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Research Article

Growth Phase Influence the Gene Expression and Metabolite Production Related to Indole-3-Acetic Acid (IAA) Biosynthesis by *Serratia plymuthica* UBCF_13

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Abstract

Background and Objective: The optimization of the indole-3-acetic acid (IAA) producing capability of *Serratia plymuthica* UBCF_13 has been intensively studied. This work tried to reveal the effect of growth phases on IAA production, gene expression and metabolite synthesis related to the IAA biosynthesis pathway. **Materials and Methods:** The growth curve and IAA production were measured every 3 hrs. The putative IAA biosynthesis pathway was investigated based on the UBCF_13 genome. To identify the possible pathway of IAA biosynthesis in UBCF_13, we applied the Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) and High-Performance Liquid Chromatography (HPLC) analysis to measure the transcript levels of each gene and indole metabolite production based on tryptophan treatment at different times of incubation. **Results:** The optimal IAA production on colorimetric assay was at 9 hrs of incubation (initial stationary phase). The level expression of *puuC*, *DDC*, *oxdA*, *amiE*, *nthA* and *nthB* have been upregulated maximum in 3 hrs of culture time (lag phase), except *tyrB* and *ipdC*. The highest transcript level of the genes was found in nitrile hydratase genes (*nthA* and *nthB*) and indole-3- acetamide (IAM) has been detected as the only intermediate in the crude extract of UBCF_13 thus the IAM pathway may be used to produce IAA. The maximum IAA production on HPLC analysis was found at 21 hrs of incubation (late stationary phase). **Conclusion:** This study gives a new insight that the best time to measure gene expression and intermediates related to the IAA biosynthetic pathway in bacteria was found at a specific growth phase.

Key words: Growth curve, HPLC, PGPB, IAA biosynthesis pathway, qRT-PCR, gene expression, luria bertani

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Auxin is a vital hormone for controlling the growth and development of plants which is also found and synthesized by microorganisms^{1,2}. Plant Growth-Promoting Bacteria (PGPB) is known as a group of bacteria with various capabilities to promote the growth of the plant and one of its capabilities is producing indole-3-acetic acid (IAA)³. One of the phyloplane bacteria that can synthesize IAA is *Serratia plymuthica* UBCF_13. UBCF_13 is a strain of the Biotechnology Laboratory, Faculty of Agriculture, Universitas Andalas Collection which was isolated from *Brassica juncea* L. leaves⁴. The IAA production from this strain is known to have a positive effect on the growth of chilli⁵. Optimization of IAA production from UBCF_13 was investigated for the first time in luria bertani (LB) medium and optimal production was gained with the addition of 200 µg mL⁻¹ tryptophan at 48 hrs of duration culture⁵. The next optimization of culture media for IAA production was found in the yeast mannitol (YM) medium with the addition of 300 µg mL⁻¹ tryptophan but the optimal culture duration for IAA production in the YM medium needs to be evaluated.

The genes related to the IAA biosynthesis pathway of UBCF_13 have not been studied yet. The IAA biosynthesis pathway could be a tryptophan-dependent or tryptophan-independent pathway. The tryptophan-dependent pathway consists of tryptamine (TAM), indole-3-acetamide (IAM), indole-3-acetaldoxime (IAOx)/indole-3-acetonitrile (IAN) and indole-3-pyruvate (IPyA) pathway^{6,7}. Some *Serratia* strains use the IPyA pathway to synthesize IAA⁸⁻¹³. The putative genes related to IAA production could be detected by the genome mining method and continued with RT- qPCR to validate the expression level of the gene and chromatography analysis to confirm intermediate compounds related to IAA biosynthesis. These methods have been applied to verify the IAA biosynthesis pathway in *Arthrobacter pascens* ZZ21, *Rhizobium tropici* CIAT 899, *Variovorax boronicumulans* CGMCC 4969 and *Enterobacter* spp.¹⁴⁻¹⁷.

However, studies regarding the effect of the growth phase of bacteria on the expression level of genes and metabolites related to IAA production have not been reported. Therefore, in this study, we carried out genome mining to obtain data on genes related to IAA production. Based on the treatment of tryptophan addition at several culture times, we also used qRT-PCR and HPLC to analyze the gene expression and intermediate compound related to IAA biosynthesis in UBCF_13. The objective of this study is to find the best time for UBCF_13 to produce IAA, identify the IAA biosynthesis

pathway in UBCF_13 and find the effect of different growth phases on the transcript level of genes and metabolites production related to IAA biosynthesis.

MATERIALS AND METHODS

Study area: This study was conducted from November 25, 2020 to August 19, 2022. The study was carried out at the Biotechnology Laboratory, Department of Agrotechnology, Faculty of Agriculture, Universitas Andalas, Padang. The HPLC analysis was carried out at Biota Sumatera Laboratory, Universitas Andalas, Padang.

Chemicals: The IAA 98%, IAM 98%, TAM 97% and IAN 98% were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tryptophan (TRP), acetonitrile gradient grade for liquid chromatography and acetic acid 100% for HPLC were purchased from Merck Millipore (Darmstadt, Germany). Ethyl acetate and methanol for HPLC were purchased from Smart Lab (Banten, Indonesia).

Bacterial strain and growth conditions: *Serratia plymuthica* UBCF_13 was isolated from leaves of *Brassica juncea* L. and stored in Biotechnology Laboratory Collection (Universitas Andalas) under accession number: KX394779⁴. The bacteria were pre-cultivated on LB agar (HiMedia, India) and incubated for 24 hrs at room temperature. The pre-cultivated bacteria were inoculated into LB broth and incubated for 16 hrs at 28°C with a shaking incubator at 160 rpm.

