

Optimization of Medium Components and Genes Expression Involved in IAA Biosynthesis by *Serratia plymuthica* UBCF_13

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Abstract

Serratia plymuthica UBCF_13 produces the maximum level of indole-3-acetic acid (IAA) in yeast mannitol medium. However, the impact of medium ingredients on gene expression and metabolites related to IAA synthesis remains unclear. Therefore, Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) was used to assess the effects of culture medium optimization components on the genes involved in IAA production. High-Performance Liquid Chromatography (HPLC) has been employed to investigate the impact of medium constituents on the metabolites associated with the biosynthesis of IAA. The highest IAA production was found in sucrose and yeast extract as the carbon and nitrogen sources (134.6 µg/mL). It was also discovered that calcium carbonate and magnesium sulfate are significant inorganic salts for UBCF_13 in the production of IAA. Most genes showed higher levels of expression when sucrose was used as the carbon source, including *ipdC*, *nthA*, *puuC*, *amiE*, *oxdA*, *tyrB* and *nthB*. Furthermore, the expression of *tyrB*, *puuC*, *DDC* and *oxdA* was upregulated in response to calcium carbonate, while *puuC*, *nthA*, *nthB* and *amiE* expression levels were elevated in the presence of magnesium sulfate. Indole-3-acetamide (IAM) was identified with HPLC as an intermediate product in several optimized culture media, with the highest IAA concentration (429.79 µg/mL) which was observed in yeast sucrose medium. Thus, sucrose played a pivotal role in IAA biosynthesis, and yeast sucrose medium supplemented with complete inorganic salts emerged as the optimal medium for IAA production by UBCF_13.

Keywords: HPLC, Indole-3-acetic acid, Medium optimization, Plant growth promoting bacteria, Quantitative reverse transcription-PCR

Introduction

Plant growth-promoting bacteria (PGPB) constitute a group of bacteria that are capable to enhance plant growth through the production of phytohormones, siderophores and antifungal metabolites [1]. *Serratia plymuthica* is recognized as one of the PGPB species having symbiotic relationships with plants, functioning both as rhizosphere and phyllosphere bacteria [2-4]. Several mechanisms are employed by *S. plymuthica* to promote plant growth including the production of antifungal compounds [5-7], siderophore compounds [8-10], phosphate solubilization [11], antibacterial agents [12], osmoprotectants [13] and phytohormones.

IAA stands out as one of the most prevalent and extensively studied phytohormones synthesized by PGPB [1]. Numerous investigations have demonstrated that IAA synthesized by PGPB contributes to enhance plant resilience against stresses such as drought and salinity [14-18]. IAA produced by PGPB also increased the growth and metabolite production of aromatic and medicinal plants [19]. Notably, various strains of *S. plymuthica* possess the capacity for IAA production, including *S. plymuthica* AS13 [20], *S. plymuthica* G3 [5], *S. plymuthica* M24T3 [21], *S. plymuthica* DT8 [22], *S. plymuthica* EDC15 and *S. plymuthica* EEP5 [23].

S. plymuthica UBCF_13 is one of the IAA-producing strains which augments the growth of various Solanaceae plants [24]. In the previous studies, numerous factors aimed at optimizing IAA synthesis by UBCF_13 have been explored. It was determined that YM medium supplemented with 300 µg/mL tryptophan and incubated for 9 h (for colorimetric assay) and 21 h (for HPLC assay) provided the optimal culture condition [25,26]. However, further investigation into medium components is warranted, as nutrients play a crucial role in influencing secondary metabolite production by microorganisms [27,28]. The IAA production by plant growth promoting rhizobacteria (PGPR) from *Stevia rebaudiana* is significantly influenced by the carbon and nitrogen sources [29]. In another study, it was found that maltose and a combination of yeast extract and tryptone increased the IAA production of *Pseudarthrobacter* sp. NIBRBAC000502770 [30]. Inorganic salt, such as sodium chloride, has a positive effect on enhancing the IAA production by *Rhizobium* sp. and *Streptomyces fradiae* NKZ-259 [31,32].

