reference no KR261097).

Fig 2 Showing the Phylogenetic tree representing the position of *Bacillus megaterium* KD235 (Sequence submitted to NCBI reference no. KR 261097)

2.4 Optimization of biotransformation rate at Shake flask using CCD

To find out the effects of optimum growth conditions such as initial pH on biotransformation in shake flask cultures, the different initial pH was adjusted (4.5-9.0) by addition of 1.0 M NaOH or HCl. The temperature change (25-45°C) was conducted in the temperature controlled shaking incubator. The biotransformation time (24 to 144 hrs.) after the addition of the substance was examined. The experiments at shake flask culture level were performed in a 250 ml Erlenmeyer flask containing 50 ml media after inoculating with the *Bacillus megaterium* KD235 culture. The pH and agitation rates were controlled at 6.5 and 120, respectively at 30°C. After 24 hours inoculation, 0.10ml of the already prepared substrate solution betulin (0.5 mg/ml) was added to each flask and these flasks were maintained under the same cultivation conditions for additional 144 hours. Culture control was run with the inoculation of microorganism, while with the addition of the equal amount of dichloromethane instead of betulin. So, there was microorganism growing while no substrate biotransformation took place in culture control. While substrate control was run without the inoculum that means betulin was present but our biocatalyst (i.e. *Bacillus megaterium* KD235) was absent but rest of the other culture conditions were the same with the biotransformation experiment.

2.5 Purification and identification of BETULINIC ACID as biotransformed metabolite

2.5.1 Liquid-liquid extraction

Liquid-liquid extraction is an important separation technology, with a wide range of applications in the modern process industry. It is a process of transferring a solute from one liquid phase to another immiscible or partially miscible liquid in contact with the first. The two phases are chemically quite different, which leads to a separation of the components according to their distribution or partition between the two phases, normally one organic and one water. The extraction process is based on different solubilities of components in to immiscible, or partially miscible, liquids such as water with chloroform, or water with ethyl acetate / dichloro methane / dichloro ethane and other organic solvents.

Both liquids have to mixed thoroughly, together and subsequently separated from each other again. To achieve high purities and yields of our desired product, it is necessary to operate with multiple stages or we can say that the extraction should be done repeatedly with different ratios of the solvents as well different combinations of these solvents. For successful separation by liquid-liquid extraction, all components must meet certain specifications. Here the main criteria for the extractant are a favorable partition coefficient, a high selectivity and an easy separation from the extracted product. The basic conditions for the pair of liquids are a low mutual solubility and a difference in density, which is the driving force for the motion of the droplets. The viscosity and interfacial tension are additional important parameters.

2.5.2 Chromatographic analysis

The extracts were concentrated and chromatographed on TLC plates. Substrate controls were composed of sterile medium to which betulin was added and were incubated without the microorganism. Thin-layer chromatography (TLC) analysis were carried out on pre-coated Silica UV254 plates (E.Merck, Darmstadt, Germany). The adsorbents used for column chromatography were Silica Gel 60 Å (70 to 230 meshes) and lipophilic Sephadex LH-20. The solvent system used for TLC was CHCl₃-methanol (8:2 v/v) and visualization of the spots on TLC plates was performed with anisaldehyde-H₂SO₄ spray reagent. The spots were

visualized by spraying a plate with anisaldehyde-H₂SO₄ spray and then heating it at 110°C for 3 min in an oven.

2.5.3 ESI-MS analysis

In addition to conform the structural changes and product formation and structural elucidation in our substrate, ESI-MS was performed from JNU, New Delhi India. The ESI-MS Peaks shown difference in their values as shown in figure 3 (A) and (B) that itself indicates that the biotransformation taken place.

(a) was identified as betulin. Except for the medium composition and of betulin, there was a new peaks found at the retention time of 12.84 min (fig 4b) occurred which should be the transformed products of betulin i.e. betulinic acid. The productivity of betulinic acid after biotransformation was calculated as the percentage of the amount of betulinic acid formed against the total amount of betulin added in the transformation medium. We collected these fractions and did ESI-MS which confirmed that product of peak at 3.14 min is betulin and 12.84 min is betulinic acid. That result also indicates towards the confirmation of the activity of our biocatalyst (*Bacillus megaterium* KD235) is efficiently converting substrate to its derivative betulinic acid which is a significant molecule.