Growth curve and IAA production based on differences in culture duration: Growth curve analysis was created by adding 1 mL of pre-inoculated bacteria and incubating on LB medium and YM broth medium (HiMedia, India) with and without tryptophan for 24 hrs, 28°C in a shaking incubator at 160 rpm. The bacterial density was calculated every 3 hrs with absorbance at 600 nm. This analysis was conducted with three biological replicates.

The IAA production based on culture duration was evaluated in YM medium with the addition of 300 µg mL⁻¹ tryptophan for 24 hrs at 160 rpm (28°C) with three biological replicates. The IAA production was calculated periodically every 3 hrs according to the colorimetric assay by Gordon and Weber¹⁸ with some modifications. The bacterial cultures were centrifuged for 10 min at 10000 rpm and 2 mL of supernatant was added with 4 mL of Salkowski reagent. The solution was incubated for 30 min in darkness and the absorbance was calculated at 530 nm. The absorbance value

Table 1: Primers used in the RT-qPCR analysis

Gene	Primer	Sequence (5' to 3')	Amplicon size (bp)
<i>tyrB</i>	tyrB_F	GAA GTT TGA CGC CAT GTT GAG C	132
	tyrB_R	ATC ACC TTG ACC ACT TCA TCC C	
<i>ipdC</i>	ipdC_F	GGC AAC AGA TCC AGG ACT TCT T	124
	ipdC_R	CAC AGC GAC TGA ACG ATA AAC C	
<i>puuC</i>	puuC_F	TTC TTA CCC TGG TGG ATA ACG C	91
	puuC_R	TGC CGT TCG TCG TTA AAG GA	
<i>nthA</i>	nthA_F	GTC TGT ACG CTT TGC TCC TGT TA	136
	nthA_R	CGC CGA TGT TCA GAC CAA ACT	
<i>nthB</i>	nthB_F	GCG GTC ACT TCA ATG TCG ATA TG	149
	nthB_R	GGA AAG CTC ATC GGC GGT AAT A	
<i>DDC</i>	ddc_F	CTG GAG GAT TTC TGG TTA CGC T	133
	ddc_R	TTT GGT CTG TGG CAC TAA CCA G	
<i>amiE</i>	ami_F	CCT ACA GAT TTC CGA GAG CGA A	138
	ami_R	AAC CCG TCA ATC AGA TCG TAG G	
<i>oxdA</i>	oxd_F	CAC ACC TTC GCA TAT TCA CCA C	125
	oxd_R	GGG ATG GCA GTT GAT GTA CTC A	
<i>gyrA</i>	gyrA_F	CGG TGG AAG ATG AAG AGT TGG A	99
	gyrA_R	CGG CTT CAG AAT CAT CGT CAA C	

of the sample was then compared with the standard curve formed from the IAA standard ($5\text{--}100\ \mu\text{g mL}^{-1}$) to obtain the IAA concentration of the sample.

Genome mining and qRT-PCR of genes related to IAA biosynthesis in UBCF_13:

Genome mining of genes that are related to IAA biosynthesis from the genome of *S. plymuthica* UBCF_13 (RefSeq: NZ_CP068771.1)¹⁹ refers to Batista *et al.*²⁰, with modifications. The predicted genes were confirmed using blastx (NCBI) to get the putative function and BlastKoala (<https://www.kegg.jp/blastkoala/>) to find the KEGG Orthology (KO) number.

qRT-PCR of genes related to IAA biosynthesis in UBCF_13 based on different culture duration:

To analyze the effect of tryptophan addition and culture duration on the expression of predicted genes related to IAA biosynthesis, *Serratia plymuthica* UBCF_13 was cultured in YM medium with and without $300\ \mu\text{g mL}^{-1}$ tryptophan for 3, 6, 9, 12 and 21 hrs at 160 rpm, 28°C . A total of 5 mL of each culture was taken and centrifuged at $13000\times g$ for 3 min and then the cells were used to attain the total RNA. The total RNA was extracted using SV total RNA isolation system kit (Promega). Then the cDNA was constructed from the extracted RNA using ReverTra AceTM qPCR RT master mix with gDNA remover (Toyobo). The cDNA was subjected as the DNA template for reverse transcription-quantitative PCR (RT-qPCR) with THUNDERBIRDTM SYBRTM qPCR mix (Toyobo) using CF $\times$ 96 Touch Real-Time PCR Detection System (Bio-Rad, USA) under cycling condition: 95°C for 1 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min melt curve at $65\text{--}95^\circ\text{C}$. The primers that were used in this study are listed in Table 1. The *gyrA* gene was used as the

housekeeping gene for normalizing the expression of target genes (*tyrB*, *ipdC*, *puuC*, *nthA*, *nthB*, *DDC*, *amiE* and *oxdA*). The reaction for qPCR analysis was performed in 20 μL total volume reactions consisting of 10 μL SYBR[®] Green Realtime PCR master mix, 1 μL of each primer ($0.3\ \mu\text{M}$), 1 μL cDNA template, 1 μL ROX reference dye and 6 μL distilled water. This experiment was carried out with three biological replicates. The gene expression was analyzed using the $2^{-\Delta\Delta\text{CT}}$ method based on Livak and Schmittgen^{21,22}.