Although studies regarding the impact of the medium components such as carbon, nitrogen and mineral sources on IAA production have been extensively analyzed, the impact of these components on the expression of the genes involved in IAA production remains unexplored. Culture medium components have been shown to regulate the production of metabolites in certain strains, thereby influencing gene expression in metabolite production [33-35]. Tryptophan has commonly been utilized in culture media to analyze the expression of genes involved in IAA synthesis [30,36-38]. The IAA biosynthesis pathway in bacteria involves both tryptophan-dependent and tryptophan-independent pathways. In general, microorganisms produce IAA through a tryptophan-dependent pathway consisting of indole-3-pyruvic acid (IPyA), tryptamine (TAM), indole-3-acetaldoxime (IAOx)/indole-3-acetonitrile (IAN) and IAM [39]. The expression of IAA biosynthesis genes in UBCF_13 has been analyzed by adding tryptophan at different incubation times. The *nthA* and *nthB* genes, associated with the IAN-IAM pathway, exhibited the highest upregulation in the medium with the addition of tryptophan after 3 h of incubation [26]. However, the impact of optimized medium components on IAA biosynthesis genes has not been studied. Thus, this study aimed to optimize the carbon, nitrogen and inorganic salt sources in the culture medium and reveal the effect of optimized medium components on the expression of IAA biosynthesis genes in UBCF_13.

Materials and methods

Bacterial strain and culture conditions

Serratia plymuthica UBCF_13 (Universitas Andalas - Biotechnology Laboratory Collection accession no: KX394779) was pre-cultured on LB agar (HiMedia, India) for 24 h at 28 °C [40]. The single colony from the previous culture was then inoculated into LB broth and incubated at 160 rpm for 16 h at 28 °C.

Optimization of medium components for IAA production

Based on our previous study [25], the optimized culture medium was yeast mannitol (YM) broth consisting of 10 g/L mannitol, 0.1 g/L sodium chloride, 0.5 g/L dipotassium phosphate, 0.2 g/L magnesium sulphate, 1 g/L yeast extract and 1 g/L calcium carbonate and added with 300 µg/mL tryptophan. In this study, we used a 1 factor at 1 time method for the optimization of IAA production. Optimization was analyzed based on different carbon, nitrogen and sole inorganic salt sources. Carbon source optimization included YM medium devoid of mannitol (no carbon), YM medium with mannitol (mannitol) and substitution of mannitol with diverse sugar sources (sucrose, maltose, glucose and starch). Nitrogen source optimization entailed an optimized medium devoid of yeast extract (no nitrogen) and the replacement of yeast extract with different nitrogen sources (urea, peptone, tryptone, ammonium sulphate and potassium nitrate). The effect of sole inorganic salt from the optimized medium on IAA production was evaluated using a medium devoid of inorganic salt (no inorganic salt) and a medium containing each inorganic salt compound (dipotassium phosphate, magnesium sulphate, sodium chloride and calcium carbonate). Each experiment was conducted using a completely randomized design (CRD) with 3 biological replicates.

The pre-inoculated bacteria were added 1 mL to each treatment of optimization medium culture and incubated for 9 h at 160 rpm, 28 °C. The bacterial growth analysis was conducted by measuring the optical density using a UV spectrophotometer (V1100D, Mapada Instruments, China) at 600 nm. The colorimetric assay was used to measure the IAA production from each experiment [41]. The bacterial culture was centrifuged at 10,000 rpm at 4 °C for 10 min. The supernatant was then added with Salkowski reagent (35 % H₂SO₄ and 1 % 0.5 M FeCl₃) and incubated at 28 °C for 30 min in the dark. The absorbance of IAA from each treatment was measured with a UV spectrophotometer (V1100D, Mapada Instruments, China) at 530 nm. The IAA standard from 5 to 100 mg/mL was used to plot the standard curve for the IAA concentration quantification of each sample.

