Improvement of Bioconversion of Vitamin D_3 into Calcitriol by *Actinomyces hyovaginalis* through Protoplast Fusion and Enzyme Immobilization

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Abstract: Protoplast fusion and enzyme immobilization techniques were applied to increase calcitriol production from vitamin D_3 using *Actinomyces hyovaginalis*, a local isolate recovered from Egyptian soil, that has a potential bioconversion activity of vitamin D_3 into calcitriol. A total of sixteen protoplast hybrids, formed between *Actinomyces hyovaginalis* isolate and two *Bacillus* species (*B. thuringiensis* and *B. weihenstephanensis*) were screened for vitamin D_3 bioconversion activity. Compared to wild type isolate, four hybrids (formed between *Actinomyces hyovaginalis* isolate and *B. thuringiensis*) were found to preserve the bioconversion activity; out of which, three hybrids coded V2B, V3B and V8A exhibited higher calcitriol production. The hybrids coded V2B and V8A produced, per 1 L culture medium, about 0.5 and 0.4 mg calcitriol corresponding to 350% and 280%, respectively, increase compared to the wild type isolate. Among different alginate concentrations applied, immobilization of cell lysate of *Actinomyces hyovaginalis* isolate using 2% alginate showed 140% increase in calcitriol production from vitamin D_3 compared to the free cell lysate. Activity of the immobilized form was preserved for five repetitive uses over a period of 15 days but with a 50% decline in production occurring at the fifth use.

Keywords: Bioconversion, protoplast fusion, immobilization, vitamin D_3, calcitriol, *Actinomyces hyovaginalis.*

INTRODUCTION

Vitamin D_3 is biologically inert (prohormone) and to exert its function, it must be first activated by hydroxylation in liver and kidney [1], as shown in Figure **1**. Deficiency of formation of vitamin D_3 active forms in human body, especially calcitriol (1α, 25-dihydroxyvitamin D_3), may be due to genetic or physiological factors including hepatic and renal disorders. This causes several diseases such as rickets, osteoporosis, hyperparathyroidism and psoriasis. Synthetic calcitriol has been used clinically to treat such disease states but unfortunately, its chemical synthesis, especially regio- and stereo- selective introduction of a hydroxyl group at C-1, is a very expensive and complicated procedure [2]. Such criteria have necessitated the need to apply enzymatic chemistry using microorganisms to hydroxylate vitamin D_3 at the 1α- and /or 25- positions.

Sasaki and his coworkers were the first to apply bioconversion to hydroxylate vitamin D_3 at the 1α- and /or 25- positions [3,4]. They showed that microorganisms capable of hydroxylating vitamin D_3 compounds were found to be belonging to the order Actinomycetales. The first attempt for industrial production of calcitriol was carried out in 1994, when Takeda and his research group succeeded in the application of cyclodextrin to increase the conversion of vitamin D_3 into 25-hydroxy and 1α, 25-dihydroxy metabolites by *Amycolata autotrophica* [5]. In 2006**,** Kang and his coworkers have demonstrated that optimizing the culture conditions and the timing of substrate addition is an excellent strategy for the improvement of bioconversion of vitamin D_3 to calcitriol by *A. autotrophica* ID 9302 in a 5 L fermentor [6]. Many researchers have attempted to isolate vitamin D_3 hydroxylase (Vdh) from *A. autotrophica*, but such mission has not been successful till 2009 when Fujii and his researchers isolated Vdh, cytochrome P450 monooxygenase from the same bacterial species. Overexpression of the recombinant Vdh was carried out using a *Rhodococcus erythropolis* expression system and the protein was subsequently purified, crystallized and subjected to preliminary X-ray diffraction studies [7]. In 2010, Yasutake and his research team described the crystal structures of wild-type Vdh -isolated from *A. autotrophica* and from the highly active mutant, generated by directed evolution [8]. Later, Kang and his coworkers used resting cells of *Pseudonocardia* sp. KCTC 1029BP for the bioconversion of vitamin D_3 to calcitriol and they applied a two-step optimization process including the Plackett–Burman and the central composite designs [9]. Luo *et al.* investigated the optimal culture conditions for bioconversion of vitamin D_3 to calcifediol

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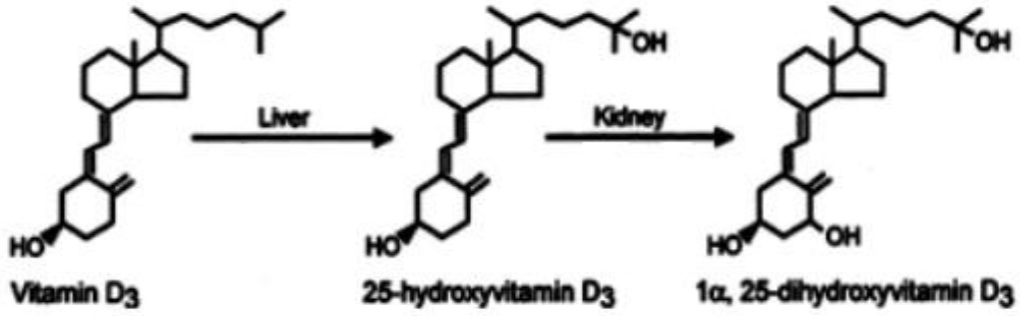

Figure 1: Metabolic activation of vitamin D_3 in liver and kidney.

