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FERMENTATION, CELL CULTURE AND BIOENGINEERING - ORIGINAL PAPER

Significance of oxygen carriers and role of liquid paraffin in improving validamycin A production

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Abstract Validamycin A (Val-A) synthesized by *Streptomyces hygroscopicus* 5008 is widely used as a high-efficient antibiotic to protect plants from sheath blight disease. A novel fermentation strategy was introduced to stimulate Val-A production by adding oxygen carriers. About 58 % increase in Val-A production was achieved using liquid paraffin. Further, biomass, carbon source, metabolic genes, and metabolic enzymes were studied. It was also found that the supplementation of liquid paraffin increased the medium dissolved oxygen and intracellular oxidative stress level. The expression of the global regulators *afsR* and *soxR* sensitive to ROS, *ugp* catalyzing synthesis of Val-A precursor, and Val-A structural genes was enhanced. The change of the activities of glucose-6-phosphate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase was observed, which reflected the redirection of carbon metabolic flux. Based on these results, liquid paraffin addition as an oxygen carrier could be a useful technique in industrial production of Val-A and our study revealed a redox-based secondary metabolic regulation in *S. hygroscopicus* 5008, which provided a new insight into the regulation of the biosynthesis of secondary metabolites.

Keywords Fermentation strategy · Oxygen carrier · Liquid paraffin · *Streptomyces hygroscopicus* · Validamycin A

Introduction

Streptomyces bacteria produce a large number of valuable secondary metabolites, which are used as antibiotics, pesticides, immunosuppressants, and germicides. Among these secondary metabolites, validamycin A (Val-A) synthesized by *S. hygroscopicus* 5008 is a widely used high-efficient antibiotic to protect rice, wheat and corn from sheath blight disease in East Asia [37]. In the medicine area, Val-A has been used to produce two antidiabetic drugs—voglibose and acarbose [11, 37]. Not only the low toxicity and the high efficiency as the agricultural antibiotic, but also the precious medicinal value attracts the attention of researchers. By the isotope labeling method, it has been found that D-glucose is the only carbon source to synthesize Val-A, and sedoheptulose 7-phosphate (S-7P) in the pentose phosphate pathway is the immediate precursor involved in the biosynthesis of Val-A [8, 24, 31]. Besides, previous studies have shown that *valABC*, *valKLMN* and *valG* have function to catalyze S-7P to Val-A and these eight genes are the entire Val-A structural genes locating in three operons by the genetic analysis of *S. hygroscopicus* 5008 [2].

The fermentation of Val-A is susceptible to some environmental factors, such as nitrogen composition in medium and fermentation temperature [16, 34]. The oxygen environment often plays an important role in the microbial metabolism and, therefore, changes in the oxygen supply and the oxygen transfer rate can cause a series of intracellular responses. High oxygen supply in adenosine fermentation of *Bacillus subtilis* was found to have an impact on the expression of genes which were involved in energy, cell differentiation and

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protein synthesis [36]. In other study, excess O_2 supply was associated with the induction of intracellular reactive oxygen species (ROS) and ROS was considered as a signaling molecule in the stimulation of pimaricin production in *S. natalensis* [3]. In terms of Val-A production by *S. hygroscopicus*, it has been proved that addition of H_2O_2 (the direct ROS inducer) could heavily affect cell metabolism and increase Val-A productivity [33]. However, most of oxidizing substances such as H_2O_2 are unstable and sometimes toxic to cells, which are not practical to apply in industrial fermentation. Other ways to improve oxygen supply condition are rarely reported. The rotation speed in *S. hygroscopicus* 5008 fermentation has been optimized and as an aerobic bacterium, *S. hygroscopicus* 5008 is inevitably subjected to oxygen transfer rate in culture to increase the Val-A production.