Detection of indole compound by HPLC analysis

Crude extract preparation: UBCF_13 was cultured under the same conditions as the qRT-PCR assay. The supernatant from each culture was collected by centrifugation at 10000 rpm for 10 min, 4°C and acidified with HCl (1N) to pH 2.7. The acidified supernatant was then extracted two times using double volumes of ethyl acetate. The ethyl acetate fraction was evaporated using a rotary evaporator (Heidolph, Germany) and the extract was dissolved in methanol and stored at -20°C ²³.

HPLC of crude extract: Indole compounds were analyzed using HPLC (Shimadzu 10A-Tvp HPLC system, Japan). Ten microliters of crude extract were injected into Shim-pack MRC-ODS RP-18 columns ($4.6\ \text{mm}\times 25\ \text{cm}$, $5\ \mu\text{m}$, Shimadzu, Tokyo, Japan) with acetonitrile: water: acetic acid (40:60:0.01%) as the mobile phase. The flow rate was $1.0\ \text{mL min}^{-1}$ and the total run time was 30 min¹⁶. The indole compounds were detected at 280 nm by comparing the retention time and co-injection with the TRP, IAM, IAA, IAN and TAM standards. The concentration of each indole compound was quantified using a standard curve.

Statistical analysis: The data were analyzed from three replicates and were subjected to One-way ANOVA for the statistical analysis of indole compounds. Duncan Multiple Range Test ($p = 0.05$) was used to compare the mean values through SPSS version 24.0.

RESULTS

Growth curve and IAA production based on differences in culture duration: Growth curves of UBCF_13 from LB medium and YM medium with and without the addition of tryptophan were shown in Fig. 1a. Bacterial growth curves on LB medium, YM medium and YM medium with the addition of tryptophan showed the logarithmic phase occurred at 3-9 hrs of incubation and stationary phase at culture durations above 9 hrs. The UBCF_13 growth curve on YM media with the addition of tryptophan has instability in cell density at the stationary phase compared to YM media.

The IAA production of UBCF_13 had been calculated every 3 hrs and presented in Fig. 1b. The highest IAA production was found at 9 hrs of culture duration ($104 \mu\text{g mL}^{-1}$). The IAA production started at the initial log phase at above 3 hrs of culture duration and reached the maximum production at 9 hrs. Afterwards, the IAA production showed no significant increment until 24 hrs. Culture duration at 9 hrs has reached the early stationary phase on the YM medium with tryptophan addition (Fig. 1a).

Identification and qRT-PCR of genes related to IAA biosynthesis in UBCF_13: The putative IAA biosynthesis pathway based on the UBCF_13 genome (Fig. 2 and Table 2) shows four possible tryptophan-dependent IAA biosynthesis pathways. The first pathway is IPyA in which the UBCF_13 genome has *ipdC* gene (QUY48270.1) that encodes indole pyruvate decarboxylase [EC:4.1.1.74]. However, two other genes related to the IPyA pathway that encode

Table 2: Identification of gene related to IAA biosynthesis in UBCF_13 genome

Putative IAA biosynthesis pathway	Gene (Product)	RefSeq from NCBI	Identity (%)	KEGG Orthology (KO) and entry numbers	Organism and Identity (Blast-Koala)
Indole-3-pyruvate (IPyA)	<i>tyrB</i> (aspartate/tyrosine/aromatic aminotransferase)	WP_013814736.1 [<i>Serratia</i>]	99.75	K00832 [EC:2.6.1.57]	<i>Serratia</i> sp. AS13 (100%)
	<i>ipdC</i> (indolepyruvate decarboxylase)	WP_212557842 [<i>Serratia plymuthica</i>]	100	K04103 [EC:4.1.1.74]	<i>Serratia inhibens</i> (96%)
	<i>puuC</i> (aldehyde dehydrogenase)	WP_212559473.1 [<i>Serratia plymuthica</i>]	100	K09472 [EC:1.2.1.99]	<i>Serratia inhibens</i> (97%)
Tryptamine (TAM)	<i>DDC</i> (aspartate aminotransferase family protein)	WP_212556539.1 [<i>Serratia plymuthica</i>]	100	K01593 [EC:4.1.1.28] [EC:4.1.1.105]	<i>Massilia umbonata</i> (34%)
Indole-3-acetaldoxime (IAOx)/ Indole-3-acetonitrile (IAN)	<i>nthA</i> (nitrile hydratase subunit alpha)	WP_212558035.1 [<i>Serratia plymuthica</i>]	100	K01721 [EC:4.2.1.84]	<i>Serratia plymuthica</i> S13 (98%)
	<i>nthB</i> (nitrile hydratase subunit beta)	WP_212558036.1 [<i>Serratia plymuthica</i>]	100	K20807 [EC:4.2.1.84]	<i>Serratia plymuthica</i> S13 (93%)
	<i>oxdA</i> (phenylacetaldoxime dehydratase)	WP_212558032.1 [<i>Serratia plymuthica</i>]	100	K13028 [EC:4.99.1.5]	<i>Serratia plymuthica</i> S13 (97%)
Indole-3-acetamide (IAM)	<i>amiE</i> (amidase)	WP_212558034.1 [<i>Serratia plymuthica</i>]	100	K01426 [EC:3.5.1.4]	<i>Serratia plymuthica</i> S13 (97%)