(25(OH)D_3) using *Pseudonocardia autotrophica* in a 50 L fermentor [10].

A previous research was conducted in our laboratory to study bioconversion of vitamin D_3 into calcitriol using local bacterial isolates collected from different soil samples in Egypt. We accomplished the bioconversion of vitamin D_3 into calcitriol with *Actinomyces hyovaginalis* species. In addition, it was found that an alternative promising bioconversion approach for vitamin D_3 into calcitriol by the *Actinomyces hyovaginalis* isolate could be applied depending on the use of cell lysate [11,12].

Based upon the previous results, the following study aimed at improving calcitriol production from vitamin D_3 by the *Actinomyces hyovaginalis* isolate using protoplast fusion and enzyme immobilization techniques. The results obtained would help for calcitriol production form vitamin D_3 for industrial applications.

MATERIALS AND METHODS

Microorganisms and Maintenance Conditions

Actinomyces hyovaginalis A11-2 (study isolate), a local isolate recovered from Egyptian soil, which, in a previous study, was proven to be of a potential bioconversion activity of vitamin D_3 into calcitriol [11]. It was maintained onto nutrient agar slants at 4°C. For stock cultures, the isolate cells were concentrated and suspended in glycerol solution (50%) [13] and the cell suspension was stored in cryogenic tube at –20°C. Two *Bacillus* isolates (available at department of Microbiology and Immunology, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt), previously identified using 16S rRNA sequencing as *B. thuringiensis* isolate B16 and *B. weihenstephanensis* isolate B65 [14], were maintained onto nutrient agar slants at 4°C and used for protoplast fusion with *Actinomyces hyovaginalis* isolate A11-2.

Vitamin D_3 and its Derivatives

Vitamin D_3 (bioconversion substrate) was kindly provided by Medical Union Pharmaceuticals (MUP), Cairo, Egypt. 1α-hydroxy vitamin D_3 (used instead of 25-hydroxy vitamin D_3 for HPLC experiments) was obtained from MinaPharm/LEO, Cairo, Egypt and 1α, 25-dihydroxy vitamin D_3 (calcitriol) was purchased from Sigma-Aldrich (St Louis, MO, USA).

Bioconversion of Vitamin D_3

Bioconversion reaction and preparation of concentrated extracts of the bioconverted products were carried out as previously reported by Sasaki *et al.* [3,4] with some modifications [11].

Analysis Techniques of Vitamin D_3 Metabolites

Thin Layer Chromatography (TLC)

Aliquots (40 µl each) of concentrated bioconversion extracts were loaded on a TLC plastic sheet (Merck, F_{254} pre-coated) against 20 µl of calcitriol standard (0.5 µg/ml). Then the TLC plate was developed ascendingly in a closed glass chamber using chloroform/methanol (95:5 mixture) as a mobile phase. Detection of spots was performed using UV light at 254 nm wavelength [4].

High Performance Liquid Chromatography (HPLC)

An aliquot (20 µl) of the concentrated bioconversion extract was analyzed by HPLC system (Agilent technologies GmbH & Co. Waldbronn, Germany) using the following conditions: Discovery/Supelco HS C18 column (4.6 mm [inner diameter] by 25 cm length) as a stationary phase; 0.1% acetic acid/acetonitrile (55:45) as a mobile phase at flow rate 1 ml/min for 10 min; and UV-VIS detector at wavelength of 254 nm. Concentrations of calcitriol in all samples were determined using calibration curve of calcitriol standard run by HPLC under the same conditions.

Improvement of Bioconversion of Vitamin D_3 into Calcitriol by Actinomyces Hyovaginalis Isolate through Protoplast Fusion

Protoplasts Preparation

Protoplasts were prepared according to procedures described by Sambrook and Russell [15] which were originally based on the work of Okanishi *et al.* [16]. Two identified *Bacillus* isolates; *B. thuringiensis* isolate B16 and *B. weihenstephanensis* isolate B65 were selected for protoplast preparation, each with *Actinomyces hyovaginalis* isolate (study isolate), as follows: A single bacterial colony was inoculated in 10 ml nutrient broth which was incubated at 30°C and 200 rpm for 24 h (*Bacillus* isolates) and for 48 h (*Actinomyces hyovaginalis* isolate). Then 2% v/v vegetative inoculum of the prepared preculture was transferred to 25 ml nutrient broth containing 5% w/v PEG 6000 and 0.5% w/v glycine and incubated at 30°C and 200 rpm for 24 h. Bacterial cells were harvested by centrifugation at 4000 rpm for 10 min and then washed twice with 10.3% w/v sucrose. Harvested bacterial cells were then suspended in 10 ml P-buffer [16,17] containing lysozyme (1 mg/ml) and incubated at 30°C for 60-90 min with periodic tilting. Formation of protoplasts was checked by microscopical examination at different time intervals. Protoplasts were harvested by centrifugation at 4°C and 2500 rpm for 10 min, then re-suspended in 2 ml P-buffer and stored at -20°C till use.