Oxygen carriers are a kind of organic reagents with the excellent ability to increase the oxygen content in fermentation broth by elevating oxygen transfer rate. Oxygen carriers are widely used in the fermentation of medicine and other biological products without cytotoxicity [14, 18]. Perfluorinated oxygen carriers were used in *Escherichia coli* to stimulate the cell growth and recombinant protein production [7, 23]. Using *n*-dodecane in *Aspergillus niger* and *A. terreus* culture, their fermentation products citric acid and lovastatin were increased with the rise of dissolved oxygen concentration [15, 32]. The addition of *n*-hexadecane in shake-flask fermentation of *Phaffia rhodozyma* significantly improved the oxygen transfer rate and led to an increase of carotenoid production [17]. Liquid paraffin is a safe, high-efficient, easily procured and cost-effective oxygen carrier, which makes it to meet demands of the fermentation industry. During fermentation of *Bacillus brevis*, liquid paraffin was found to maintain dissolved oxygen in broth and exhibit a stimulative effect on cell division, cell mass accumulation, L-asparaginase activity and L-asparaginase productivity [20]. All of these indicated that the oxygen carriers could change the intracellular oxidative level and regulate the cellular responses. However, impact of oxygen carriers on ROS generation, gene expression, enzyme activity and metabolic pathway was rarely reported in previous research. Oxygen carriers may contribute to Val-A biosynthesis and improve Val-A productivity further.

This study intended to test effects of oxygen carriers on Val-A production and investigate impact of the most efficient oxygen carrier—liquid paraffin on Val-A biosynthesis in *S. hygroscopicus* 5008. By analyzing intracellular ROS and ROS-related genes and enzyme activities, it was proved that liquid paraffin could influence the intracellular oxidative stress level. The expression of global regulatory genes and Val-A biosynthesis-related genes was assayed to verify cell response to the efficient oxygen vector. Besides, two main carbon metabolic fluxes related to the biosynthetic precursor's accumulation were monitored. Based on these data, a new insight was provided to understand the impact of oxygen carrier on Val-A biosynthesis and the results would be useful for industrial antibiotic production.

Materials and methods

Strain and cultivation

The strain *S. hygroscopicus* 5008 was used in this study. Detail of culture medium and inoculation method was consistent with the previous report [38]. Three effective oxygen carriers—*n*-dodecane, *n*-hexadecane and liquid paraffin—were sterilized at 121 °C for 20 min and put in heat oven at 40 °C overnight. Then they were added into the fermentation broth at 12 h (inoculation time) with final concentrations of 0.05, 0.1 and 0.5 % (v/v), respectively, in the medium.

Val-A production, cell growth and residual sugar analysis

Fermentation broth of 2 ml was taken for analysis of cell growth, residual sugar and Val-A concentration. The pretreatment of samples was as follows: the broth sample was centrifuged at 12,000 g for 5 min, 0.5 ml of supernatant was extracted by the same volume of chloroform, and then filtered by 0.22-μm hydrophilic filter. The filtered sample was used to determine Val-A concentration by high-performance liquid chromatography (HPLC) method [11]. As corn powder and soybean powder used in medium were insoluble, the total protein released from mycelia was determined by standard Bradford method to reflect the growth of cell [13]. The total residual sugar was determined by standard phenol–sulfuric acid method [16].

Assay of dissolved oxygen concentration (DOC) and intracellular oxidative stress level

The dissolved oxygen concentrations in flask were measured using a dissolved oxygen probe (Finesse TruDo). The DOC in conditions of 50 ml distilled water, 220 rpm and 37 °C in 250-ml glass flask with gassed air to saturation was defined as 100 %. The fermentation medium containing a lot of corn powder (100 g l^{-1}) was in a solid state after sterilization and the medium changed from solid to liquid in 24 h after inoculation of *S. hygroscopicus* 5008 with the consumption of the carbon source. The DOC of medium could be tested after one day's culture of *Streptomyces* when the medium changed from solid to liquid.

Intracellular ROS was assayed as Hu's report [10]. However, most ROS even most stable hydrogen peroxide (the common form of ROS existing in cell), could be rapidly detoxified and some researchers thought direct measurements of intracellular ROS could not represent exact oxidative stress

level [4]. Hence, both catalase gene (*catR*) expression and catalase activity were also detected to reflect intracellular oxidative stress level. Activity of catalase was assayed by ultraviolet spectroscopy according to previous report [5].