3.2 Optimal conditions for the biotransformation of betulin

In order to investigate the effect various parameters such as temperature, pH, substrate concentration, growth measurement as packed cell volume (PCV @ mg/ml) on transformation process, *Bacillus megaterium* KD235 was cultivated in the medium with different combinations set by group of experiments using Design of Expert (Trial Version 9.0.5.1) in shake flask cultures. Maximum transformation product was obtained at an initial pH 6.5, the optimal transformation temperature was investigated and found to be $30 \pm 1^\circ\text{C}$.

3.2.1 Optimization of experiments of designs and statistical analysis

All experiments were carried out in triplicate. The data analyses were performed using Design-Expert version 9.0 (Free Trial version Stat-Ease Inc., Minneapolis, MN). Treatment effect was analyzed using analysis of variance. Differences were considered to be significant at $P < 0.05$ throughout the present study. Each point is the mean of replicate experiments. The Model F-value of 111.19 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction can improve our model. The "Lack of Fit F-value" of 1.71 implies the Lack of Fit is not significant relative to the pure error. There is a 22.03 % chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good we want the model to fit. All empirical models were tested by doing confirmation runs. A ratio greater than 4 is desirable. Our ratio of 25.581 indicates an adequate signal so this model can be used to navigate the design space. From the response data analysis it was confirmed that the empirical relationship between betulinic acid productivity (%) as bioconversion and the tested variables (X_i) in the coded unit through the following regression equation

Betulinic acid productivity (%)	
=	+22.342-0.23734*A+0.10231*B-0.16286*C+0.33231*D
	-0.06458*E - 0.10031*AB + 0.11781*AC - 0.21656*AD
	-0.01156*AE - 0.18031*BC + 0.15406*BD+0.09156*BE
	-0.22531*CD - 0.05719*CE + 0.00781*DE

3.2.2 Effect of reaction time on Bioconversion

To find out the best reaction time that means the duration is most suitable to give highest yield we set the experiments in triplicate. The experiments were performed according to the

designed parameters using statistical tools. As shown in the figure 5(A), it is evident that best results were obtained at 72 hours of reaction after inoculation, which is positively supported by increase in biomass of our bacterium. Initially it increases as reaction time goes on but as after 72 to 84 hours but later on it decreases probably because of oxidation of betulin to betulinic acid on longer incubation which is also supported in the previous findings (Liu et al. 2010;Parnali et al. 2005).

Fig 5 Showing the interaction of Betulin Bioconversion with (A) Time (B) Temp.

3.2.4 Effect of reaction temperature on Bioconversion

To determine the best reaction temperature the reaction mixtures as mentioned above were incubated at various temperatures ranging from 25-45°C. It is evident from the figure 5(B) that the betulin bioconversion % was found maximum at 30°C, and as the temperature goes on increasing the bioconversion decreases. This decrease in bioconversion may be because of the reduced enzyme activity of biocatalyst or may be oxidation of betulin to Betulinic acid. It is evident from the figure that higher temperature leads to lower bioconversion because of deactivation of enzymes responsible for betulin bioconversion, this co-relationship between temperature and biotransformation *Bacillus megaterium* (Dubey et al.2011; Parnali et al. 2005).

3.2.4 Effect of Substrate on Bioconversion

To find the effect of the substrate concentration reactions were carried out in the 250 ml flask with the increasing concentration of betulin ranges from 0.5 to 4.5 mg/l. It is shown in the figure that 1.375 mg/l conc. Betulin found to be the best for the maximum bioconversion up to 24% productivity of supplied substrate with maximum biomass. But as soon the betulin concentration rises above the 1.375mg /l the biomass generation is inhibited (figure 6A) due to toxicity of betulin towards our biocatalyst. In our case microbial transformation by whole cell mass of *Bacillus megaterium* KD 235 we have achieved 22.34 % productivity while in case of the whole cell mass of cultured *Armillaria luteo-virens* Sacc ZJUQH100-6 the overall productivity has been 9.63% after the incubation of three days (Liu et al.2010).