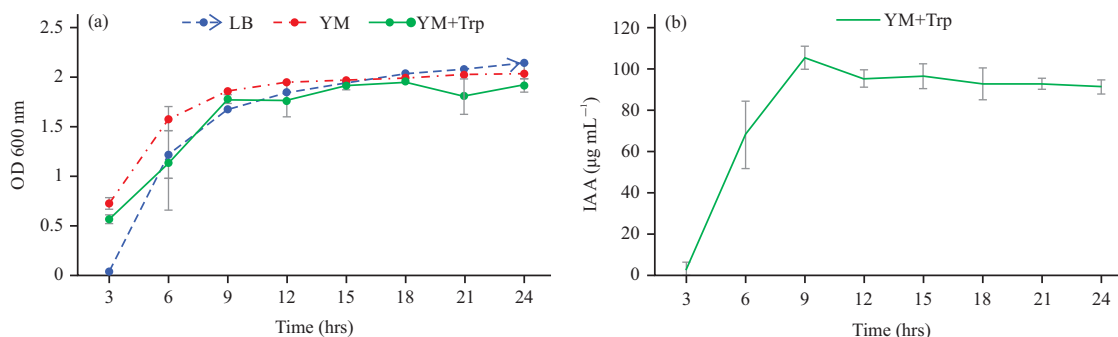


Fig. 1(a-b): Growth curves and IAA production by *Serratia plymuthica* UBCF_13, (a) Growth curve in LB medium, YM medium, and YM medium with the addition of $300 \mu\text{g mL}^{-1}$ tryptophan for 24 hrs and (b) IAA production based on differences in culture duration using YM media with the addition of $300 \mu\text{g mL}^{-1}$ tryptophan

Data were calculated as mean values ($n = 3$)

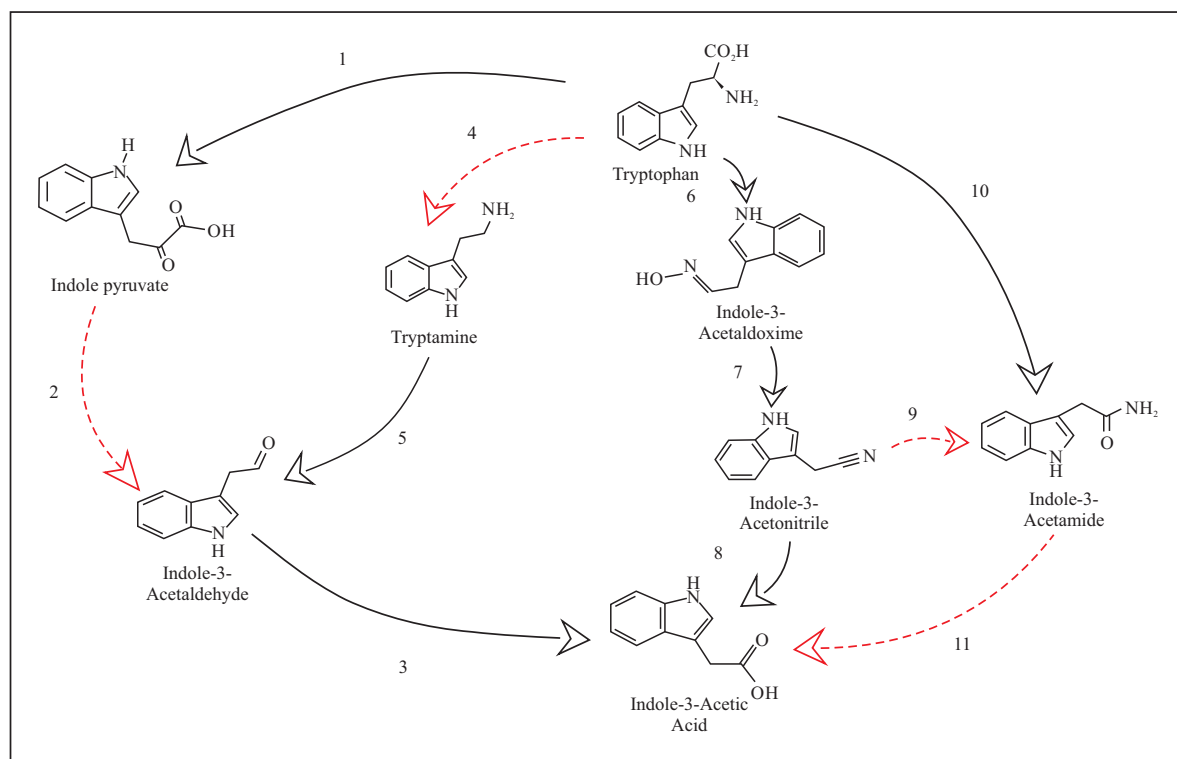


Fig. 2: IAA biosynthetic pathway based on KEGG site (No: ko00380)

Red broken lines show the genes found in the genome of *S. plymuthica* UBCF_13 (RefSeq: NZ_CP068771.1), 1: Aminotransferase, 2: Indole pyruvate decarboxylase, 3: Aldehyde dehydrogenase, 4: L-tryptophan decarboxylase, 5: Diamine oxidase, 6: Tryptophan N-monooxygenase, 7: Indole acetaldoxime/phenylacetaldoxime dehydratase, 8: Nitrilase, 9: Nitrile hydratase, 10: Tryptophan 2-monooxygenase and 11: Amidase

aminotransferase and aldehyde dehydrogenase were not obtained in the UBCF_13 genome. On the contrary, we found *tyrB* gene and *puuC* gene that respectively encode aromatic aminotransferase [EC:2.6.1.57] and aldehyde dehydrogenase [EC:1.2.1.99]. The product of these two genes might be involved in IAA biosynthesis via the IPyA pathway.

