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On the bi-enzymatic behaviour of *Saccharomyces cerevisiae*-mediated stereoselective biotransformation of 2,6,6-trimethylcyclohex-2-ene-1,4-dione

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ABSTRACT

Baker's yeast has been well-known to have the ability to reduce a variety of substrates into many optically active compounds. One of the important chemicals is (4**R**,6**R**)-4-hydroxy-2,2,6-trimethylcyclohexanone or in short, (4**R**,6**R**)-actinol, a product formed from the reduction of 2,6,6-trimethylcyclohex-2-ene-1,4-dione, (ketoisophorone). The work has successfully characterized the route of the reaction during the conversion mediated by *Saccharomyces cerevisiae* and it was also experimentally observed the importance of cofactor (NADH/NADPH) availability and stability during the biotransformation. The presence of cofactor has been proven to assist the product formation by keeping a continuous flow of hydrogen ions to and from the reduction system, while the amount of enzymes present determined the rate of both intermediates and product formed during the course of biotransformation. Results of the growing cells, particularly during the exponential phase of the growth significantly differ to that of the stationary phase system in terms of the formation of the intermediates and the final product. For the growing cell biotransformation, the route only stopped at the intermediate compound (**4**) during the course of the 30 h reaction, whereas for the stationary phase biotransformation, compound (**5**) was readily formed in a range of time between 2 and 12 h of reaction, depending on the amount of cells available and glucose supplied to the system. Within the yeast cell, there are two responsible enzymes that mediate the biotransformation; carbonyl reductase and enoate reductase that work both in parallel and sequential to produce a chiral alcohol of (4**R**,6**R**)-actinol.

1. Introduction

The application of biocatalysts to undergo chemical reactions has been widely practised by major chemical processing companies including food and pharmaceuticals [1]. This is due to the fact that biocatalysts, in the form of either enzyme or whole-cell are able to specifically target a particular bond or functional group of a chemical compound [2,3]. However, in the natural environment of a microorganism, there are intricate connections of enzymes that work both in parallel and sequential in order to keep the biological system alive. It is within these connections of enzymes that one could exploit the ability of a microorganism into becoming an important biocatalyst, which could reduce the cost of a process as well as maintain a green environment [4–6].

The reduction of ketoisophorone or 2,6,6-trimethylcyclohex-2-ene-1,4-dione (**1**) has long become an interest for its valuable products as well as its interesting mechanism through biotransformation. The reduction of (**1**) was first synthesized and reported for more than four decades ago and only recently, the mechanism of the particular reduction is about to be fully understood [7,8]. Fig. 1 shows the possible route of biotransformation of ketoisophorone leading to the final product(s); (4**R**,6**R**)-4-hydroxy-2,2,6-trimethylcyclohexanone, (**5**) ((4**R**,6**R**)-actinol) and its corresponding enantiomer (**6**).

Various species of microorganisms have been screened for better yield of compound (**5**), which is the precursor for a number of optically active carotenoids and plant hormones such as zeaxanthin and xanthoxin to name a few [9]. Zeaxanthin is a type of carotenoid from the xanthrophyll group found both in human and plants. Nowadays, zeaxanthin has been largely used as a natural food colorant as well as an active ingredient in pharmaceutical industry due to its high anti-oxidant properties [10]. It was also

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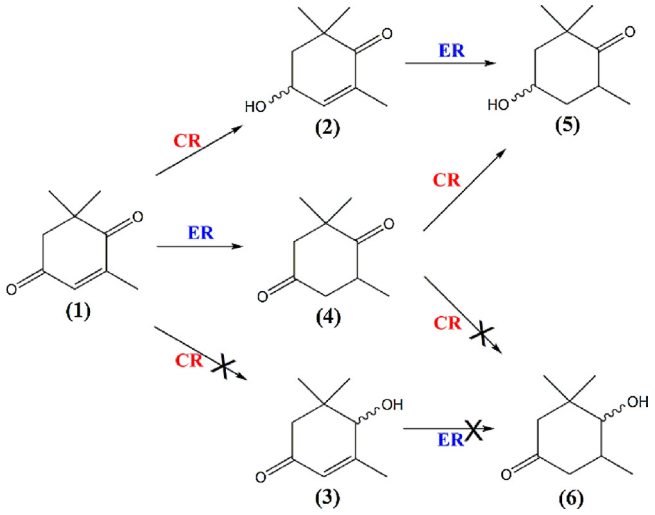

Fig. 1. Possible routes of ketoisophorone (**1**) reduction based on two enzymes naturally produced by *Saccharomyces cerevisiae*. **ER** represents enoate reductase and **CR** refers to carbonyl reductase.

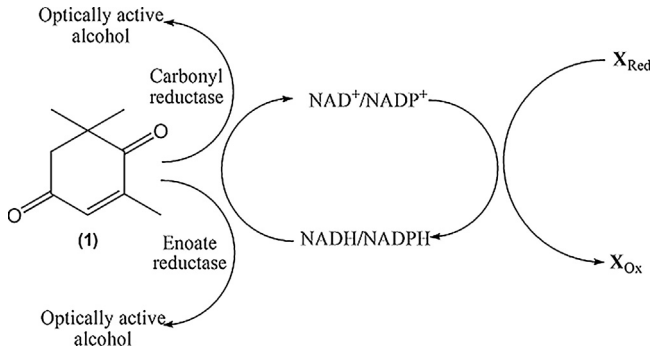

Fig. 2. The use of cofactor as hydrogen ion transfers to both reductases-mediated reductions. Compounds X_{Red} and X_{Ox} represent the reduced and oxidized X respectively, where X is a species that undergoes oxidation reaction.

reported that zaexanthin and other members of xanthrophylls family could prevent the appearance of many diseases, for instance; arteriosclerosis, cataracts, multiple sclerosis, macular degeneration and even cancer [11]. Xanthoxin on the other hand is a type of plant hormone that inhibits growth, found in abundant in plants such as tomato and spinach [12]. In the previous report by Kobayashi and co-workers, xanthoxin was also found in the extract of algae and sea grass namely; *Codium latum* and *Sargassum thunbergii*, which were collected from the coastal areas of Japan [13]. In some higher plants biosynthetic pathway, xanthoxin acts as a precursor in the formation of abscisic acid, in other words, a plant growth regulator [14].