Protoplasts Fusion

1 ml of the P-buffer containing protoplasts of *Actinomyces hyovaginalis* isolate was mixed with 1 ml protoplast suspension of each of the two *Bacillus* isolates in 10 ml fusion medium (25% w/v PEG 1500 in P-buffer) and the mixtures were shaken at 20°C and 150 rpm for 45 min. Protoplast hybrids were harvested by centrifugation at 4°C and 2500 rpm for 5 min and then re-suspended in 10 ml P-buffer.

Regeneration of Protoplast Hybrids

Five hundreds aliquots of each original hybrid suspension, its 10- and 1000-fold dilutions were transferred and separately streaked onto plate containing protoplast regeneration medium [18] which was then incubated at 30°C for 7-10 days with periodic observation and microscopic examination of the resulting growth.

Testing Vitamin D_3 Bioconversion Capability of Protoplast Hybrids

Sixteen protoplast hybrids were selected randomly from regeneration medium; coded V1A to V8A and V1B to V8B. They were screened for their vitamin D_3 bioconversion activities using wild type *Actinomyes* isolate as a control [11]. Analysis of extracts was carried out using TLC and HPLC.

Improvement of Bioconversion of Vitamin D_3 into Calcitriol through Immobilization of the Crude Cell Lysate of *Actinomyces hyovaginalis* Isolate

Immobilization

This was carried out via entrapment technique using calcium alginate polymeric matrix [19,20] as follows: A single bacterial colony was inoculated in 10 ml nutrient broth which was incubated at 30°C and 200 rpm for 48 h. Vegetative inoculum of the prepared preculture was transferred to 50 ml bioconversion medium (fructose, 15 g; defatted soybean, 15 g; NaCl, 5 g; $CaCO_3$, 2 g; K_2HPO_4, 1 g; NaF, 0.5 g; distilled water, 1000 ml; pH 7.8) at 2% v/v. Incubation was carried out at 30°C and 200 rpm for 48 h. Bacterial cells were recovered by centrifugation, suspended in 10 ml phosphate buffer (0.2 M $(NH_4)_2HPO_4$, 45 ml; 0.2 M $NH_4H_2PO_4$, 40 ml; NaCl, 1 g; NaF, 0.1 g; K_2HPO_4, 0.2 g; $MgSO_4.7H_2O$, 0.1 g; distilled water to 115 ml; pH 7.8), contained in 50 ml beaker, and lysed via sonication using probe sonicator [Hielscher Ultrasonics GmbH,Teltow, Germany] at 70% power for 3 successive times; each for 1 min with 30 sec intervals, while keeping the beaker in ice. Crude cell lysate suspension (10 ml) was mixed with appropriate volume (90 ml) of distilled water containing sodium alginate (final concentration of 1%, 2% and 3% w/v) and the mixture was added drop-wise (dripped) from a height of approximately 20 cm into 100 ml of 0.2 M calcium chloride solution with continuous stirring.

Formed beads, containing immobilized cell lysate, were left to equilibrate at room temperature for 1 h, then collected, rinsed with distilled water, left to harden in air and stored in 50 mM Tris-HCl (pH 7.4) at 4°C [21,22].

Vitamin D_3 Bioconversion by the Immobilized Crude Cell Lysate

This was carried out using free crude cell lysate as a control. Beads stored (for 1 day) were washed with distilled water, dried and transferred to separating funnel. A volume of 50 ml Tris-HCl (50 mM, pH 7.8) containing the substrate (10 mg vitamin D_3 dissolved in 250 µl 96% ethanol) was added to the funnel and incubation was carried out at 28°C for 3 h. The reaction solution, covering and surrounding the beads, was

withdrawn, extracted and analyzed, for calcitriol production, using HPLC. The beads left were washed once with distilled water (50 ml) and once with 50 mM Tris-HCl (50 ml), then re-stored in Tris-HCl (50 mM, pH 7.4). The previous steps were repeated after 2, 5, 9 and 15 days of beads preparation.