The second pathway is TAM which is supported by the existence of the *DDC* gene encoding aspartate aminotransferase family protein (blastx results) or aromatic-L-amino-acid/L-tryptophan decarboxylase [EC:4.1.1.284.1.1.105] (blastKoala result) but the gene encoding diamine oxidase [EC:1.4.3.22] was not found. In IAOx/IAN pathway, there are *nthA* and *nthB* genes encoding nitrile hydratase subunit alpha (QUY48501.1) and nitrile hydratase subunit beta (QUY48502.1). UBCF_13 also has *oxdA* that encodes phenylacetaldoxime dehydratase [EC:4.99.1.5] which has a similar role to indole acetaldoxime dehydratase [EC:4.8.1.3]. However, the UBCF_13 genome did not contain the genes that encode tryptophan N-monooxygenase [EC:1.14.14.156] and nitrilase [EC:3.5.5.1]. The last putative pathway is IAM

which is proved by the presence of *amiE* encoding amidase (QUY48500.1) but the *IAAM* gene encoding tryptophan 2-monooxygenase [EC:1.13.12.3] was not detected in UBCF_13.

qRT-PCR of genes related to IAA biosynthesis in UBCF_13 based on different culture duration:

The expression of eight putative genes involved in IAA biosynthesis by UBCF_13 was analyzed on YM media as control and YM media with the addition of tryptophan as treatment with culture durations of 3, 6, 9, 12 and 21 hrs (Fig. 3). In the 9 hrs of culture duration, the highest expression level of the genes related to IAA biosynthesis was only found in *tyrB* by 7 folds (Fig. 3a). The level expression of *ipdC* (Fig. 3b) showed low increment with no more than 2 folds in all culture duration. In the *puuC*, *DDC* and *oxdA* (Fig 3c-e), the level of gene expression increased at 3 hrs of culture duration by 7, 4 and 1.5 folds, respectively. Moreover, the expression of the *nthA* gene (Fig. 3f) showed an increase of 13 folds at 3 hrs of culture duration and 7 folds at 6 hrs of culture duration. The *nthB* gene expression (Fig. 3g) showed an increase in the treatment