The application of whole-cell Baker's yeast type-II, (*Saccharomyces cerevisiae*) has shown a substantial productivity, particularly on the product's regio- and stereoselectivity as compared to other organisms such as *Thermomonospora curvata* and *Bacillus stearothermophilus* [8,15–17]. Within these microorganisms, there are two important enzymes responsible for the formation of both products (**5**) and (**6**). Carbonyl reductase or commonly known as alcohol dehydrogenase (ADH), that mediates the reduction of aldehyde and ketone to alcohol, while enoate reductase, normally termed as the Old Yellow Enzyme, (OYE) [18–20], which catalyzes the reduction of the C=C bond are the two enzymes that stereoselectively reduced ketoisophorone (**1**). On separate works on enzyme characterization, both carbonyl reductase and enoate reductase have been successfully purified and characterized from different range of microorganisms. However, the main focus was on the yeast genus including Baker's yeast as well as other species such as *Saccharomyces carlsbergensis*, *Kazachstania* sp., *Kluyveromyces marxianus*, *Candida* sp. and *Zygosaccharomyces* sp. to name a few [21–24].

The possible routes of ketoisophorone (**1**) biotransformation using different types of microorganisms are presented in Fig. 1. However, the particular scheme only refers to Baker's yeast-mediated reduction, since the presence of compounds (**3**) and (**6**) were not observed during the course of biotransformation. In the earlier work of Leuenberger et al., *S. cerevisiae* was first used as a biocatalyst in a number of experimental set-ups. The highest fermentation volume of 200 L was used with the formation of intermediate (**4**) at 60 g/L [7]. The work was later continued by many Japanese researchers, applying different types of microorganisms ranging from bacteria, fungi, different species of yeasts and plant cells. Yamazaki et al. first reported the fungal-mediated reduction of ketoisophorone (**1**) in 1988 [8]. Their observation showed that the mycelium of *Aspergillus niger* released a plethora of enzymes during the growth period, which resulted in the formation of compounds (**1**)–(**6**) shown in Fig. 1. Prior to the work of Yamazaki, a thermophilic bacterium from the species of *Thermomonospora curvata* was used as the biocatalyst to perform the biotransformation. The reaction however stopped only until compound (**4**) with the concentration of 0.0065 mol/L (1.0 g/L) at a maximum cell growth of 1.5 g/L [15]. The other compounds existed only at a trace level within the reaction medium. The use of thermophile was further extended with the combination of two different microorganisms. *Bacillus stearothermophilus* was chosen to link with the *T. curvata* system into a sequential biotransformation procedure. The reaction then continued where compound (**4**) was further reduced into compound (**5**) at a concentration of 1.5 g/L [16,25,26].

From the selected biocatalysts listed above, it could be summarized that the rate of product formation is totally governed by the two enzymes responsible in the reduction of ketoisophorone (**1**). However, for a reduction reaction in particular, the case is rather challenging when the product formation is also dependent on the amount of cofactor available within the cells [27–29]. The cofactor that coupled the ketoisophorone (**1**) reduction is shown in Fig. 2. Compounds X_{Red} and X_{Ox} represented in the diagram are the reduced and oxidized formed of X respectively, that occur during the oxidation process. Since oxidation could take place by many substrates within the cell, therefore, X represents an arbitrary compound that undergoes such oxidation reactions.

The aim of this work is mainly to investigate the effect of cofactor stability and enzymes availability towards biotransformation leading to the formation of (**R**)-4-hydroxy-2,2,6-trimethylcyclohexanone, (**5**). The work also monitors the amount of enzymes present within the cells and how they could eventually affect the route of the biotransformation. The formation of (**R**)-4-hydroxy-3,3,5-trimethylcyclohexanone (**6**) was not followed during this investigation due to the fact that *S. cerevisiae*-mediated biotransformation of ketoisophorone (**1**) gave no trace of the compound with no apparent peak on the chromatogram as separately reported by Wada and Buque-Taboada et al. [30–32]. The outcome of this work would provide a guideline to biochemists as well as molecular biologists into carrying out molecular work on genes expression for further product optimization.

2. Materials and method

2.1. Microorganism

Baker's yeast type-II (*Saccharomyces cerevisiae*) in dried form was purchased from Sigma-Aldrich, USA.

2.2. Chemicals

Standard ketoisophorone (**1**) (98%), potassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), D-(+)-glucose (>99.5%), Tris-HCl, bovine serum albumin (BSA), nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADPH), sodium hydroxide (NaOH) and ethyl acetate were purchased from Sigma-Aldrich, USA. Luria-Bertani (LB) growth medium which includes; yeast extract, peptone and sodium chloride (NaCl) were also purchased from Sigma-Aldrich, USA. Pure enantiomer of (**S**)-4-hydroxy-2,6,6-trimethylcyclohex-2-enone, (**2**) (>98%) and 2,2,6-trimethylcyclohexane-1,4-dione, (**4**) (>95%) were purchased from AAP Pharma Technologies (India) and Angene International Limited (Hong Kong) respectively.

2.3. Growth of Baker's yeast Type-II, Saccharomyces cerevisiae

The growth of *S. cerevisiae* was carried out in shake-flasks. LB medium as the growth nutrient was prepared with 10 g sodium chloride, 10 g peptone and 5 g yeast extract dissolved in 1 L deionized water. 5 g/L glucose was added as an additional carbon source to the medium in a separate system for comparison purposes. 250 mL of the prepared medium was measured and poured into a baffled shake-flask. The flask was autoclaved at 121 °C for 20 min. Cooled medium was then ready to be inoculated with dried yeast cells. 0.25 g of dried Baker's yeast was measured and added to the 250 mL LB medium resulting into 1 g/L initial cell concentration. The procedure was carried out aseptically in a laminar flow cabinet to ensure that the growth is free from contamination. Fermentation was carried out in an incubator-shaker (IKA, KS4000i Control, Korea) at 37 °C with the shaking speed of 150 rpm.