RESULTS AND DISCUSSION

Improvement of Bioconversion of Vitamin D_3 into Calcitriol by *Actinomyces hyovaginalis* Isolate through Protoplast Fusion

Out of sixteen protoplast hybrids selected randomly from regeneration medium and screened for their vitamin D_3 bioconversion activity, four protoplast hybrids showed positive results regarding bioconversion of vitamin D_3 into calcitriol, as determined by TLC and HPLC analyses (Figures **2-4**). Quantification of calcitriol amount revealed that 3 protoplast hybrids produced higher amounts of calcitriol from vitamin D_3 than that produced by the wild type *Actinomyces hyovaginalis* isolate (Figure **5**).

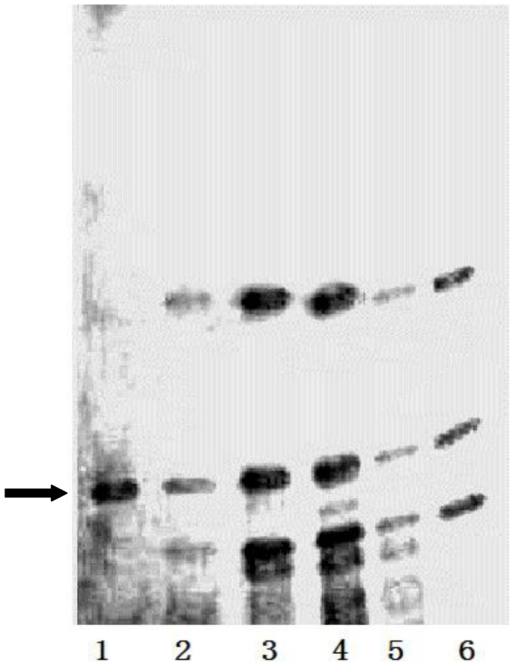

Figure 2: TLC analysis profile, as photographed under UV light (254 nm), of developed concentrated extracts of wild type *Actinomyces hyovaginalis* isolate and some protoplast hybrids against calcitriol standard where lane 1: calcitriol standard (calcitriol band is marked by an arrow), lane 2-6: concentrated extracts of wild type *Actinomyces hyovaginalis* isolate and protoplast hybrids V2B, V3B, V8B and V8A, respectively.

Protoplast fusion has been an important tool for strain improvement through bringing genomes of two different cells in one hybrid strain with the aim of combining desired properties of two different strains. Many researchers have applied protoplast fusion technique to improve the phenotypic characters of different microbial isolates and/or increase the production of valuable secondary metabolites [23-25].

Based upon that, we tried, in this study, to apply protoplast fusion to improve bioconversion of vitamin D_3 into calcitriol using a local potential *Actinomyces hyovaginalis* isolate. Strain improvement by construction of intergeneric hybrids with *Bacillus* species has been established before [26,27]. From this point of view, intergeneric protoplast fusion between *Bacillus* species and the study *Actinomyces hyovaginalis* isolate has been applied in attempt to construct hybrids with improved vitamin D_3 bioconversion capabilities. Results of this study revealed that out of sixteen protoplast hybrids selected and screened for vitamin D_3 bioconversion activity, four hybrids (coded V2B, V3B, V8B and V8A) showed positive results regarding production of calcitriol from vitamin D_3. TLC analysis of the concentrated extract of each of the four hybrids showed a spot with Rf value comparable to that of calcitriol standard (Figure **2**). This was further confirmed by HPLC where the analysis profile of the concentrated extract of each of the previously mentioned hybrids showed a peak at a retention time (3.2 min) matching that of calcitriol standard. Figure **4** illustrates the HPLC analysis profile of the concentrated extract of one of the positive protoplast hybrids; coded V2B as a representative example.

Quantification of calcitriol amounts produced by wild type *Actinomyces hyovaginlais* isolate and each of the four hybrids was performed. As shown in Figure **5**, the protoplast hybrid coded V8B showed less production of calcitriol than that of wild type isolate (which produced about 0.14 mg calcitriol per 1 L bioconversion medium). Whereas, the three hybrids coded V2B, V3B and V8A showed higher production of calcitriol than that of wild type isolate. The increments in calcitriol production by the three previously mentioned hybrids (V2B, V3B and V8A) compared to that of the wild type isolate were 3.5, 1.4 and 2.8 fold, respectively, under the same culture and reaction conditions.