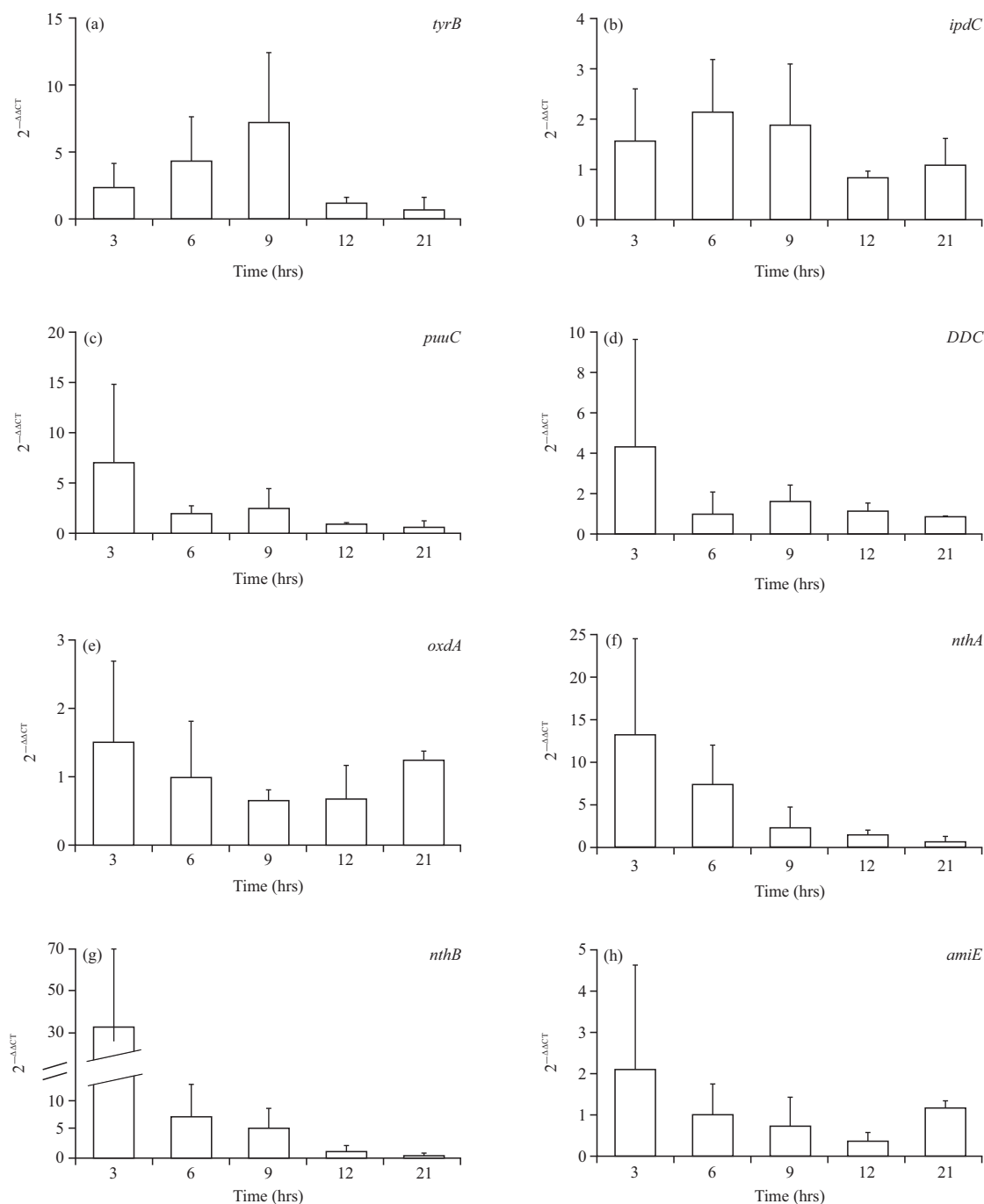


Fig.3(a-h): Analysis of gene expression involved in IAA biosynthesis of UBCF₁₃ based on different culture duration in YM medium with the addition of 300 μg mL⁻¹ tryptophan, (a) Relative level expression of *tyrB*, (b) Relative level expression of *ipdC*, (c) Relative level expression of *puuC*, (d) Relative level expression of *DDC*, (e) Relative level expression of *oxdA*, (f) Relative level expression of *nthA*, (g) Relative level expression of *nthB* and (h) Relative level expression of *amiE*. Error bars represent standard deviations from three biological replicates.

with the addition of tryptophan about 32 folds at 3 hrs of culture duration and 7 folds at 6 hrs of culture duration. The increase of gene expression in 3 hrs of culture duration was

also found in *amiE* by 2 folds (Fig. 3h). In general, almost all of the genes demonstrated the highest expression level at 3 hrs of culture duration.

Identification of indole compound by HPLC: The indole compounds related to the genes involved in the IAA biosynthesis of UBCF_13 were specifically analyzed using HPLC. The retention times of TRP, IAM, IAA, IAN and TAM were 5.713, 6.770, 7.784, 12.870 and 15.521 min, respectively. The concentration of TRP, IAM, IAA, IAN and TAM was $100 \mu\text{g mL}^{-1}$ (Fig. 4a). TRP and IAA were detected in the crude extract after 3, 6, 9, 12 and 21 hrs of incubation. However, IAM was not detected in 3 hrs of culture duration but was found in 6, 9, 12 and 21 hrs of incubation (Fig. 4b-f). The other indole compounds such as IAN and TAM were not identified from all crude extracts. Therefore, the possible IAA

biosynthesis pathway of UBCF_13 based on HPLC analysis was the IAM pathway.

As shown in Table 3, the concentration of TRP decreased from 3 hrs until 9 hrs of incubation but significantly increased from 9 hrs until 21 hrs of incubation. In the 6 hrs of incubation, IAM started to be synthesized and the amount of IAM significantly increased from 9-12 hrs of culture duration. The IAA showed an increase in concentration from 3 hrs until 21 hrs of incubation. According to HPLC analysis, the maximum IAA concentration was calculated at 21 hrs of culture duration and the concentration was 2.5 times higher than the IAA screened from the colourimetric assay (Fig. 1b).