Samples from the fermentations were collected every hour in order to observe the cell growth and later a profile was plotted for both systems without and with the addition of glucose. The fermentation was left to continue for about 6 days (154 h).

In order to determine the cell mass throughout the course of fermentation, 1 mL of sample from the culture was withdrawn and centrifuged at 4000 rpm for 10 min. Prior to centrifugation, 1 mL-size of empty plastic Eppendorf tubes were dried in an oven and weighed (dried-tube weight). Supernatant from the centrifuged sample was removed and the cell pellet was dried at 60 °C for 1 h. The dried cell within the tube was again weighed and the difference between the tube with cell pellet and that with the empty ones were calculated, which gives a measurement in terms of g_{dcw}/L.

2.4. Shake-flask biotransformation

2.4.1. Growing cell biotransformation

The method was carried out in order to observe the cell capability in reducing ketoisophorone (**1**) into its respective products during the growth period. This will provide a wider perspective in terms of enzymes responsible for the conversion, cofactor availability and inhibition effect by the substrate (**1**) should there be any. By referring to the standard growth profiles of *S. cerevisiae* obtained previously, fermentations were again repeated with a standard LB medium and LB medium with additional 5 g/L glucose at 37 °C and 150 rpm shaking speed. Growth is basically defined as cell division where microorganisms actively divide themselves and later produce an exponential curve within the profile. The point where this exponential curve begins marked the point where 0.2 g/L (≈1 mM) of substrate (**1**) was introduced into the medium. As the fermentation continued, samples were withdrawn from the shake-flasks every hour to check for substrate consumption, products formation and cofactor availability and stability.

2.4.2. Stationary phase biotransformation at different glucose concentrations

For a stationary phase biotransformation, phosphate buffer was used as an aqueous medium for cell suspension. Phosphate buffer was prepared at pH 7 at a concentration of 0.1 M. 10.7 g of K_2HPO_4 and 5.24 g of KH_2PO_4 were added and dissolved with 1 L dionized water. 1 M sodium hydroxide was used to adjust the solution to the required pH. The buffer solution was prepared with the addition of 3 different glucose concentrations (5, 10 and 15 g/L). This would provide an additional source of carbon especially for cofactor recycling during the bioconversion process. 5 g_{dcw}/L Baker's yeast was prepared in 250 mL buffer solution prior to biotransformation (with conditions similar to the growing cell biotransformation). After 30 min of cells inoculation, 0.2 g/L ketoisophorone (**1**) was added into the buffer solution and samples were withdrawn every hour in order to check for substrate degradation, products formation and cofactor availability and stability.

2.4.3. Stationary phase biotransformation at different cell concentrations

The experiment to determine the effect of different cell concentrations was also carried out in 250 mL phosphate buffer. 3 different cell concentrations (7, 10 and 15 g_{dcw}/L) were chosen with the addition of 5 g/L glucose. 0.2 g/L ketoisophorone (**1**) was introduced into the cell suspension 30 min after the inoculation with biotransformation conditions of 37 °C and 150 rpm shaking speed. Samples were withdrawn every hour in order to determine the remaining substrate, the amount of products and the cofactor level during the biotransformation.

2.5. Analytical methods

2.5.1. Substrate and products quantifications

Samples were withdrawn at least 2 times at every hour. For a 0.5 mL sample withdrawn from the biotransformation medium, 0.5 mL ethyl acetate was added and the mixture was vigorously vortexed for at least 5 min. Sample was then centrifuged at 4000 rpm for 10 min (Eppendorf 5702R, Germany) to separate the aqueous and the organic phase. Once the separation was complete, the top layer (organic phase) was removed and kept in a sample vial for gas chromatographic analysis.

2.5.2. Cofactor (NADH/NADPH) analysis

Another 1 mL sample was taken from the reaction medium and subjected to centrifugation for 10 min at 4000 rpm in a 1 mL Eppendorf tube. The supernatant resulted after centrifugation was removed and the cell pellet remained at the base of the tube was added with 1 mL solution of BSA and Tris-HCl buffer. The mixture was vortexed for 5 min until the pellet was completely suspended in the buffer. The suspended cell was then undergone sonication process in order to break open the cell membrane. Sonication using Misonix Sonicator 3000 (Cole-Palmer, USA) was carried out in ice (<5 °C) for 10 cycles (5 s on and 5 s off) at the sonication amplitude of 8 μm and later centrifuged at 4000 rpm for 10 min to remove the cell debris. The supernatant was carefully transferred into a quartz cuvette and quickly checked for absorbance using a UV–vis spectrophotometer (Cary 60, Agilent Technologies, USA) with the absorbance wavelength of NADH/NADPH set at 340 nm. The method of cofactor quantification separately reported by Walker and Rover Jr. with their co-workers was used with slight modifications to suit with the yeast cells used in the experimentation [33,34].

2.5.3. Gas chromatographic analysis

Organic samples extracted from the biotransformation method previously described in subsection 2.5.1 were kept in glass vials

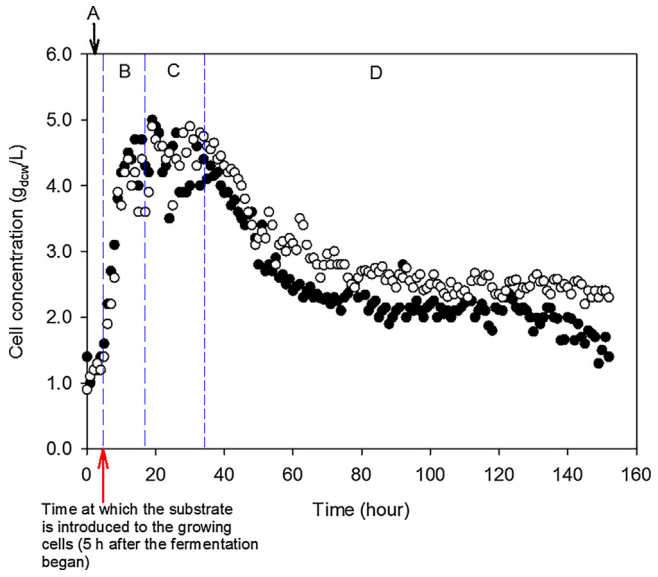

Fig. 3. Growth profile of *Saccharomyces cerevisiae* with different phases denoted as (A) lag phase, (B) exponential phase, (C) stationary phase and (D) death phase. (○, growth with 5 g/L glucose, ●, growth without 5 g/L glucose).