Fig 6 Showing interaction of Betulin Bioconversion with (A) Substrate conc.(B) pH

3.2.5 Effect of pH on Bioconversion

To validate the pH of reaction mixture we performed the experiments according to designed conditions at varying pH ranging from 4.5 to 8.5 using various buffers. As shown in figure 9 as the pH increases the bioconversion increases and gives the best results on pH 6.5 (figure 6B). As we proceed to higher pH above than 7.5 the bioconversion decreased and shows that betulin bioconversion is prone to pH change towards alkalinity. This decrease in bioconversion is because of the oxidation of betulin to betulinic acid.

3.2.6 Effect of surfactant

The effect of surfactant is not that much studied so far. Here we studied the effect of addition of different concentration of Triton-X100. It has been reported that by increasing the availability of the substrate to catalyst the conversion can be achieved to a better extent (Kumar et al.2006). Betulin and BA acid are strongly non polar compound, that's why addition of non-ionic surfactant can help to improve the solubility of betulin which become available to our biocatalyst and reduces the problem of conversion to its derivative product as betulinic acid (Liu et al.2010).

Fig 7 Showing interaction of Betulin Bioconversion with surfactant

4. Result and Discussion

Day by day new infections are observed because of the multi drug resistance and lower efficacy of the available treatments. Therefore, novel potent antiretroviral agents with different targets on cheaper prices are still urgently required. Two lupane-type triterpenes betulin and betulinic acid (BA), exhibit diverse pharmacological activities, including anti-HIV. From the chemically synthesized betulin derivatives, "Bevirimat" [3-O-(3',3'-dimethylsuccinyl) betulinic acid] shown remarkable anti-HIV-1 activity against primary and drug-resistant HIV-1 isolates, (Kashiwada et al. 1996; Kanamoto et al. 2001). This represented a pioneer in a class of anti-HIV compounds termed maturation inhibitors (MIs). Bevirimat has recently succeeded in Phase IIb clinical trials (Li et al. 2003). The present work deals with the microbial conversion of betulin to betulinic acid that has better pharmaceutical significance. As betulin has lower solubility and more toxicity than betulinic acid it has been suggested to make derivatives of betulin to be used for pharmaceutical applications. Here we investigated the bioconversion of betulin into its better pharmaceutical derivative betulinic acid (BA) which is product of importance. Biochemically this bioconversion of betulin into its respective derivative occurred due to the redox activity of microorganisms. We isolated 289 microorganisms from industrial sites and these were screened for monooxygenase activity out of them, few strains were found positive and out of these positive strains *Bacillus megaterium* KD235 was found most appropriate strain which was showing maximum biocatalytic power. Experiments were performed with *Bacillus megaterium* ACBT03 by Dubey et al. has also shown the use of *Bacillus megaterium* for bioconversion of plant alkaloids (Dubey et al. 2008, 2011). Betulin is toxic chemical and its toxicity is clearly observed with growing *Bacillus megaterium*. To study this effect experiments in triplicate were performed with gradually increased concentration of betulin (0.25mg/l, 1.0mg/l, 1.375 mg/l, 2.5 mg/l, 4mg/l). It was observed that as concentration of betulin is increased in medium it hinders the growth of *Bacillus megaterium* KD235. In all experiments lag phase for all concentrations is approximately similar while log phase varies with change in concentration. As the concentration of betulin is increased it affects the growth of *Bacillus megaterium* negatively. Bioconversion of betulin into betulinic acid through *Bacillus megaterium* was studied with different concentrations. In this experiment concentrations of betulin were kept at 0.1 mg/ml, 0.25mg/l, 1.0mg/l, 1.375 mg/l, 2.5 mg/l, 4mg/l. All the experiments were performed in triplicate to avoid error in experiments. The bioconversion rate of betulin into betulinic acid was increased initially from 0.01 to 1.375 mg/l concentration of betulin and after that productivity was reduced. It was observed that at 1.375 mg/l betulin concentration the productivity was about ~22%. Possible mechanism for this bioconversion process might be depending on two factors one is availability of betulin to catalyst (*B. megaterium*) and second is availability of carboxylase enzyme (that corresponds to amount of viable bacterial cells in the medium) to the substrate. The slight increased concentration of betulin hinders growth of bacteria but not so much and this reduction was adjusted by catalytic power of monooxygenase enzyme (turn over number) and more betulinic acid was produced from more betulin acid and upto 1.375 mg/l of betulin, bioconversion of betulin was increased. Further high concentration of betulin concentration reduced growth of bacteria to the level that it could not be compensated from carboxylase and it resulted in reduction of bioconversion with increased concentration of betulin.