The effect of medium components optimization on gene expression related to IAA biosynthesis

Quantitative real-time PCR (qRT-PCR) was applied to evaluate the effect of medium component optimization on gene expression. The SV Total RNA isolation system kit (Promega, USA) was used to extract the total RNA and the ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (Toyobo, Japan) was used to create the cDNA. The THUNDERBIRD™ SYBR™ qPCR Mix (Toyobo, Japan) was used to run the qRT-PCR on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). The cycling condition and primers used in this study refer to Yusfi *et al.* [26]. This experiment was conducted in a triplicate. The transcript level data was evaluated using the $2^{-\Delta\Delta CT}$ method [42].

Detection of indole compound by HPLC analysis

The samples were prepared based on the significant treatments from the previous optimization process. The UBCF_13 was grown in yeast extract - sucrose with complete inorganic salt (sucrose), sucrose with complete inorganic salt (no nitrogen), yeast sucrose with magnesium sulphate and yeast sucrose with calcium carbonate for 21 h. Crude extract preparation was done according to Yusfi *et al.* [26]. The sample was centrifuged at 10,000 rpm for 10 min, 4 °C then the supernatant was acidified to pH 2.7 with HCl (1N). The samples were extracted twice with 2 volumes of ethyl acetate. A rotary evaporator (Heidolph, Germany) was used to evaporate the fraction of ethyl acetate for 15 min at 40 °C, 150 rpm and 240 mbar vacuum pressure. Methanol was used to dissolve the extract. The extract was evaluated by high performance liquid chromatography (HPLC; Shimadzu 10A-Tvp HPLC system, Japan) equipped with Shim-pack MRC-ODS RP - 18 column (Shimadzu, Tokyo, Japan). Acetonitrile: Water: Acetic acid (40:60:0.01 %) was used as the mobile phase. The total run time was 30 min at a flow rate of 1.0 mL/min [43]. The indole compounds were identified at the wavelength of 280 nm. Verification and quantification were analyzed by comparing the retention time and the standard curve of indole standards from Yusfi *et al.* [26].

Statistical analysis

The data were analyzed using analysis of variance (ANOVA) and the Duncan Multiple Range Test (significance = 0.05) was used for mean separation through SPSS version 24.0 software. GraphPad Prism 9 was used to construct each graph.

Results and discussion

Optimization of medium components for IAA production

In the previous studies, the production of IAA by UBCF_13 has been optimized through various factors, including tryptophan concentration, pH, medium type and incubation time [24-26]. In this study, the optimization of IAA production was conducted by carbon, nitrogen and sole inorganic salt sources in the YM medium. A correlation between bacterial density and IAA production was observed in the optimization of several carbon sources, namely mannitol, sucrose, glucose and maltose (**Figure 1**). A positive correlation was also found between the optimal carbon source for bacterial growth and IAA production in *Pseudarthrobacter* sp. NIBRBAC000502770 [30]. Uniquely, the IAA production from glucose was higher than maltose and starch where both of them were di- and polysaccharides composed of glucose [44,45]. Chandra *et al.* [29] observed that IAA production from monosaccharides was higher than polysaccharides and disaccharides. However, in UBCF_13, the highest IAA concentration was found in sucrose which was grouped as a disaccharide consisting of glucose and fructose. The concentration of IAA in sucrose (134.6 µg/mL) was increased by 11.26 µg/mL compared to mannitol as the carbon source of basal medium, YM (**Figure 1**). Sucrose has also been identified as the optimal carbon source for IAA production in various microorganisms, including *Rhodospiridium paludigenum* DMKU-RP301, *Klebsiella pneumoniae* OM-17.2, *Providencia* sp. (7MM11), *Enterobacter cloacae* MG00145, *Bacillus megaterium* MQ-2.5, *Pseudomonas bereopolis* CP-18.2 and *Paenibacillus cineris* TP-1.4 [46-49]. Most PGPB produced the highest IAA in the media with mono- and disaccharide carbon sources. PGPB, which produces higher IAA in disaccharide carbon sources, may be influenced by the number of membrane transport systems for sugar uptake and a more complex enzymatic system for carbohydrate degradation [50,51]. These findings suggest that the optimal type of carbon source for IAA production varies among bacteria based on their specific enzymatic systems for carbohydrate degradation [51].