for chromatographic analysis. Standards were performed prior to samples analysis using 7820A Gas Chromatography, (Agilent Technologies, USA) equipped with flame ionization detector and a capillary column (0.25 μm, 0.25 mm 30 m, MEGA-DEX DMT Beta, Italy). The oven was initially set at 120 °C for 2 min and ramped to 210 °C at a rate of 2 °C/min. The injector temperature was maintained at 220 °C with helium as a carrier gas with a flow rate of 1.0 mL/min. Pure ketoisophorone (**1**), (***S***)-4-hydroxy-2,6,6-trimethylcyclohex-2-enone, (**2**) and 2,2,6-trimethylcyclohexane-1,4-dione, (**4**) were prepared according to standard concentrations in ethyl acetate prior to injection. 1 μL of each component was analyzed in triplicates with individual calibration curves quoted at 95% confidence interval based on five independently prepared standard solutions (0.1–6.0 g/L, $R^2 > 98\%$).

3. Results and discussion

3.1. Biotransformation of ketoisophorone (*1*) during the growth phase of Saccharomyces cerevisiae

Prior to carrying out biotransformation during the growth phase of the cells, standard fermentation of *S. cerevisiae* was conducted in a shake-flask. A standard LB medium was prepared as previously described and 1 g_{dcw}/L cell was inoculated in a 250 mL medium. The culture was left in an incubator-shaker set at 37 °C and 150 rpm for 30 min before the first sample was withdrawn for growth quantification. The period of 30 min was given for the cells to be completely mixed and suspended in the medium as well as adapted to the controlled environment. A similar set of culture was also prepared using LB medium with the addition of 5 g/L glucose. 3 sets of fermentation cultures were run in order to obtain consistent phases as depicted in Fig. 3. This phase diagram, particularly the exponential phase of the yeast growth (region B), shows that the cells were actively dividing until a period where the cell division stopped and reached a stationary phase. From the growth phase diagrams (medium with and without 5 g/L glucose), the time at which the phase shifts from one phase to the other is approximately similar for each set with different of only ±1 h. Fig. 3 provides a clear indication of the exact time where the substrate should be introduced into the growing cell. Since the introduction of glucose in the growth medium is only to provide an additional carbon source

Table 1
Specific growth rates of the growing *S. cerevisiae* without and with the addition of 5 g/L glucose.

Growth medium content	Specific growth rate μ (h^{-1})
Without glucose	0.078
With glucose	0.129

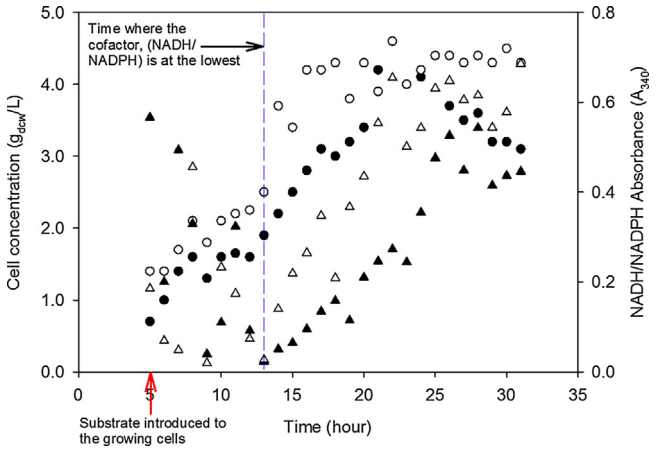

Fig. 4. Cofactor stability in a biotransformation during the exponential phase of *S. cerevisiae* (○, cell growth with 5 g/L glucose, ●, cell growth without 5 g/L glucose, Δ, cofactor absorbance from growth with 5 g/L glucose, ▲, cofactor absorbance from growth without 5 g/L glucose).

to the cell, therefore, the phases did not differ much during the period of 6 days of fermentation. For both fermentations, the specific growth rates, μ for medium without and with 5 g/L glucose are 0.078 and 0.129 h^{-1} respectively as listed in Table 1. These values apparently show that the additional glucose in the growth medium does not give much effect to the growth curve until at the very end of the fermentation period.

From the growth phase diagram obtained, the lag phase (region A) was observed where the cell took approximately 5 h to stabilize and adjust to the surrounding new environment. Due to this reason, biotransformation was only started by introducing the substrate (**1**) at the fifth hour after the fermentation began. Samples were then taken every hour to check for substrate consumption, product(s) formation, cell growth as well as cofactor availability and stability. Fig. 4 apparently represents the growth of cells and the availability of NADH/NADPH within the cells (in the form of absorbance, A_{340}) at any particular times during biotransformation. The cell seems to correspond considerably well to the substrate as the biotransformation progresses. It was earlier reported by Buque-Taboada et al. that the substrate (**1**) would only affect the cells for concentration above 80 mM (12.17 g/L) [32]. Therefore, the substrate concentration used in the present study would not show any inhibition and toxicity effects as that observed in the previous investigation.

As the cells increase in density, the cofactor detection through the UV–vis also follows the same trend. However, the only difference observed is on the effect of glucose added to the medium, where it stabilizes the cofactor (NADH/NADPH) produced by the cells. A sudden decrease of cofactor was observed after about thirteenth hour after the reaction started. This could be due to the fact that the cells were still adjusting themselves after the addition of ketoisophorone (**1**) into the medium and a sudden plunge of cofactor absorbance provides a clear picture of the sudden use of the available cofactor for substrate conversion before the cells need to produce more for other cellular application. This is supported by the appearance of 2,2,6-trimethylcyclohexane-1,4-dione, (**4**) at fifteenth and eleventh hours of biotransformation without and with glucose respectively (Fig. 5(A and B)).