5. Conclusions

From the last 30 years, Betulinic acid has been a promising new chemotherapeutic agent for the treatment of cancer and HIV infections. There are very few studies available on the bioprocess optimization studies for terpene like betulin and betulinic acid. The available

literature represents the extraction, birch plant biology but not more work found on microbial transformation of betulin and betulinic acid. Here morphological and biochemical characterization of 289 isolates were done, 03 out of them (KD190, KD 235 and KD 271), were screened for further analysis which shows that these isolates could be suitable bacteria for biotransformation of betulin to betulinic acid. Among the isolates, KD 235 shown about 22% productivity (1.375 mg/l used as substrate) in to their respective derivatives i.e. betulinic acid at 250 ml shake flask and 5 liter bioreactor level betulin as a sole carbon source with control. Biotransformation was confirmed by carrying HPLC analysis using C18 Column under flow rate set at 1.0 ml/min, mobile phase was composed of acetonitrile-methanol and detection wavelength set at 210 nm. This is a pioneer attempt to carry microbial transformation of the betulin as a starting molecule and to give product as betulinic acid through a prokaryotic system i.e. *Bacillus megaterium* KD235 other researchers used fungi for similar biotransformation (Liu et al.2010; Mao et al.2012; Min Li et al.2013). For, betulinic acid as a product through microbial conversion the reaction parameters have to be optimized according to high stability of strain, high yield of the product and low production cost. In the present work we optimized physical process parameters as temperature, pH, Incubation duration, as well surfactant as another significant factor for the process. While in the present study the most significant factors are the concentration of betulin and the surfactant concentration. As our studies indicates that 1.375 mg/l betulin and 1.25 % of Triton X100, are the best suitable parameters for microbial transformation to give its derivative as betulinic acid. But our result also indicates that pH, incubation duration and glucose are also similarly important parameters for the process. For glucose as the additional carbon source, results indicate as lesser significance as upto 4.5 % it helps in the biomass generation during the process but above that concentration its effect is not that much significant. However, we worked on the bioprocess development of the betulin biotransformation but further studies are required to be done on molecular biology and enzymatic pathway elucidation of the same. Present work resulted that betulin could be biotransformed with the help of bacterial also to give betulinic acid as a significant compound for pharmacological applications having anti-cancer and anti-HIV activity. Betulinic acid has been considered as a very significant candidate for the clinical treatment of various forms of cancers and HIV-1 as well this has a pharmaceutical potential in combating type 2 diabetes mellitus and obesity by efficiently regulating the various enzymes and hormones involved in the absorption and metabolism of carbohydrates and lipids. We have found that betulinic acid and its derivatives can be formed by means of a transformation with *Bacillus megaterium* KD 235. *Bacillus megaterium* is a suitable and interesting microbial model for evaluating the biotransformation of betulin. However, further structure identification and functionality evaluation of transformed products should be considered. This work is in progress in our lab.

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Contributions

Author 1 and 2 performed all experiments, authors 3 and 4 supported for instrumental analysis of ESI-MS, and NMR, while author 5 and 6 helped in improvising the manuscript. All authors have no conflicts of interest. All the Authors agreed to submit the manuscript.

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Compliance with Ethical Standards

Ethics Declarations

This research does not include any studies or trials with human participants or animals.

Consent to Participate

All the authors agreed to participate in the scientific work.

Consent for Publication

All the authors agreed to submit the manuscript.

Competing Interests

The authors declare that they have no competing interests.