Assay of gene expression by quantitative real-time PCR (qRT-PCR)

Total RNA was extracted by Trizol (Invitrogen, USA) and treated with gDNA Eraser (Takara, Japan). Subsequently, reverse transcription was achieved with Prime Script® RT Enzyme Mix I (Takara, Japan). The transcriptional level of genes was determined by qRT-PCR on PowerPac Universal (Bio-Rad, USA) with SYBR® Premix Ex Taq™ II (Takara, Japan). The PCR conditions were pre-denaturation at 95 °C for 5 min followed by 40 cycles of 15 s amplification at 95 °C, 15 s denaturation at 60 °C and 30 s annealing at 72 °C. For each gene, the fermentation samples without addition of the oxygen carriers were defined as the expression level of 1.0, and results were expressed as the fold increase of mRNA level over the control samples. The sequences of primer pairs are listed in Table S1.

Assay of enzyme activities in carbon metabolism

The activities of glucose-6-phosphate dehydrogenase (G6PD) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) that reflected carbon metabolic flux were determined as reported [16, 25].

Statistical analysis

The data were the average of three independent samples. The error bars indicated the standard deviation (SD) from the mean of triplicates. The data were statistically compared using one-way analysis of variance (ANOVA), and significant differences were analyzed by Student's *t* test ($p < 0.05$). To validate the best oxygen carrier to increase the Val-A production, Tukey's test was also used to compare the difference between three oxygen carrier treatment groups and the control. All these analyses were carried out with SPSS software (SPSS Inc., USA).

Results and discussion

Fermentation profiles with the addition of oxygen carriers

Some nontoxic oxygen carriers were added into fermentation broth, respectively. Three efficient additives *n*-dodecane, *n*-hexadecane and liquid paraffin were found while some additives had no influence, such as silicone oil, oleic acid and Tween 80. As shown in Fig. 1, the highest Val-A