Improvement of Bioconversion of Vitamin D_3 into Calcitriol through Immobilization of the Crude Cell Lysate of *Actinomyces hyovaginalis* Isolate

A previous study was conducted, successfully, to utilize *Actinomyces hyovaginalis* study isolate cell

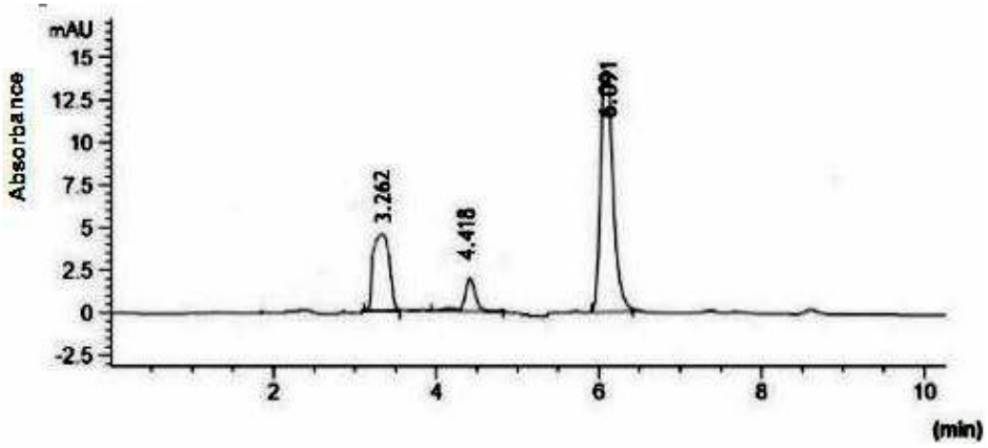

Figure 3: HPLC analysis profile of vitamin D_3 and its derivatives, showing peak at retention time of 3.2 min corresponding to calcitriol, peak at retention time of 4.4 min corresponding to 1α-hyrdoxy vitamin D_3 and peak at retention time of 6.1 min corresponding to vitamin D_3.

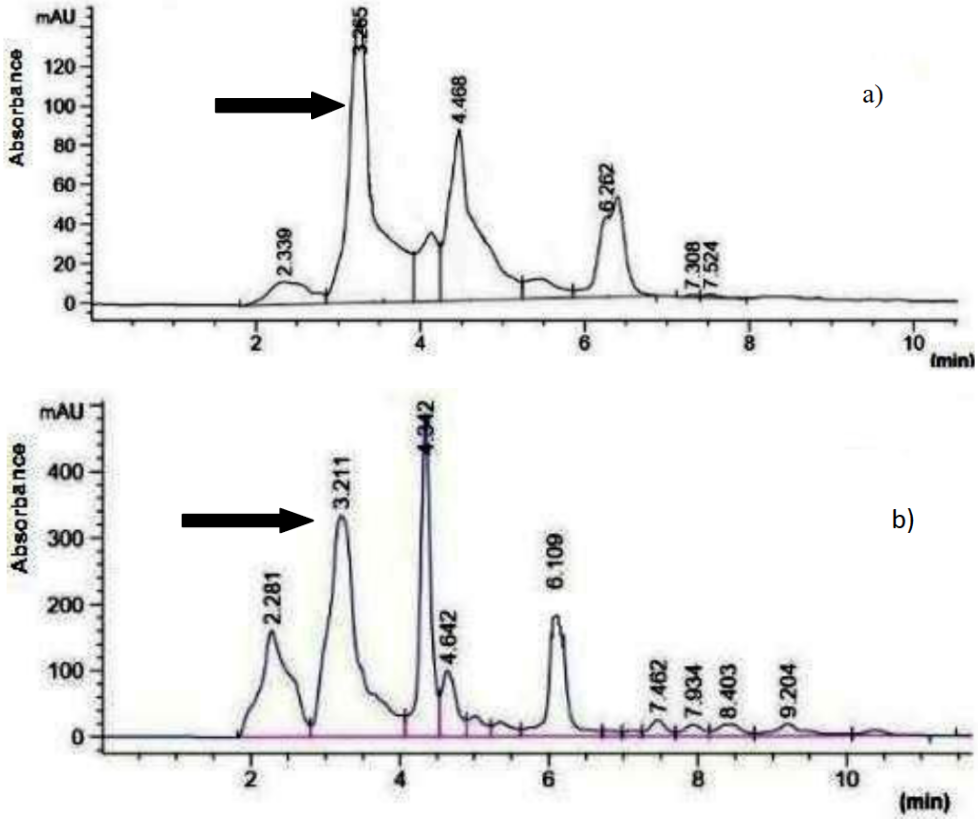

Figure 4: HPLC analysis profile of vitamin D_3 bioconversion concentrated extract produced by the wild type *Actinomyces hyovaginalis* isolate; **a**) and by the protoplast hybrid V2B, as a representative example; **b**) showing peak at retention time of 3.2 min (corresponding to calcitriol) marked by an arrow. Reaction conditions: agitation of 200 rpm, temperature of 28°C, incubation for 96 h.

lysate to bioconvert vitamin D_3 into calcitriol, indicating that the enzyme(s) responsible for the bioconversion activity is/are confined to the cell lysate [12]. Enzyme immobilization technique has become more important in industry and biotechnology as it offers numerous advantages such as recovery of biocatalyst for reuse for the same reaction with longer half-lives and less degradation. Also, immobilization increases enzymes stability under different operational conditions, heat, organic solvent and extreme pH values. Furthermore, it has also helped to prevent the contamination of the substrate with enzyme/protein or other compounds, which decreases purification costs. These benefits of immobilized enzymes have made them highly