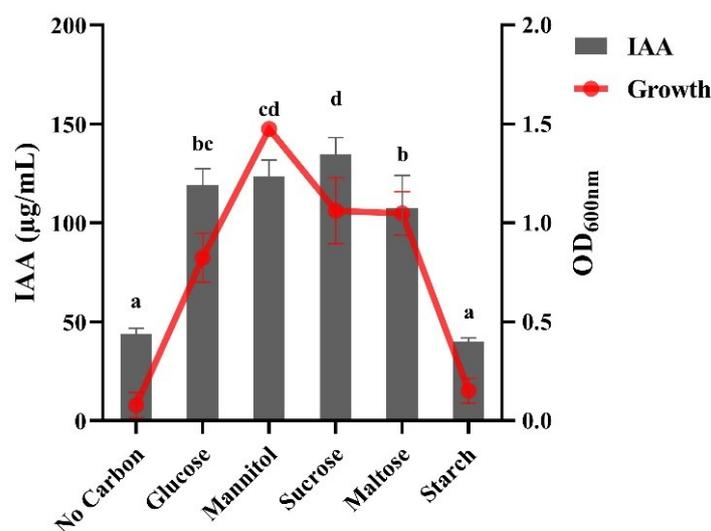


Figure 1 The effect of carbon source on bacterial growth and IAA production by UBCF_13. Each culture was added 300 µg/mL tryptophan. Values were means of 3 replicates ± SD. Lowercase letters represent significant differences among treatments by DMRT (alpha = 0.05 %).

Furthermore, the IAA production from different nitrogen sources did not correlate with the cell growth of UBCF_13 (**Figure 2**), consistent with the findings of Ham *et al.* [30]. Several nitrogen sources that have been applied in this study were categorized as organic (peptone and tryptone) and inorganic nitrogen (urea, potassium nitrate and ammonium nitrate). The IAA yield of urea and ammonium sulphate significantly decreased, which referred to the presence of the ammonium compound. Ammonia, as the nitrogen source in the culture medium, regulates and inhibits the production of several secondary metabolites [52]. **Figure 2** illustrates that bacterial growth was inhibited in the medium without nitrogen. Interestingly, although the IAA production in this medium remained high, the IAA concentration did not surpass it (**Figure 1**). Yeast extract, an organic nitrogen source containing vitamins and growth factors, promotes bacterial growth and enhances tryptophan availability for IAA synthesis [53]. In some similar studies, the yeast extract as the optimal nitrogen source for IAA production in *Bacillus* isolates from *Vigna radiata*, *Enterobacter* sp. DMKU-RP206 and *Pseudomonas fluorescens* E9 was identified [53-55].

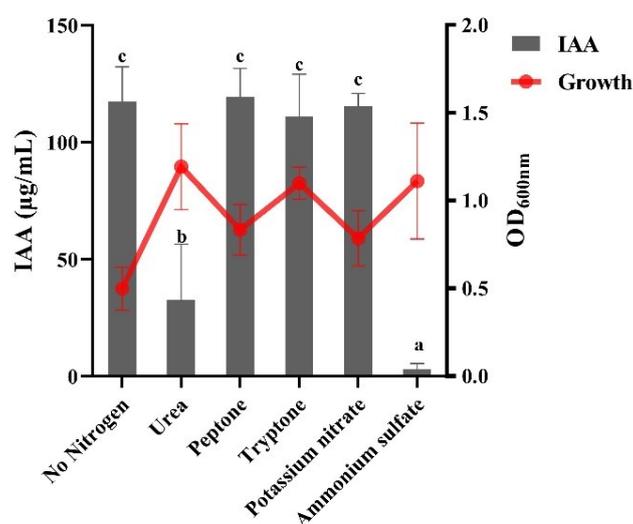


Figure 2 The effect of nitrogen source on bacterial growth and IAA production by UBCF_13. Each culture was added 300 µg/mL tryptophan. Values were means of 3 replicates ± SD. Lowercase letters represent significant differences among treatments by DMRT (alpha = 0.05 %).