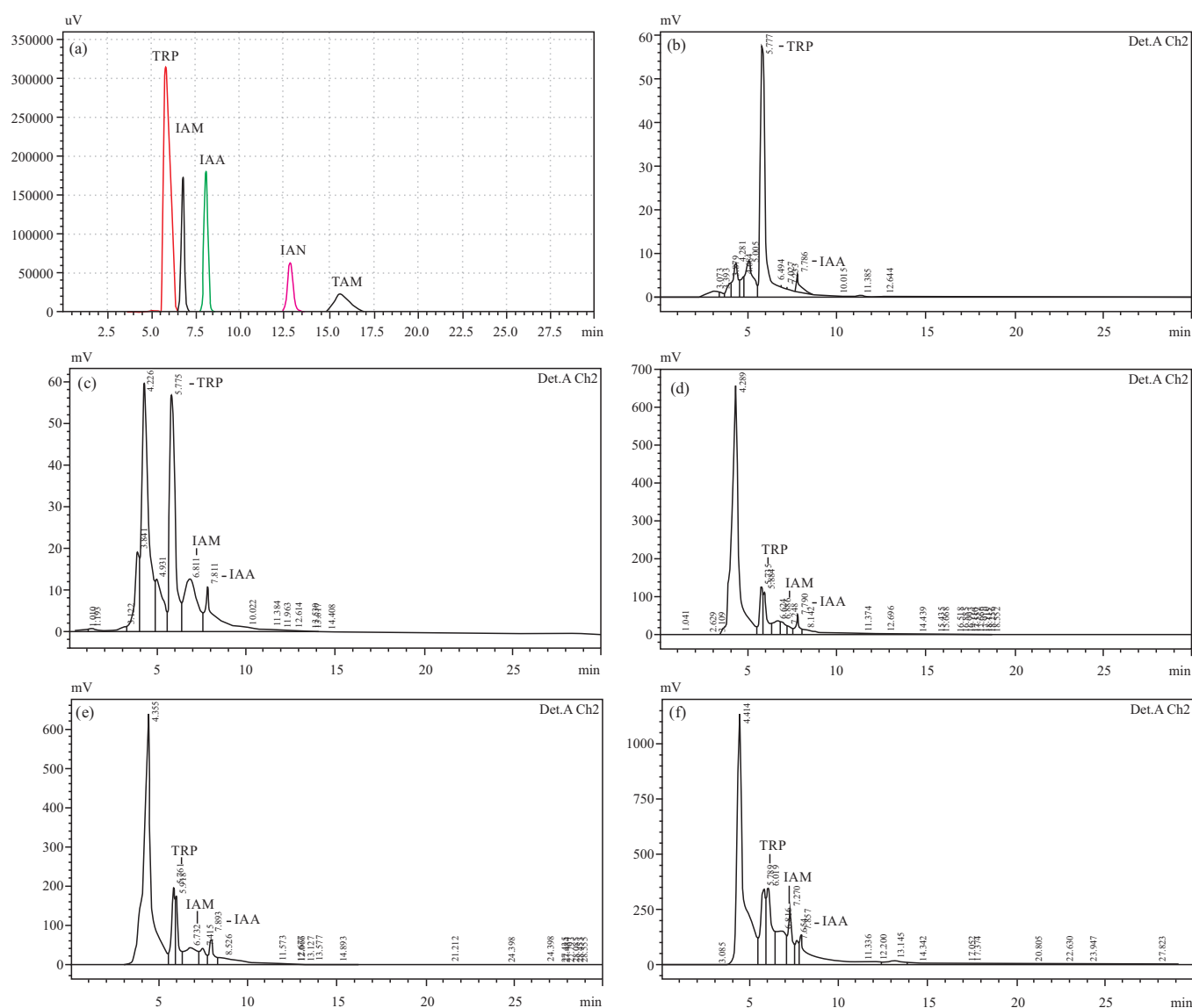


Fig. 4(a-f): HPLC analysis of indole compound produced by UBCF_13, (a) Indole compounds standard, (b) Crude extract after 3 hrs of incubation, (c) 6 hrs of incubation, (d) 9 hrs of incubation, (e) 12 hrs of incubation and (f) 21 hrs of incubation

Table 3: Indole compound concentration ($\mu\text{g mL}^{-1}$) from HPLC analysis based on differences in culture time

Compound	3 hrs	6 hrs	9 hrs	12 hrs	21 hrs
TRP	81.04 ^a	69.58 ^a	63.77 ^a	116.46 ^a	207.62 ^b
IAM	-	23.80 ^a	31.67 ^a	244.77 ^b	239.83 ^b
IAA	5.45 ^a	18.55 ^{ab}	47.35 ^{ab}	178.50 ^{bc}	241.92 ^c

Data were calculated as mean values ($n = 3$) and different lowercase letters indicate significant differences between treatments based on DMRT ($\alpha = 0.05\%$)

DISCUSSION

PGPB with IAA producing ability need specific time incubation to maximize the production. In this study, we used *Serratia plymuthica* UBCF_13 as IAA-producing-PGPB and generated the growth curve in LB and YM as the best media to produce IAA⁵. The stationary phase on the LB medium (Fig. 1a) was faster than Aisyah *et al.*⁵, at 16 hrs of culture duration. The growth curves on the YM medium with tryptophan showed unstable cell numbers after the initial stationary phase. Various effects of tryptophan on bacterial growth were found in some studies. The addition of tryptophan increased the growth of *Lysinibacillus fusiformis* UD270, *Neptunomonas* sp. BPy-1 and *Neptunomonas* sp. BZm-1 but inhibited the growth of *Enterobacter asburiae* STY10 and *Pseudomonas* Pt13^{17,24-26}.

The IAA production was also monitored every 3 hrs for 24 hrs on a YM medium containing tryptophan and showed that 9 hrs of culture duration was the best time to produce IAA (Fig. 1b). IAA is a secondary metabolite produced in the stationary phase in which the condition of the culture medium has lacked nutrients²⁷. A previous study showed the optimal duration of UBCF_13 to produce IAA was 48 hrs⁵. The differences in the media composition may affect the time at which bacteria enter the stationary phase, thereby affecting the optimal time for IAA production.

To identify the IAA biosynthesis pathway in UBCF_13, we found *tyrB*, *ipdC*, *puuC*, *DDC*, *oxdA*, *nthA*, *nthB* and *amiE* as the putative genes related to the IAA biosynthesis pathway (Fig. 2 and Table 2). The *tyrB*, *ipdC* and *puuC* are involved in the IAA biosynthesis pathway via the IPyA pathway while the *DDC* and *oxdA* are involved in TAM and IAOx/IAN pathways, respectively. The *nthA* and *nthB* are involved in the IAA biosynthesis pathway that connected the IAN pathway to the IAM pathway. The *amiE* was the gene required in the last step to produce IAA via IAM pathway²⁸. IAA biosynthesis pathway in *Serratia* species commonly used IPyA pathway. Based on genome mining, the IPyA pathway was found as the only pathway for IAA biosynthesis of *Serratia plymuthica* G3, *Serratia marcescens* UENF-22G1, *Serratia marcescens* S217 and *Serratia* sp. ZM^{9,11-13}.

To validate the expression of genes related to the IAA pathway, qRT-PCR analysis was conducted at different growth

phases consisting of 3 hrs as the lag phase, 6 hrs as the exponential phase, 9 hrs as the highest IAA production time (Fig. 1b), 12 and 21 hrs as the early to mid-stationary phase and late stationary phase, respectively (Fig. 1a). The result shows the expression of all genes increased maximum at 3 hrs of incubation except *tyrB* and *ipdC* (Fig. 4) which reinforces the hypothesis of the growth phase affects the expression of IAA biosynthesis-related genes. This result is also supported by data published by Smith *et al.*²⁹, who found that the upregulation for the expression of several genes related to the metabolism of *E. coli* can be influenced by the growth phase.