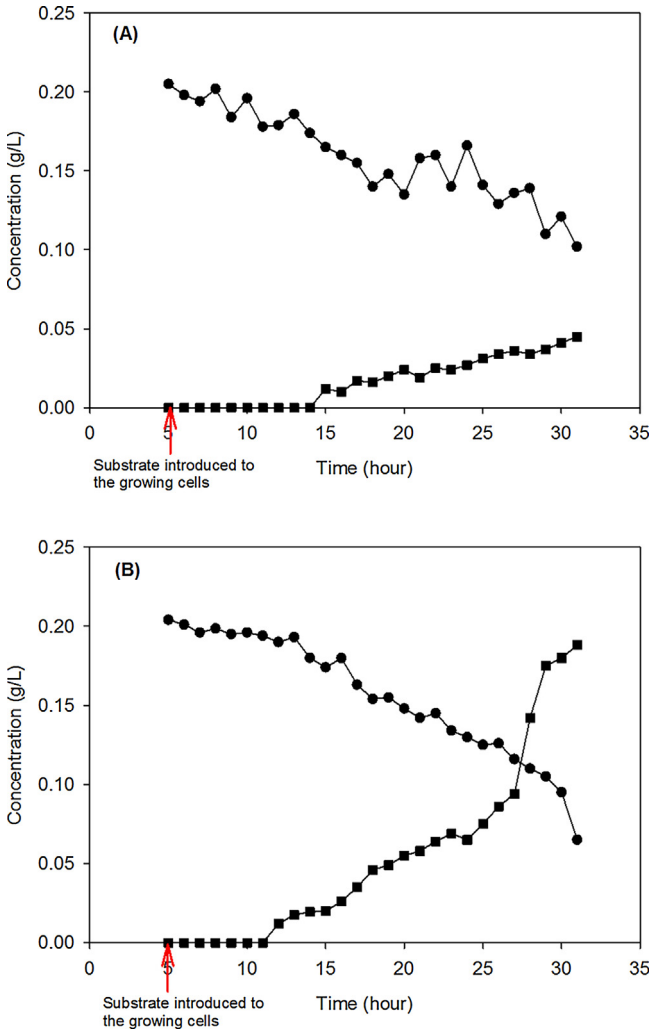

Fig. 5. Biotransformation of 2,6,6-trimethylcyclohex-2-ene-1,4-dione (**1**) (●) with the formation of intermediate 2,2,6-trimethylcyclohexane-1,4-dione (**4**) (■) in growth media (A) without 5 g/L glucose and (B) with 5 g/L glucose.

Throughout the course of biotransformation, either with the LB medium with or without glucose, the yeast cells seem to produce only the intermediate (**4**) after about 30 h of reaction. It was also observed that with the maximum cell density of approximately 5 g_{dcw}/L, the organism could only reduce the substrate (**1**) into 2,2,6-trimethylcyclohexane-1,4-dione, (**4**), with enoate reductase dominating the group of proteins formed during the growth period. This observation is similar to that observed by Hori et al. on the biotransformation of ketoisophorone (**1**) with *T. curvata* as a biocatalyst, where only compound (**4**) appeared for a 30 h period of fermentation [15].

3.2. Biotransformation of ketoisophorone (*1*) with variable glucose concentrations for the maintenance of cofactor regeneration

In all biotransformation involving redox reaction, one of the important aspects is the transfer of hydrogen ion (H^+) to and from the main reaction. The hydrogen transfer process from an oxidized system to that of the reduced system is carried out by a cofactor and the generation of cofactor is dependent on the most basic cellular metabolism of glucose breakdown or glycolysis [35]. Therefore the aim of varying glucose concentration is mainly to look into the ability of the cells to generate more cofactor (NADH/NADPH) in order to

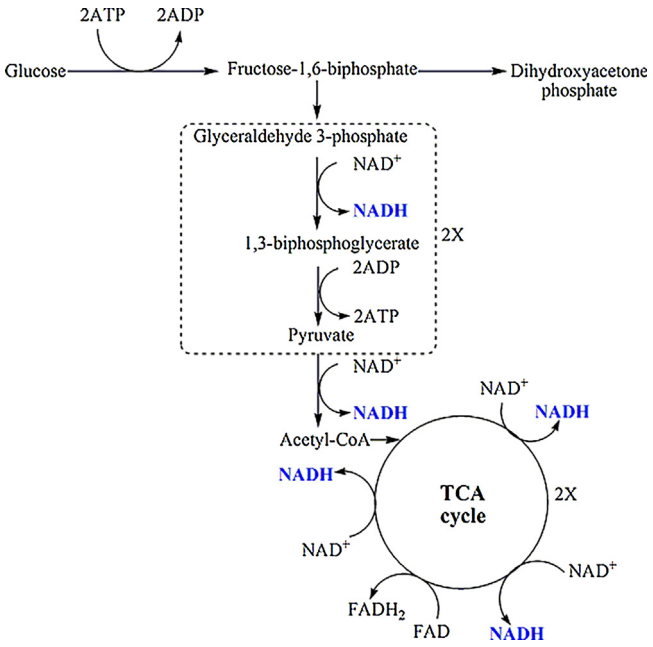

Fig. 6. A simplified version of the glycolysis steps combined with the TCA cycle showing the theoretical formation of cofactor (NADH) in yeasts/bacteria.

link between the redox reactions and later conclude that the compound is also important in determining the rate of the reaction [36]. Fig. 6 provides a simplified version of the glycolysis steps and that connected to the TCA (tricarboxylic acid) or the Kreb's cycle, which are the main source of cofactor regeneration. Theoretically, with the total of 10 cofactor molecules formed from each glucose structure, the additional glucose could therefore maintain the availability and stability of cofactor needed for the redox biotransformation.

This experiment utilized phosphate buffer as the aqueous medium for cell suspension, where the main purpose is to remove all the nutrients required for cell division and growth could then be suppressed. Since the maximum cell growth was approximately 5 g_{dcw}/L in the previous experiments, thus, the amount of yeast inoculated in the medium was also of the same amount.