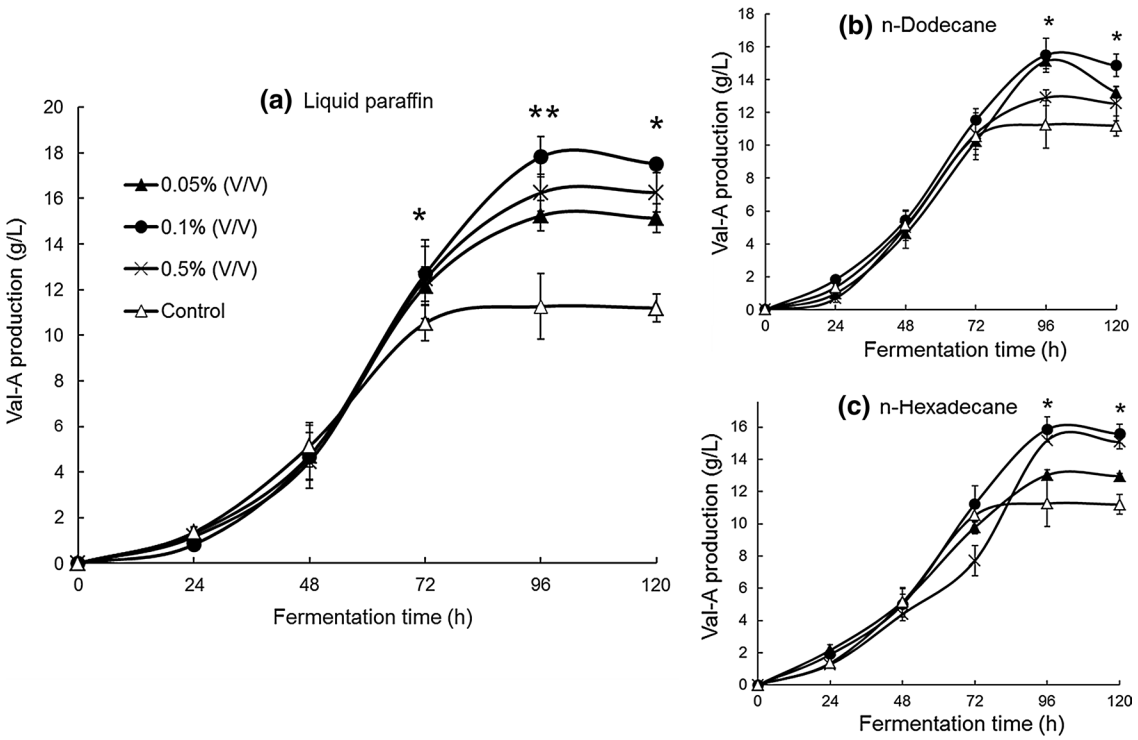

Fig. 1 Val-A production curves of *S. hygroscopicus* in different conditions of oxygen carriers

production with three additives was as follows: liquid paraffin 17.82 ± 0.88 g l^{-1}, *n*-dodecane 15.50 ± 1.03 g l^{-1}, and *n*-hexadecane 15.87 ± 0.77 g l^{-1}. The data of three oxygen carriers were significantly better than the control in Val-A production ($p < 0.05$), and liquid paraffin treatment group had extremely significant difference (p value 0.001, $p < 0.01$) in particular (Table S2). The liquid paraffin group had the highest yield of Val-A, so liquid paraffin was chosen for the further study. As for the optimal concentration, when more than 1 % (v/v) concentration of oxygen carriers were added, both cell growth and Val-A production were inhibited. After comparing different addition concentrations, three important gradients were shown in Fig. 1 and 0.1 % (v/v) was regarded as the best concentration of liquid paraffin, in which Val-A production increased by 58 % to 17.82 g l^{-1}. The improvement of Val-A production with addition of liquid paraffin is statistically significant ($p < 0.01$). The function of liquid paraffin to enhance Val-A production was also verified in 5-L bioreactors, in which addition of liquid paraffin brought the increase of Val-A production (31 %) and the improvement of DOC (7 %). When the concentration of oxygen carrier in broth was below 0.1 %, Val-A production was promoted with the increasing amount of oxygen carrier. Until reaching 0.1 %, adding oxygen carrier persistently would have a negative effect on Val-A production. This dose-dependent effect also existed in other oxygen carrier application examples, such as *n*-hexadecane in *Mortierella alpina* ME-1 fermentation and *n*-dodecane in *A. niger* [22, 32]. There was a solid-state fermentation process before 24 h in the fermentation of *S. hygroscopicus* 5008, and in this case, excess of oxygen carrier would cover the fermentation medium, which inhibited oxygen transmission and impaired the growth and metabolism of aerobic bacteria instead of improving oxygen transfer rate.

With the improvement of Val-A production, liquid paraffin was selected to further investigate its impact on cell growth and carbon utilization. In terms of the final results of cell growth (Fig. 2a), liquid paraffin did not bring more cell mass of *S. hygroscopicus*, which suggested enhancement of Val-A production was not due to more cell division. However, at 72 h, the intracellular proteins of the treatment with 0.1 % liquid paraffin addition and the control were 6.33 and 5.93 g l^{-1}, respectively, which were significantly different, while at 48 h and at 96 h, there was no difference between parameters of the treatment group and the control. Hence, from 48 to 72 h, samples with 0.1 % liquid paraffin addition presented a higher intracellular protein accumulation rate (0.070 g l^{-1} h^{-1}) than the control (0.055 g l^{-1} h^{-1}) and the difference disappeared at 96 h. Meanwhile, carbon utilization was detected as shown in Fig. 2b. Although liquid paraffin addition did not cause more carbon source to be used finally, it significantly increased the carbon utilization rate from 24 to 96 h. Combined with the above results, it was found that enhancement of Val-A production was not because of higher cell concentration or more carbon utilization, but because of more efficient biosynthesis in *S. hygroscopicus* 5008. Besides, the 72 h was the time when fermentation samples with liquid paraffin addition began to produce more Val-A than the control (Fig. 1). Thus, the addition of the appropriate amount of liquid paraffin could bring the sustained high productivity of Val-A from the end of log phase to decline phase.