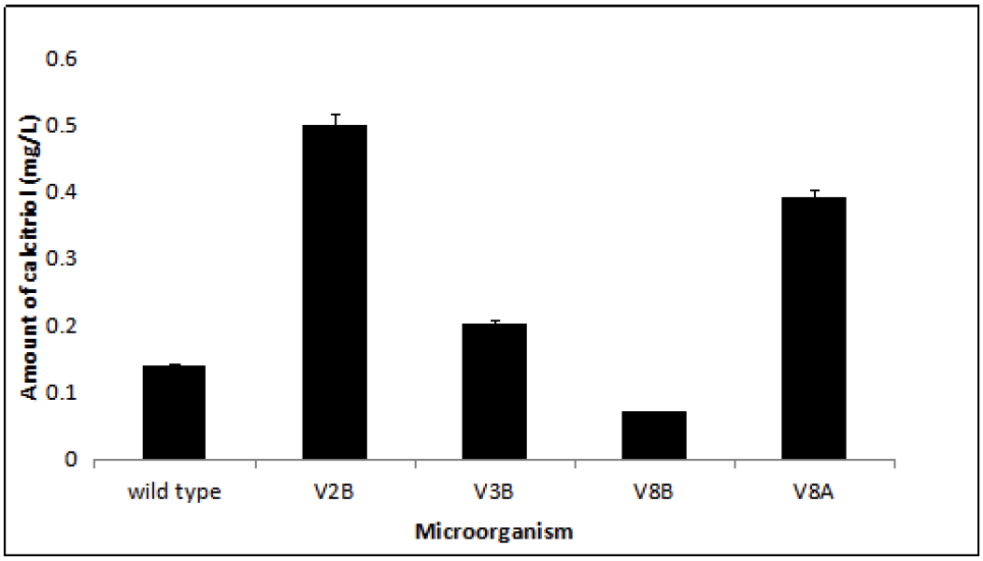

Figure 5: Calcitriol amounts produced from vitamin D_3 by the wild type *Actinomyces hyovaginalis* isolate and some protoplast hybrids. Reaction conditions: agitation of 200 rpm, temperature of 28°C, incubation for 96 h. The wide bars are the means of three readings and the vertical error bars indicate the standard deviation of the data.

applicable to a range of evolving biotechnologies [20,22,28].

So, in this study, application of cell lysate immobilization to improve calcitriol production from vitamin D_3 was carried out via entrapment in calcium alginate beads [20]. Different concentrations of alginate were tested for immobilization of crude cell lysate of wild type *Actinomyces hyovaginalis* isolate. All immobilized forms were tested for vitamin D_3 bioconversion activity, at different reaction times. The results were compared with that obtained using free cell lyaste (Figures **6-8**). HPLC analyses of the concentrated extracts of the cell lysate either in the free or immobilized form, using different sodium alginate concentrations (1%, 2% and 3% w/v), were carried out and the data showed positive results regarding production of calcitriol. All profiles showed a peak at a retention time (3.2 min) matching that of calcitriol standard (Figures **6**, **7**).

As shown in Figure **8**, quantification of the amounts of calcitriol produced by free and immobilized cell lysate of wild type *Actinomyces hyovaginlais* isolate revealed that 1% w/v alginate-immobilized cell lysate, 1 day after its preparation, resulted in nearly equivalent calcitriol production as that produced by free cell lysate. Unfortunately, observable leakage of cell lysate from 1% w/v alginate-immobilized beads started to occur on day 5 of their storage causing calcitriol production to fall by about 25% and further to 40% on day 9 of storage, compared to day 1. Testing the bioconversion activities of immobilized forms, 1 day after their preparation, showed that, among the 3 alginate

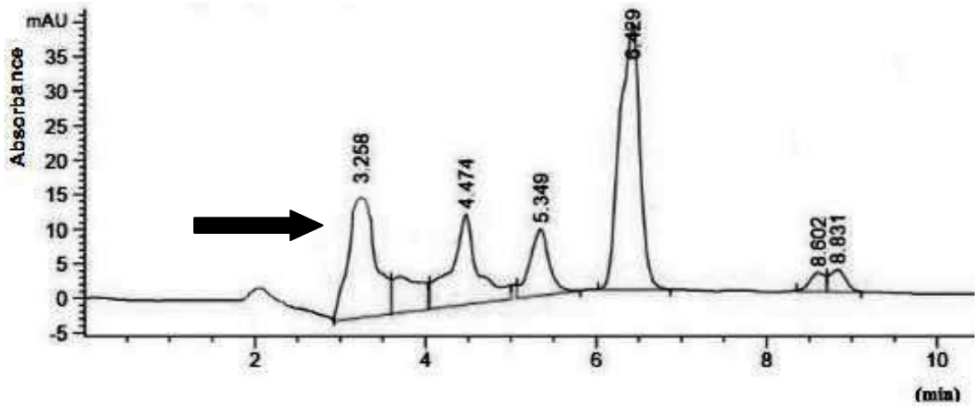

Figure 6: HPLC analysis profile of vitamin D_3 bioconversion concentrated extract of free crude cell lysate of the wild type *Actinomyces hyovaginalis* isolate showing peak at retention time of 3.2 min (corresponding to calcitriol) marked by an arrow. Reaction conditions: temperature of 28°C, incubation for 3 h.