The growth of UBCF_13 increased upon the addition of magnesium sulphate and calcium carbonate as the sole inorganic salt in the medium culture (**Figure 3**). Conversely, bacterial growth decreased in media lacking inorganic salt, containing dipotassium phosphate, or comprising sodium carbonate, leading to undetectable levels of IAA production. Significantly, IAA production was markedly elevated in the medium supplemented with magnesium sulphate (80 $\mu\text{g}/\text{mL}$) and calcium carbonate (43 $\mu\text{g}/\text{mL}$). Both inorganic salts are crucial for bacterial development and thus enhance biomass production [56,57]. The effect of sole inorganic salt has been previously studied on yeast mannitol medium, revealing IAA production only in the presence of magnesium sulphate [58]. Therefore, the increase of IAA amount in calcium carbonate addition was predicted due to the interaction between calcium carbonate and sucrose. In this study, the addition of sodium chloride did not influence the IAA production in UBCF_13. On the contrary, in the other studies, it was found that sodium chloride was an essential inorganic salt to induce the IAA production of *Enterobacter* sp. DMKU-RP206, *Rhizobium* sp., *Pseudarthrobacter* sp. NIBRBAC000502770, halotolerant bacteria, *Bacillus circulans* E9 [30,32,53,59,60].

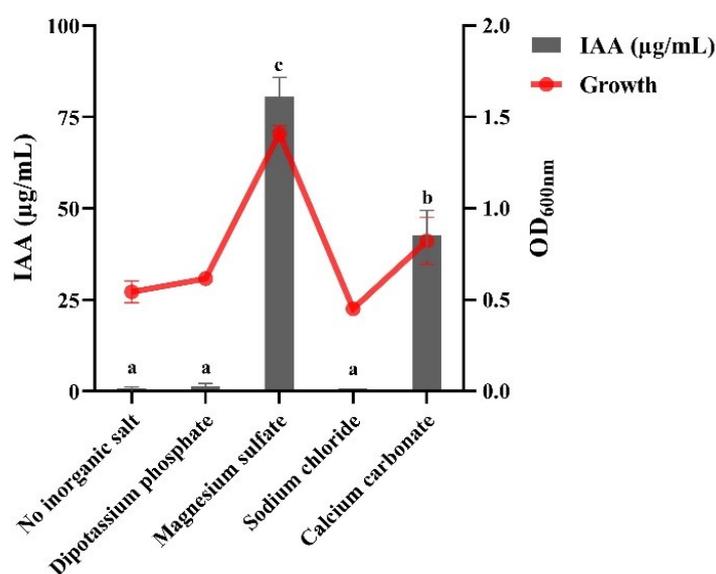


Figure 3 The effect of sole inorganic salt source on bacterial growth and IAA production by UBCF_13. Each culture was added 300 $\mu\text{g}/\text{mL}$ tryptophan. Values were means of 3 replicates \pm SD. Lowercase letters represent significant differences among treatments by DMRT ($\alpha = 0.05$ %).