Effect of liquid paraffin on DOC and intracellular oxidative stress level

Oxygen carriers could accelerate oxygen transport directly to microorganisms by forming a new interfacial area between gas and liquid phases [26–28]. Sometimes the addition of oxygen carriers can also change the viscosity of the medium and provide the carbon source in industrial production when the concentration of oxygen carriers is as high as 5 %. However, the used concentration of oxygen carriers is less than 1 % in this study and, therefore, its contribution to the viscosity and carbon source is negligible. The function of liquid paraffin to increase the dissolved oxygen is the key factor to influence the fermentation of

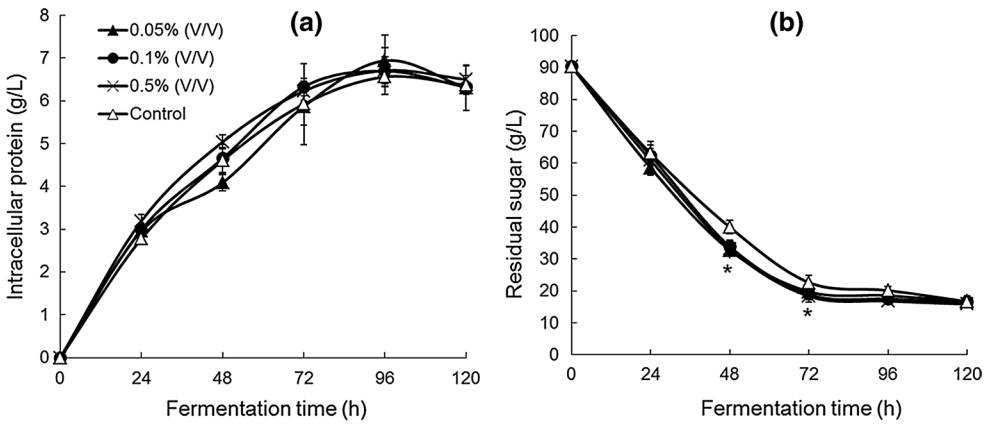

Fig. 2 Cell growth (**a**) and sugar utilization (**b**) curves in different liquid paraffin addition conditions

Table 1 Effect of liquid paraffin on DOC and intracellular ROS in Val-A production by *S. hygroscopicus* 5008 in shake-flask culture (37 °C, 220 rpm)

	DOC (%)[a]		ROS (μmol g^{-1})[b]		Val-A (g l^{-1})[b]	
	24 h	72 h	24 h	72 h	24 h	72 h
Control	11	72	2.18	2.07	1.35	10.54
0.1 % Paraffin	25	81	3.15	2.20	0.82	12.68