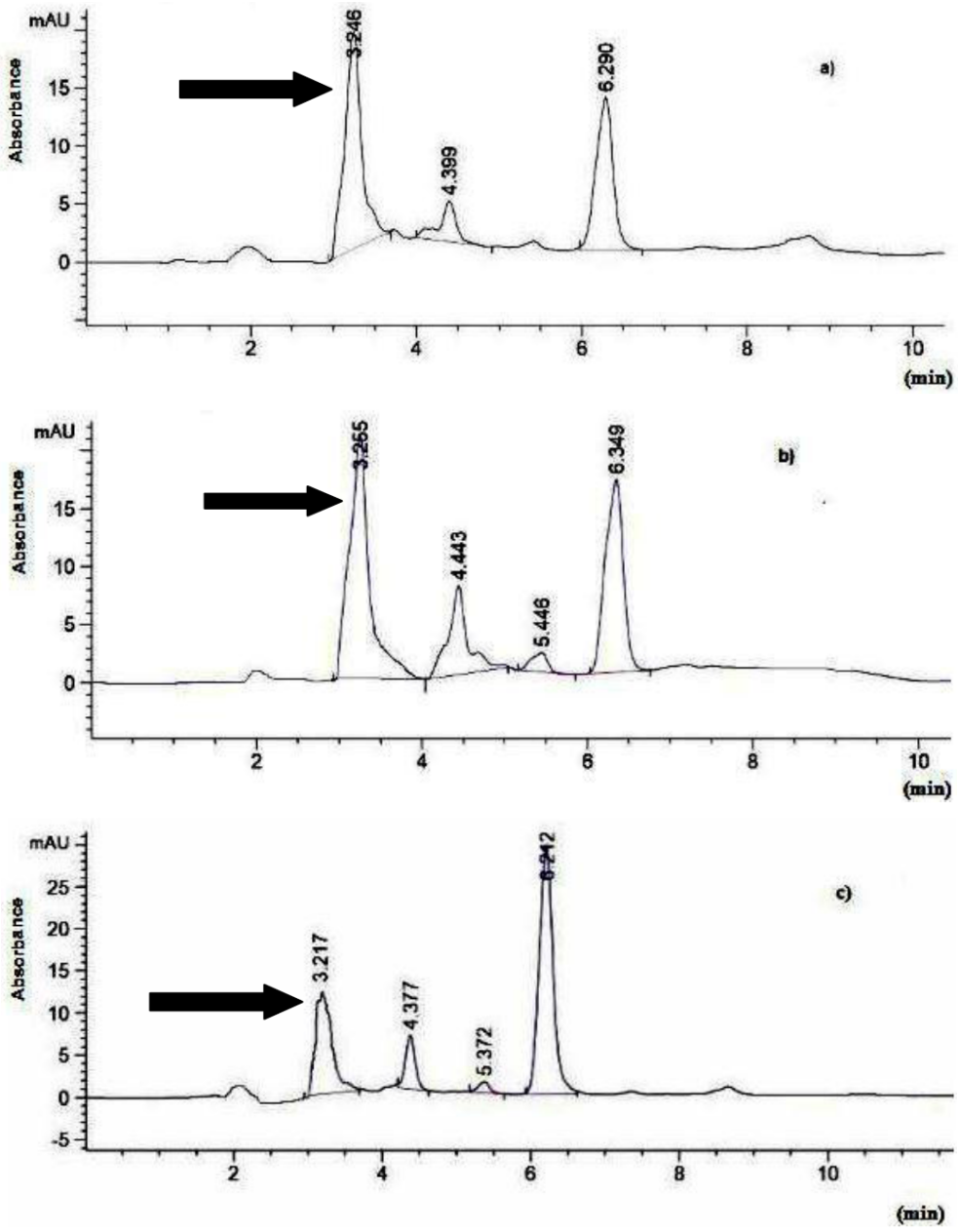

Figure 7: HPLC analysis profiles of vitamin D_3 bioconversion concentrated extracts of crude cell lysate, of the wild type *Actinomyces hyovaginalis* isolate, immobilized by different alginate concentrations (1, 2 and 3% w/v) 1 day after preparation. **a**), **b**) and **c**); immobilized forms at 1, 2 and 3% w/v alginate concentrations, respectively. Peak at retention time of 3.2 min (corresponding to calcitriol) is marked by an arrow. Reaction conditions: temperature of 28°C, incubation for 3 h.