The highest expression level of the *tyrB* gene was at 9 hrs of culture duration (Fig. 3a). The aromatic amino acid transferase from *tyrB* and *aspC* were homologs hence the indole-3-pyruvic acid could be produced by aromatic amino acid aminotransferase from *tyrB* and *aspC*^{30,31}. The *puuC* showed the highest expression in 3 hrs of time culture (Fig. 3c). On the other hand, in *Arthrobacter pascens* ZZ21, the expression of *puuC* did not increase related to tryptophan addition and may not be involved in the IAA biosynthesis pathway¹⁵. Although *tyrB* and *puuC* showed higher level expression than *ipdC*, the expression of the *ipdC* is the most important in the IPyA pathway. The level expression of *ipdC* showed low upregulation (Fig. 3b) and we need to detect the indole-3-acetaldehyde in UBCF_13 culture to confirm if the IPyA pathway is being used in UBCF_13.

In the TAM pathway, the *DDC* encodes L-tryptophan decarboxylase which contributes in the first step to convert L-tryptophan to tryptamine based on the KEGG pathway (https://www.genome.jp/kegg-bin/show_pathway?ko00380). The *DDC* showed the maximum upregulation at 3 hrs of incubation (Fig. 3d). L-tryptophan decarboxylase gene expression in *Paenonia lactiflora* Pall. is also influenced by time which was upregulated maximum at 02.00 AM and downregulated at 05.00 PM³². Another IAA biosynthesis, IAOx/IAN pathway is frequently encoded by *oxdA* and nitrile hydratase genes in one operon³³. The *oxdA* gene has been found in some bacteria such as *Burkholderia cenocepacia* and various *Rhodococcus* species³⁴⁻³⁶. The *oxdA* showed a small upregulation at 3 hrs of incubation (Fig. 3e). However, the nitrile hydratase gene consisting of *nthA* and *nthB* presented the highest transcript levels among all genes and the best expression time found in 3 hrs of incubation (lag

phase) (Fig. 3f-g). The nitrile hydratase enzyme could convert indole-3-acetonitrile to indole-3-acetamide³⁷. A different result was found in *Pseudomonas* sp. UW4 transformant showed that the transcript level of phenylacetaldoxime dehydratase was higher than nitrile hydratase³⁸. In *Variovorax boronicumulans* CGMCC 4969, the study of genes encoding nitrile hydratase also showed no significant effect on expression levels at 6 and 12 hrs of culture duration¹⁶. The last gene that involves in the IAM pathway is *amiE* which showed a similar result to *oxdA* with a low increment of the level transcript at 3 hrs of incubation (Fig. 3h). The low transcript level of the amidase gene is also found in *Variovorax boronicumulans* CGMCC 4969 based on IAN and cobalt addition and *Arthrobacter pascens* ZZ21 based on tryptophan addition^{15,16}. In UBCF_13, the *nthA* and *nthB* are presumed to have the most important role in IAA biosynthesis.

The genomic and transcriptomic analysis data was further confirmed with a metabolomic assay to detect intermediate production related to the IAA biosynthesis pathway in UBCF_13. From all standards, only IAM has been found in the extract of UBCF_13 (Fig. 4). The IAM started to be produced at the log phase and reached the highest concentration at the mid-stationary phase. Meanwhile, IAA has been detected since the lag phase and the maximum production was found at the late stationary phase. The concentration of tryptophan also increased at the stationary phase (Table 3). According to HPLC analysis, the IAA concentration was 2.5 times higher than the IAA screened from the colourimetric assay (Fig. 1b). A similar result was also found in *Micrococcus aloeverae* DCB-20 which the IAA quantification by HPLC was 1.5 times higher than the colorimetric method³⁹. The concentration of tryptophan also increased at the stationary phase. It was indicated that UBCF_13 could synthesize tryptophan by itself and also have a tryptophan-independent pathway to produce IAA. Another study from *Enterobacter xiangfangensis* BWH6 showed a similar result with UBCF_13 which the concentration of tryptophan was decreased from 1-2 days and increased from 2-3 days¹⁷. The change of concentration metabolite related to IAA production through HPLC analysis has been studied from the extract of *Enterobacter* sp. Cbg70 and Zch 127 showed the concentration of tryptophan decreased from 2-4 days but the concentration of IAA increased significantly. The Cbg70 isolate also produced IAM and IPyA and Zch 127 also had IAM and IAN with a low improvement of concentration from 2-4 days⁴⁰. Further optimization for IAA production by UBCF_13 needs to be carried out by other factors such as carbon source, nitrogen source and inorganic salt to find the optimum medium culture composition.

CONCLUSION

The growth curve has been analyzed and maximum IAA production was found at 9 hrs of time culture (initial stationary phase) based on colorimetric assay and 21 hrs of time culture (late stationary phase) based on HPLC analysis. According to the genome mining data, the putative IAA biosynthesis pathway in UBCF_13 was IPyA, TAM and IAM pathways. Analysis of the level expression of *puuC*, *nthA*, *nthB*, *DDC*, *amiE* and *oxdA* genes showed the maximum increment at 3 hrs of culture duration which belongs to the lag phase. The high-level expression of both *nthA* and *nthB* genes and the high production of IAM detected by HPLC suggest that the IAM pathway may be used in the IAA biosynthesis of UBCF_13.

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