[a] Average of two independent determinations

[b] Average of three independent determinations

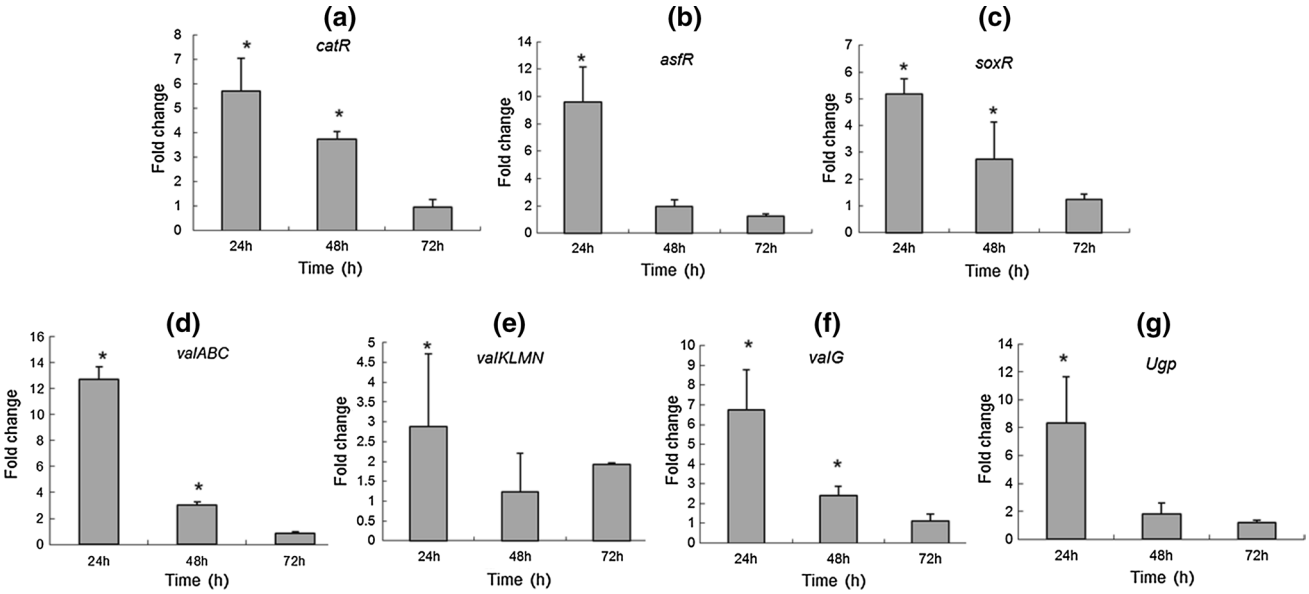

Fig. 3 Transcriptional level of catalase gene (**a**), global regulatory genes (**b**, **c**), and Val-A biosynthesis-related genes (**d**, **e**, **f**, **g**) with 0.1 % liquid paraffin addition compared with the control

Val-A. Therefore, the dissolved oxygen and intracellular ROS were estimated. As shown in Table 1, the oxygen carrier elevated the dissolved oxygen and induced the intracellular ROS, along with the promotion effect lasting from log phase to stationary phase.

However, some findings suggest that the direct measurements of intracellular ROS could not represent the exact oxidative stress level [4], because most ROS even the relatively stable H_2O_2 could be rapidly detoxified. Hence, the transcriptional level of catalase gene (*catR*) and the activity of catalase were also detected to reflect intracellular oxidative stress level. As shown in Fig. 3a, the expression of *catR* showed evident enhancement in fermentation samples with 0.1 % liquid paraffin. It was 5.7-fold higher at 24 h and 3.5-fold higher at 48 h than the control. Data in Fig. 4a showed cells in 0.1 % liquid paraffin addition condition had much higher activity of catalase from 24 to 72 h than the control. At 24 h, the catalase activity of treated sample (84.16 U mg^{-1}) was nearly threefold as high as that of the control. Those results suggested that addition of liquid paraffin caused an increase of intracellular ROS and microbial cell responded to this situation by raising catalase gene expression and catalase activity to keep a balance of intracellular ROS level. This phenomenon of oxidative stress change is in accordance with research in other bacteria [1]. In *Bacillus* sp. F26, increase of dissolved oxygen concentration in broth during the exponential growth phase directly increased activity of catalase [35]. In *Blakeslea trispora*, excess air in the flask increased specific activity of catalase and influenced cell morphology [19].

Response of global regulatory genes and Val-A biosynthesis-related genes

ROS was reported to influence some regulons [3, 36], which drove researchers to analyze the expression of many global regulators and Val-A biosynthesis-related genes. Several global regulators were found to have response to liquid paraffin addition. First found in *E. coli*, *soxR* was known as a global regulator to respond to change of oxidative stress [9]. Recent studies in *Streptomyces* suggested that *soxR* expression in response to oxidative stress was involved in regulation of secondary metabolism [6]. By binding to promoter regions of actinorhodin biosynthesis