References

1. Afzal M, Kazmi I, Semwal S, Al-Abbasi FA, Anwar F. (2014) Therapeutic exploration of betulinic acid in chemically induced hypothyroidism. *Mol Cell Biochem.*; 386, 27-34.
2. Aiken, C., & Chen, C. H. (2005). Betulinic acid derivatives as HIV-1 antivirals. *Trends in molecular medicine*, 11(1), 31-36.
3. Alakurtti S., Mäkelä T., Koskimies S., Yli-Kauhaluoma J. (2006) Pharmacological properties of the ubiquitous natural product betulin. *Eur J Pharm Sci.* 29,1-13.
4. Bastos DZ, Pimentel IC, Jesus DA. et al. (2007) Biotransformation of betulinic and betulonic acids by fungi. *Phytochemistry*, 68, 834-39.
5. Castro AJ, Frederico MJ, Cazarolli LH, Bretanha LC, Tavares Lde C, Buss Zda S, et al. (2014) Betulinic acid and 1,25(OH)₂ vitamin D3 share intracellular signal transduction in glucose homeostasis in soleus muscle. *Int J Biochem Cell Biol.* 48,18-27.
6. Csuk R (2014) Betulinic acid and its derivatives: a patent review (2008-2013). *Expert Opin Ther Pat.*, 24, 913-23.
7. Dan C, Guoling Z, and Weidong Y, (2007) Solubilities of Betulin in Fourteen Organic Solvents at Different Temperatures *J. Chem. Eng. Data*, 524, 1366-1368.
8. Dubey K.K., Haque S., Jawed A., Singh B.P., Behera B.K. (2010) Construction of recombinant Escherichia coli for enhanced bioconversion of colchicine into 3-demethylated colchicine at 70 l bioreactor level. *Process Biochem*, 45, 1036-1042.
9. Dubey, K.K., Ray, A.R., Behera, B.K., (2008) Production of regiospecific derivatives of colchicine through microbial transformation and scale-up process development. *Process Biochem*, 43, 251-257.