concentrations applied for immobilization of cell lysate, 2% w/v concentration produced immobilized beads with the highest amount of calcitriol production, which was 1.4 fold of that produced by free cell lysate. On the other hand, 3% w/v alginate immobilized cell lysate produced less amount of calcitriol which was 0.75 fold of that produced by free cell lysate, when measurements were carried out 1 day after beads preparation in both cases. This was probably due to hindrance of substrate (vitamin D_3) and/or product (calcitriol) diffusion by the high alginate concentration used [20]. For 2% and 3% w/v alginate-immobilized cell lysate, caclitriol production declined by about 25% and 18% respectively, for beads stored up to 9 days.

CONCLUSION

Calcitriol production from vitamin D_3 by *Actinomyces hyovaginalis* isolate could be improved using protoplast fusion technique with *Bacillus* isolate(s). Calcitriol production, using 50 ml bioconversion medium, was increased from 7 µg (by wild type *Actinomyces hyovaginalis* isolate) to about 20

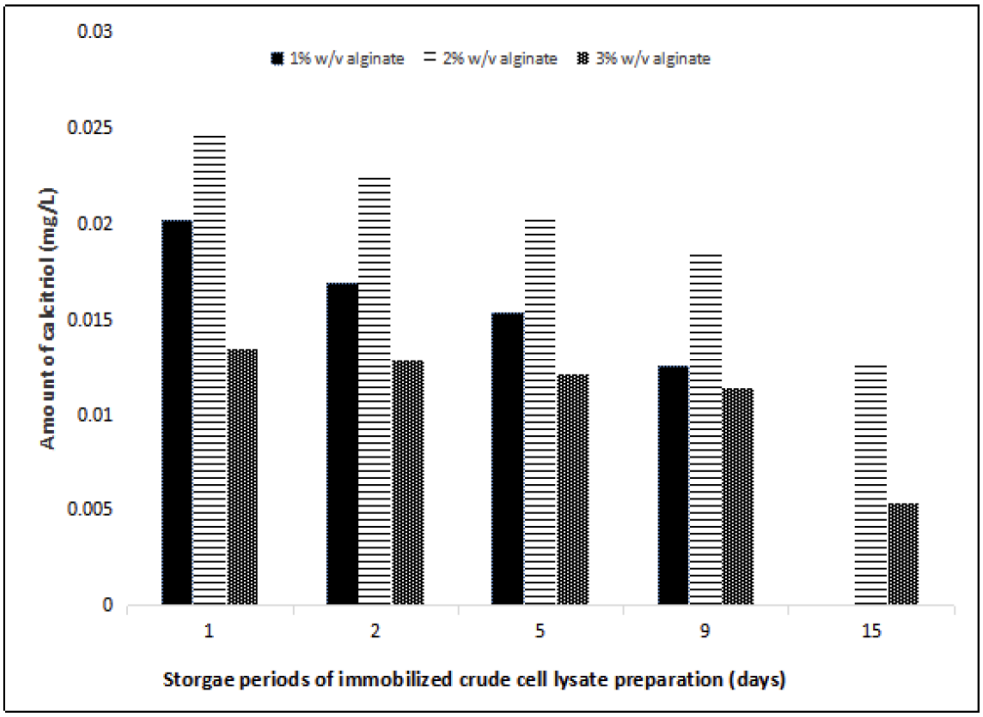

Figure 8: Calcitriol amounts produced from vitamin D_3 by crude cell lysate, of the wild type *Actinomyces hyovaginalis* isolate, immobilized by different alginate concentrations (1, 2 and 3% w/v) after different storage periods.

µg and 25 µg by protoplast hybrids coded V8A and V2B, respectively, under the same culture and reaction conditions. More studies should be done to further improve bioconversion of vitamin D_3 into calcitriol by the mentioned protoplast hybrids using different culture/physiological conditions.

An approach to produce calcitriol from vitamin D_3, using the immobilized form of crude cell lysate of *Actinomyces hyovaginalis* isolate, via entrapment technique, appeared to be successful. Among different alginate concentrations applied, 2% w/v alginate-immobilized cell lysate was proven to be the best for bioconversion of vitamin D_3 into calcitriol. Compared to free cell lysate of the study isolate, immobilization using 2% w/v alginate caused 140 % increase in calcitriol production from vitamin D_3. Moreover, after preparation, stored beads could be used up to 4 times over a period of 9 days without a major decline in the bioconversion activity. Reusability of immobilized cell lysate (containing vitamin D_3 converting enzymes) is an important criterion to be implemented in industry which, in turn, will have a great economic impact.

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