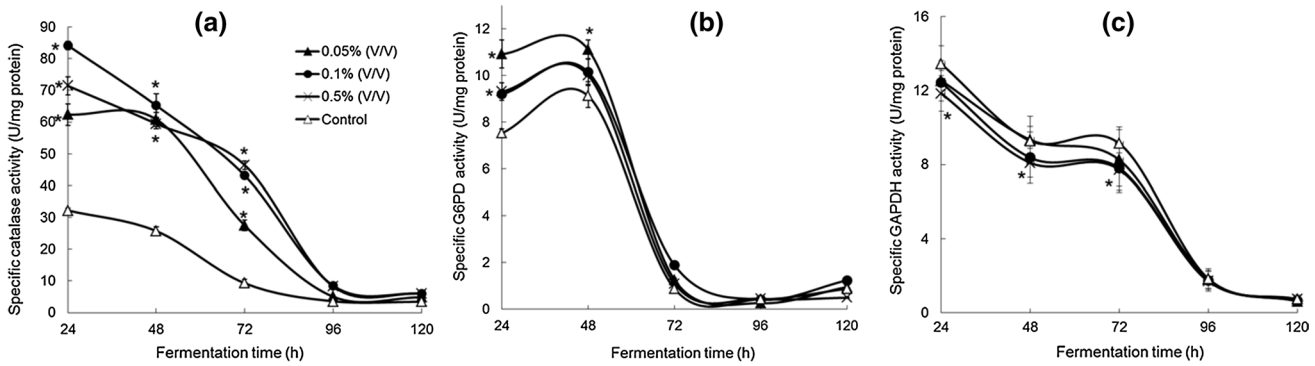

Fig. 4 Activities of catalase (**a**), G6PD (**b**) and GAPDH (**c**) in different liquid paraffin addition conditions

genes, SoxR was active immediately in actinorhodin production in *S. coelicolor* [30]. In *Streptomyces*, *afsR* was another widely existed redox-sensitive global regulatory gene which was proved to regulate a wide variety of secondary metabolites' production [21]. Kanth found when excess of peroxide and superoxide existed in *S. peucetius*, expression of *afsR* would be significantly raised [12], and regulation in *afsR* gene further led to improved production of actinorhodin and undecylprodigiosin.

By comparison and analysis of genes in NCBI, we found that there were some global regulators existing and controlling the adaptive responses to oxidative stress in *S. hygroscopicus* 5008, including *afsR*, *gntR*, *lysR*, *marR*, *soxR*, *tetR*, and *whiB*. After analyzing these global regulators, the expression of two important redox-sensitive global regulators was found to be influenced by addition of liquid paraffin. As shown in Fig. 3b, c, transcriptional level of *afsR* and *soxR* was significantly enhanced in fermentation with 0.1 % liquid paraffin. Compared to the control, up-regulation of *afsR* and *soxR* could reach 9.6-fold and 5.2-fold at 24 h, respectively. The time of global regulators' up-regulation was consistent with that of oxidative stress level's increase. It was inferred that *afsR* and *soxR* made immediate responses for the change of oxidative stress. The two regulons had more important function of withstanding the change of intracellular oxidative stress than the other oxidative stress-related regulons in *S. hygroscopicus* 5008.

In *S. hygroscopicus* 5008, *valABC*, *valKlMN* and *valG* were whole structural genes for Val-A biosynthesis [2] and *ugp* encoded UDP-glucose pyrophosphorylase. This enzyme catalyzed UDP-glucose to UTP and UDP-glucose was the substrate of glycosylation reaction from validoxylamine A to Val-A. Over-expression of *ugp* was reported to contribute to a higher Val-A productivity [39]. As shown in Fig. 3d–g, significant differences of transcriptional level of Val-A structural genes and *ugp* gene existed between the liquid paraffin treatment and the control. Among three operons, *valABC* had the most pronounced up-regulation, which was more than 12.5-fold increase at 24 h. At the same time, up-regulation of *valKLMN* and *valG* reached 2.9-fold and 6.8-fold, respectively. Besides, as Val-A biosynthesis-related gene, *ugp* got an eightfold enhanced expression at 24 h. Transcriptional analysis showed up-regulation of all Val-A biosynthesis-related genes evidently happened in exponential growth phase when global regulators made response to oxidative stress change. Hence, we inferred that in this period the Val-A biosynthesis pathway was influenced by global regulators. Up-regulation of Val-A biosynthesis-related genes contributed to accumulation of Val-A biosynthetic enzymes. When fermentation switched to the stationary phase, cells could rapidly synthesize secondary metabolites with more enzymes. That should be one reason for enhancement of Val-A production and acceleration of sugar utilization rate in liquid paraffin addition condition from the log phase to the stationary phase.