Fig. 7 provides a clear picture of the progress of the biotransformation with three different glucose concentrations. The consumption of ketoisophorone (**1**) was apparently observed after the fourth hour of reaction, however, the intermediate product, (**4**) was only detected 4 h later (Fig. 7(A)). The delay of the product formed could be due to the insufficient enoate reductase to carry out the conversion of substrate (**1**). The problem of inadequate cofactor could be ruled out as the compound was considerably stable with only minor fluctuations at the absorbance value of approximately ±0.25. The reaction was then prolonged for another 9 h where an apparent additional peak was observed on the chromatogram. The position and retention time of the peak were later confirmed with that obtained by Yamazaki et al., which to be (**R**)-4-hydroxy-2,2,6-trimethylcyclohexanone, (**5**) [8]. Since there was no standard compound purchased for (**R**)-4-hydroxy-2,2,6-trimethylcyclohexanone, the mol percent (mol%) of the compound detected by the gas chromatograph was used instead. This result also gives a preliminary information about the existence of carbonyl reductase at the very end of the biotransformation. Upon comparing the three amounts of product (**5**) in (mol%) (data shown separately in Fig. 7(A–C)), they were approximately similar (with concentration of 12 ± 2 mol%). The only difference between the three glucose concentrations is the rate at which the products were formed. On the other hand, compound (**R**)-4-hydroxy-3,3,5-trimethylcyclohexanone, (**6**) was not observed throughout the

Table 2
The initial rates of product(s) formation during the biotransformation of 2,6,6-trimethylcyclohex-2-ene-1,4-dione (**1**) in phosphate buffer at different glucose concentrations.

Glucose concentration (g/L)	Initial rate of products formation	
	2,2,6-trimethylcyclohexane-1,4-dione, (**4**) (g/L-h)	(**R**)-4-hydroxy-2,2,6-trimethylcyclohexanone, (**5**) (mol%/h)
5	5.2×10^{-4}	0.37
10	4.0×10^{-4}	0.91
15	9.0×10^{-4}	0.84

Table 3
The initial rate of intermediates and product formations during the biotransformation of 2,6,6-trimethylcyclohex-2-ene-1,4-dione (**1**) in phosphate buffer at different cell concentrations.

Cell concentration (g/L)	Initial rate of products formation		
	(***S***)-4-hydroxy-2,6,6-trimethylcyclohex-2-enone, (**2**) (g/L-h)	2,2,6-trimethylcyclohexane-1,4-dione, (**4**) (g/L-h)	(**R**)-4-hydroxy-2,2,6-trimethylcyclohexanone, (**5**) (mol%/h)
5[a]	0	5.2×10^{-4}	0.37
7	1.5×10^{-2}	8.4×10^{-3}	0.87
10	2.4×10^{-3}	1.4×10^{-2}	1.04
15	0	8.9×10^{-3}	1.60

[a] Results quoted from the biotransformation of substrate using 5 g_{dcw}/L cell concentration with 5 g/L glucose concentration in buffer solution (Table 2).

course of reaction as there was no apparent peak detected on the chromatogram as earlier reported by Leuenberger and Buque-Taboada with their co-workers, utilizing the same microorganism [7,31,32].

The rates of product formation are listed in Table 2. Even though the rates were calculated in different units, the speed could however explain about the enzymes that mediate the biotransformation, in other words, the activity of the particular enzyme. The more enzymes available in the cells, the faster a reaction proceeds. This could be observed from the formation of product (**4**) in Table 2 at 5.2×10^{-4} g/L-h for 5 g/L glucose with a slight drop when glucose was doubled in another set of reaction. Since the amount of glucose was twice that of the cells, the extra carbon source could probably be used by the cells to generate more enzymes, and in this case, the carbonyl reductase used to reduce one of the C=O bonds from the structural formula. This is clearly depicted in the higher rate of formation of product (**5**) in 10 g/L glucose as compared to that of 5 g/L. A similar observation could also be seen for higher glucose concentration of 15 g/L. Compound (**4**) was formed at a rate more than twice that of the 10 g/L glucose concentration, which result could be deduced that enoate reductase was generated in greater amount from the excess carbon source provided [37]. An excess amount of glucose was also used to produce more carbonyl reductase, which resulted in a more or less the same concentration of product (**5**) in all three glucose concentrations.

3.3. *Effect of different cell concentrations during the biotransformation of ketoisophorone (**1**)*

A part from monitoring the effect of glucose concentrations towards product formation, the amount of cells at a particular instance would also give a different progress of biotransformation. This effect could be slightly seen in the earlier reaction utilizing the growing-cell system. The result shown in Fig. 5(B) of the growing cell biotransformation clearly shows such effects where the gradual increase of cells would also stabilize the cofactor level for the H^+ transfer processes (Fig. 4).

Due to these observations, a separate set of experiments was conducted to closely monitor the progress of biotransformation at different amount of cell concentrations. The first cell concentration of 7 g_{dcw}/L was used to carry out the reduction of ketoisophorone (**1**). A progress similar to that of the 5 g_{dcw}/L cells was observed with the appearance of a trace of (***S***)-4-hydroxy-2,6,6-trimethylcyclohex-2-enone, (**2**) in the 7 g_{dcw}/L cells, which was not observed in the low concentration of cells. By referring to Fig. 8(A), the intermediate detected at approximately eleventh hour of the reaction shows that enough carbonyl reductase exists within the cells to perform the reduction of the C=O functional group [38]. The formation of (**S**)-4-hydroxy-2,6,6-trimethylcyclohex-2-enone, (**2**) from the biotransformations using 7 and 10 gdcw/L cells listed in Table 3 with the rates of 1.5×10^{-2} and 2.4×10^{-3} g/L-h respectively, proved that higher amount of cells could provide enough carbonyl reductase to mediate the reduction of the ketone functional group. Moreover, with the amount of cells exceed that of the glucose concentration, the biotransformation seems to maintain its production of compound (**5**) (Fig. 8(A and B)). Despite the deteriorating cofactor stability after the eighth hour of reaction, product (**5**) was still increasing as a result of the gradual decrease of both intermediates (**2**) and (**4**). This is due to the fact that there were still cofactor molecules available to transfer hydrogen ions to the reduction system. Based on this observation, it could be hypothesized that biotransformation could still proceed with the minimum amount of cofactor for the cells to maintain their functions. However, with such bare minimum cofactor available, the rate of product formed would not be as high as that with the additional glucose.