10. Feng Y, Li M, Liu J, et al. (2013) A novel one-step microbial transformation of betulin to betulinic acid catalysed by *Cunninghamella blakesleeana*, *Food Chemistry*, 136, 73-79.
11. Fulda, S., Friesen, C., Los, M., Scaffidi, C., Mier, W., Benedict, M., Nunez, G., Krammer, P.H., Peter, ME., Ebatin, K. (1997) Betulinic acid triggers CD95 (APO-1/Fas)- and p53-independent apoptosis via activation of caspases in neuro ectodermal tumors. *Cancer Res.*, 57, 4956-4964.
12. Fulda, S.; Debatin, K.(2005) Sensitization for anticancer drug-induced apoptosis by betulinic acid. *Neoplasia*, 7, 162–170.
13. Gao M, Lau PM, Kong SK.(2014) Mitochondrial toxin betulinic acid induces in vitro eryptosis in human red blood cells through membrane permeabilization. *Arch Toxicol.*, 88, 755- 68.
14. Gheorgheosu D, Duicu O, Dehelean C, Soica C, Muntean D. (2014) Betulinic acid as a potent and complex antitumor phytochemical: a minireview. *Anticancer Agents Med Chem.*, 14, 936- 45.
15. Godugu C, Patel AR, Doddapaneni R, Somagoni J, Singh M. (2014) Approaches to improve the oral bioavailability and effects of novel anticancer drugs berberine and betulinic acid. *PLoS One.*;9:e89919.
16. Holz-Smith SL, Sun I, Chen CH. et al. (2001) Role of human immunodeficiency virus (HIV) type 1 envelope in the anti-HIV activity of the betulinic acid derivative IC9564. *Antimicrob. Agents Chemother.* 45, 60–66.
17. Jin KS, Oh YN, Hyun SK. (2014) Betulinic acid isolated from *Vitis amurensis* root inhibits 3-isobutyl-1-methylxanthine induced melanogenesis via the regulation of MEK/ERK and PI3K/Akt pathways in B16F10 cells. *Food Chem Toxicol.* 68, 38-43.
18. Kim HI, Quan FS, Kim JE, et al. (2014) Inhibition of estrogen signaling through depletion of estrogen receptor alpha by ursolic acid and betulinic acid from *Prunella vulgaris* var. lilacina. *Biochem Biophys Res Commun.* 451, 282-87.
19. Kim HI, Quan FS, Kim JE, Lee NR, Kim HJ, Jo SJ, et al. (2014) Inhibition of estrogen signaling through depletion of estrogen receptor alpha by ursolic acid and betulinic acid from *Prunella vulgaris* var. lilacina. *Biochem Biophys Res Commun.*, 451: 282-7.
20. Kumar, A.K. and Goswami, P. (2006) Functional characterization of alcohol oxidases from *Aspergillus terreus* MTCC 6324. *Appl Microbiol Biotechnol*, 72, 906-911.
21. Li L, Du Y, Kong X, Li Z, Jia Z, Cui J, et al. (2013) Lamin B1 is a novel therapeutic target of betulinic acid in pancreatic cancer. *Clin Cancer Res.* 19, 4651- 61.
22. Lingaraju MC, Pathak NN, Begum J. et al. (2015) Betulinic acid attenuates lung injury by modulation of inflammatory cytokine response in experimentally- induced polymicrobial sepsis in mice. *Cytokine.* 71, 101-08.
23. Liu, J., Fu, M. L., & Chen, Q. H. (2010) Biotransformation optimization of betulin into betulinic acid production catalysed by cultured *Armillaria luteo-virens* Sacc ZJUQH100-6 cells. *Journal of Applied Microbiology*, 110, 90-97.
24. Mao DB, FengYQ, BaiYH et al. (2012) Novel biotransformation of betulin to produce betulone by *Rhodotorula mucilaginosa*, *J of the Taiwan Instt. Chem. Engg.* 43, 825–829.
25. Park, SY.; Kim, HJ.; Kim, KR. (2014) Betulinic acid, a bioactive pentacyclic triterpenoid, inhibits skeletal-related heavy met events induced by breast cancer bone metastases and treatment, *Toxicol Appl Pharmacol.* 275,152-62.
26. Parnali, C.; Samir A.K.; John M.P.; et al. (2000) Biotransformation of the Antimelanoma Agent Betulinic Acid by *Bacillus megaterium* ATCC 13368, *Applied and Environmental Microbiology*, 66, 93850–3855.

27. Quan et al., Quan HY, Kim do Y, Kim SJ, Jo HK, Kim GW, Chung SH. (2013) Betulinic acid alleviates non-alcoholic fatty liver by inhibiting SREBP1 activity via the AMPK/mTOR-SREBP signaling pathway. *Biochem Pharmacol.* 85, 1330- 40.
28. Saxena BB, Zhu L, Hao M. et al. (2006) Boclysinated betulonic acid: a potent, anti-prostate cancer agent. *Bioorg Med Chem Lett*, 14, 6349- 58.
29. Tiwari, R.; Puthli, A.; Mishra, KP. (2014) Betulinic acid-induced cytotoxicity in human breast tumor cell lines MCF-7 and T47D and its modification by tocopherol. *Cancer Invest.* 32, 402-408.
30. Xu T, Pang Q, Zhou D, Zhang A, Luo S, Wang Y, et al. (2014) Proteomic investigation into betulinic acid-induced apoptosis of human cervical cancer HeLa cells. *PLoS One.*; 9(8): e105768.
31. Yi J, Xia W, Wu J, Yuan L, Wu J, Tu D, et al. (2014) Betulinic acid prevents alcohol-induced liver damage by improving the antioxidant system in mice. *J Vet Sci.* 15, 141-8.
32. Yogeswari, P., and Sriram, D. (2005) Betulinic acid and its derivatives: a review on their biological properties. *Current Medicinal Chemistry.* 12, 657-666.
33. Zhao GJ, Tang SL, Lv YC, Ouyang XP, He PP, Yao F, et al. (2013) Antagonism of betulinic acid on LPS mediated inhibition of ABCA1 and cholesterol efflux through inhibiting nuclear factor-kappa B signaling pathway and miR-33 expression. *PLoS One.*; 8(9): e74782.