Effect of liquid paraffin on key enzyme activities in carbon metabolism

As mentioned in previous study, both *afsR* and *soxR* could regulate carbon metabolic flux in *Streptomyces* [30], and ROS as an intracellular signal also played an important role in regulating carbon metabolism. As cells' main pathway to provide reducing power, pentose phosphate pathway (PPP) produced most of reduced nicotinamide adenine dinucleotide phosphate (NADPH). When confronted with increasing oxidative stress, its metabolic activity would be stimulated to keep a balance of internal environment [29]. As the main metabolic pathway to produce Val-A precursor S-7P, activity of PPP was directly related to Val-A biosynthesis. Meanwhile, glycolytic pathway, the main energy metabolic pathway, kept competitive with PPP and its flux would also affect S-7P accumulation and Val-A biosynthesis.

To make a comprehensive understanding on carbon metabolism, activities of key enzymes in two pathways were analyzed. G6PD and GAPDH were rate-limiting enzymes in

the regulation center of PPP and glycolysis pathway, respectively. As shown in Fig. 4b, G6PD activity of liquid paraffin addition samples was significantly enhanced from 24 to 48 h, and then back to the same level as the control at 72 h. In Fig. 4c, GAPDH activity presented no difference between the liquid paraffin addition samples and the control at 24 h. But from 48 to 72 h, the activities of liquid paraffin addition samples were evidently inhibited. Thus, at the initial stage of fermentation, activity of GADPH was not impaired by addition of liquid paraffin. Vigorous activity of glycolysis pathway could provide enough energy for cell to synthesize primary metabolites. When fermentation entered the middle phase from 48 to 72 h, fluctuation of G6PD and GAPDH activities reflected a redirection of metabolic flux from glycolysis to PPP. During that period, secondary metabolism replaced primary metabolism as main metabolic type in cells. Flux enhancement in PPP could provide more precursors and intensify the whole pathway for Val-A biosynthesis.

Conclusions

Based on the results, we proposed a model to comprehensively represent the effect of liquid paraffin on Val-A production during the fermentation process (Fig. 5). Addition of liquid paraffin in medium would create a high dissolved oxygen fermentation environment which elevated intracellular oxidative stress level and induced intracellular ROS generation. Responding to increased oxidative stress, redox-sensitive global regulators up-regulated Val-A biosynthesis-related genes. Driven by the demand of reducing power, PPP was stimulated to provide plenty of precursors S-7P. Along with up-regulation of Val-A biosynthesis-related genes, excess accumulation of precursors led to an enhancement of Val-A productivity. Thus, the addition of liquid paraffin could activate a redox-sensitive regulation and lead to the enhancement of Val-A production in *S. hygroscopicus* 5008. This work revealed for the first time that addition of liquid paraffin was a useful strategy to enhance Val-A production. This simple and effective strategy would be of reference value to not only Val-A production but also other antibiotics' production on the industrial scale. Additionally, the information obtained in this study provided a new insight into the role of oxygen carriers in the biosynthesis of secondary metabolites.

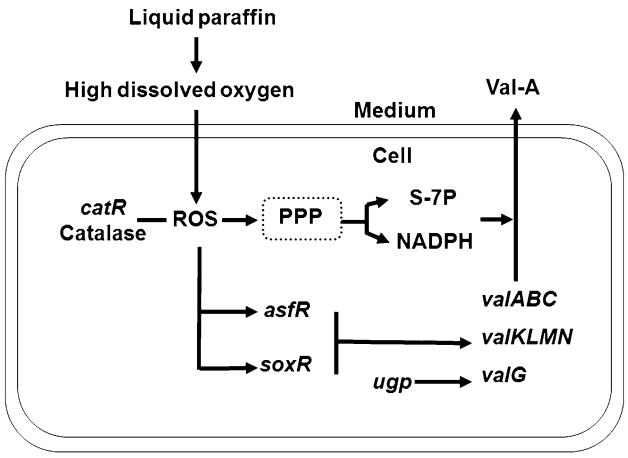

Fig. 5 Proposed signal transduction cascade of *S. hygroscopicus* induced by liquid paraffin

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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