The effect of even higher cell concentration, in the case of 15 g_{dcw}/L gave a rather conclusive observation on the behaviour of this complex bi-enzymatic biotransformation. Fig. 8(C) provides an interesting observation of the dynamics of the 2-enzyme system. It is commonly known that for a system with higher cell concentration, the result of biotransformation would be improved in terms of product formation and the rate that the product is formed, which of course conformed by the current observation on the formation of (**R**)-4-hydroxy-2,2,6-trimethylcyclohexanone, (**5**). However, by looking at the progress of both intermediates (**2**) and (**4**), it is apparent that intermediate (**2**) was only detected in traces amount (similar results for 10 g_{dcw}/L cell concentration) as compared to intermediate (**4**), where the accumulation was observed until the end of the experiment. It is obvious from the plots that there are two possibilities which could be deduced based on these intermediate compounds. Firstly, the traces amount of intermediate (**2**) detected

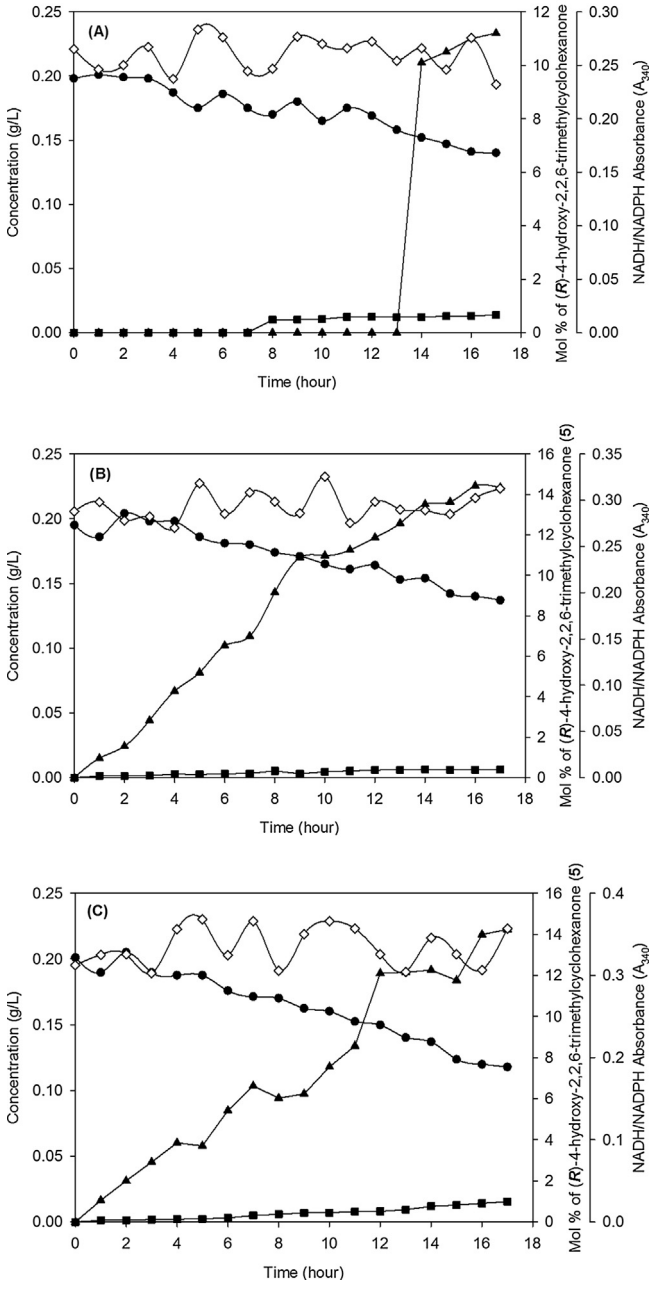

Fig. 7. Profiles of *S. cerevisiae*-mediated biotransformation of 2,6,6-trimethylcyclohex-2-ene-1,4-dione (**1**), (●) in phosphate buffer, forming an intermediate, 2,2,6-trimethylcyclohexane-1,4-dione (**4**), (■) and the final product (**R**)-4-hydroxy-2,2,6-trimethylcyclohexanone (**5**), (▲). The stability of cofactor (NADH/NADPH) during the course of reaction is given by (◊). (A) 5 g/L glucose, (B) 10 g/L glucose and (C) 15 g/L glucose.

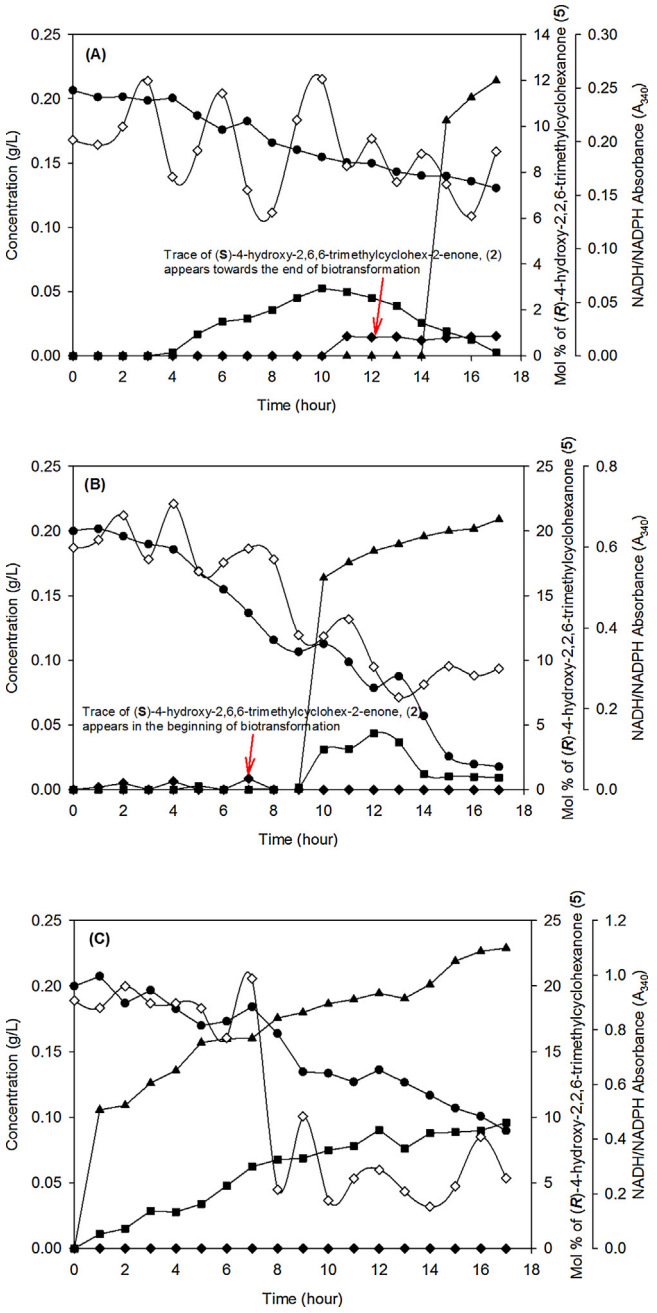

Fig. 8. Profiles of *S. cerevisiae*-mediated biotransformation of 2,6,6-trimethylcyclohex-2-ene-1,4-dione (**1**), (●) in phosphate buffer, forming intermediates, 2,2,6-trimethylcyclohexane-1,4-dione (**4**), (■) and (**S**)-4-hydroxy-2,6,6-trimethylcyclohex-2-enone, (**2**), (♦) and the final product (**R**)-4-hydroxy-2,2,6-trimethylcyclohexanone (**5**), (▲). The stability of cofactor (NADH/NADPH) during the course of reaction is given by (◊). (A) 7 g_{dcw}/L cells, (B) 10 g_{dcw}/L cells and (C) 15 g_{dcw}/L cells.

through the gas chromatographic analysis could be due to either there was no or low activity of carbonyl reductase or the conversion of intermediate (**2**) into the final product (**5**) was relatively fast that it would not be able to accumulate within the reaction medium, in other words, the conversion was fast enough that once the intermediate was formed, the enoate reductase available would immediately convert it into product (**5**).

Secondly, for the accumulated intermediate (**4**) in the reaction medium, the readily available enoate reductase from the cell would convert substrate (**1**) at a relatively higher rate of conversion, which continuously increased the concentration of (**4**) in the reaction medium. In addition, the rate of conversion of intermediate (**4**) to the final product (**5**) was considerably slow, which could be related to the slow activity of the existing carbonyl reductase within the cells. These possibilities could be depicted in a form of diagram given in Fig. 9. The diagram gives a clear scenario of the behaviour of the bi-enzymatic biotransformation system discussed above. Even though the final compound (**5**) was continuously accumulated as the reaction progresses, the system could still be optimised in terms of the availability and improved activity of carbonyl reductase. Moreover, the reduced stability of cofactor as shown in Fig. 8(C) could also be improved by perhaps introducing a continuous feeding strategy of glucose throughout the course of biotransformation.

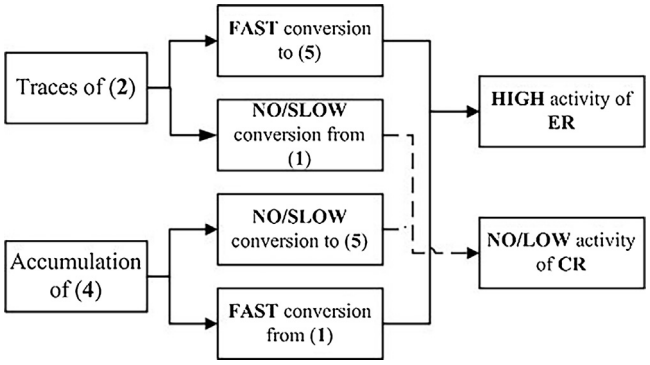

Fig. 9. The possible scenarios of the bi-enzymatic behaviour at higher concentration of *S. cerevisiae*. **ER** represents enoate reductase and **CR** represents carbonyl reductase. Compound (**1**) is the substrate 2,6,6-trimethylcyclohex-2-ene-1,4-dione, compound (**2**) refers to (**S**)-4-hydroxy-2,6,6-trimethylcyclohex-2-enone, compound (**4**) refers to 2,2,6-trimethylcyclohexane-1,4-dione and compound (**5**) represents the final product (**R**)-4-hydroxy-2,2,6-trimethylcyclohexanone.

4. Conclusions

The reduction of ketoisophorone (**1**) into a useful chiral intermediate for the synthesis of carotenoids and other pharmaceutical compounds using Baker's yeast type-II as the biocatalyst provides a complex and interesting reaction mechanism. The interaction between cofactor recycling and enzymes formation within the yeast cells is interesting and important information for a bi-enzymatic system to be carefully checked and understood prior to genes expression and cloning. Since the reaction is highly dependent on the cofactor (NADH/NADPH) availability, therefore, carbon source, such as glucose must be provided to ensure a continuous product formation. Despite of the fact that cofactor is only required in the recycling of the hydrogen ions within the redox system, its present in excess through glucose metabolism could eventually stabilize the system and consequently prolong the biotransformation for certain period of time. The present work also showed that the growing cell failed to continue the reduction towards the final product (**5**), as cells were concentrating on dividing themselves with only enoate reductase formed during the course of the growth phase. It is suspected that carbonyl reductase would only form once the cell reached the stationary phase with a continuous supply of glucose, which work is still currently in progress. Additionally, the amount of biocatalyst also plays an important role in the biotransformation reaction as it readily provides the initial enzyme concentration, for substrate conversion.

For a biotransformation system that depends on two types of enzymes, the information on which protein exists first and the amount present within the cell is essential in determining which genes needs to be enhanced in order to optimize the system [39]. Since the results also relate the carbon source to that of the proteins formation, therefore, the energy landscape in protein folding should also be taken into account in the analysis of reaction rate and products formation [40]. The finding from this work is believed to be the first reported work in understanding the behaviour of the in-vivo bi-enzymatic ketoisophorone biotransformation system.

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