Overall, the highest IAA production (134.6 $\mu\text{g}/\text{mL}$) by UBCF_13 was found in yeast sucrose medium consisting of 10 g/L sucrose, 0.1 g/L sodium chloride, 0.5 g/L dipotassium phosphate, 0.2 g/L magnesium sulphate, 1 g/L yeast extract and 1 g/L calcium carbonate with 300 $\mu\text{g}/\text{mL}$ tryptophan in 9 h incubation. IAA production in the range of approximately 100 $\mu\text{g}/\text{mL}$ has also been reported in several studies employing different media compositions. Bhutani *et al.* [54] and Lebrazi *et al.* [32] found that *Bacillus aryabhatai* MBN3 and *Rhizobium* sp. produced IAA maximum at 92.03 $\mu\text{g}/\text{mL}$ and 116.42 $\mu\text{g}/\text{mL}$, respectively in yeast mannitol broth. *Streptomyces plicatus* PT2 achieved a maximum IAA production of 110.29 $\mu\text{g}/\text{mL}$ in a basic mineral medium supplemented with 4 mg/L L-tryptophan and tomato root extract [61]. Additionally, Chandra *et al.* [29] observed the highest IAA production (104 $\mu\text{g}/\text{mL}$) by isolate CA1001 in nutrient broth with dextrose as the carbon source. *Pseudomonas boreopolis* CP-18.2 and *Klebsiella pneumoniae* OM-17.2 also produce IAA at approximately 120 $\mu\text{g}/\text{mL}$ in Burk's medium with 1 % NaCl and sucrose as the carbon source [49]. The utilization of sucrose as the carbon source for IAA production was also applied in *Providencia* sp. and *Enterobacter cloacae* MG00145. *Providencia* sp. achieved the highest IAA production (89.22 $\mu\text{g}/\text{mL}$) in medium containing sucrose, urea and tryptophan (0.1 %) after 24 h of incubation [48]. Meanwhile, *Enterobacter cloacae* MG00145 maximally produced IAA (30 $\mu\text{g}/\text{mL}$) in nutrient broth supplemented with 0.5 % sucrose and 0.1 % calcium nitrate [47]. Besides sucrose, the best carbon source in IAA production media varies in various PGPB strains. *Pseudarthrobacter* sp. NIBRBAC000502770 produced IAA (24.73 $\mu\text{g}/\text{mL}$) in medium consisting of yeast extract, NaCl, tryptone, L-tryptophan and maltose as the carbon source [30]. Mannose and mannitol as the carbon source

with ammonium chloride, potassium nitrate and tryptophan as the medium composition for *Bacillus velezensis* to produce IAA (56.60 µg/mL) [62]. Pea flour medium (PYM) and alginate medium have also been applied as the medium culture for IAA production by *Bacillus circulans* E9 (7.81 µg/mL) and *Vibrio* sp. 9.32 µg/mL, respectively [60,63].

The effect of medium components optimization on gene expression related to IAA biosynthesis

The effect of carbon source on the expression of IAA synthesis genes has been investigated (**Figure 4**). The relative expression of *tyrB* slightly increased by 1.5 folds in sucrose (**Figure 4(A)**). The expression levels of *ipdC* and *puuC* were the highest under the effect of sucrose (**Figures 4(B)** and **4(C)**). However, in *DDC*, the maximum transcript level was found in mannitol by 9 and 3.2 folds higher than no carbon (**Figure 4(D)**). The expressions of *oxdA*, *nthA*, *nthB* and *amiE* were significantly increased in sucrose which was 2.3 folds, 5.4 folds, 4.7 folds and 11.6 folds higher than no carbon, respectively (**Figures 4(E)** and **4(H)**). It was observed that almost all gene expressions were elevated when sucrose was utilized as the carbon source. In glucose, the upregulation was found in *oxdA*, *nthA* and *nthB*, while in maltose and starch, the relative expression increases were not observed across all genes.

Moreover, based on the effect of the nitrogen source, it can be observed that the expression of *tyrB* was down-regulated in urea and was not upregulated significantly in other nitrogen sources (**Figure 5(A)**). The expression of *ipdC* was downregulated considerably in urea, peptone, potassium nitrate and ammonium sulphate (**Figure 5(B)**). A similar result with *tyrB* was also found in *puuC*, where the expression level was not upregulated significantly among all treatments (**Figure 5(C)**). The expression of *DDC* was elevated in potassium nitrate by 2.2 folds compared to no nitrogen (**Figure 5(D)**). None of the nitrogen sources significantly influenced the upregulation of *oxdA* expression level (**Figure 5(E)**). The expression of *nthA* was significantly upregulated in peptone with 14.3 folds higher than no nitrogen (**Figure 5(F)**). In *nthB*, the expression level only increased in potassium nitrate by 2.9 folds higher than no nitrogen (**Figure 5(G)**). The maximum increment of *amiE* expression level was found in urea by 3.7 folds compared to no nitrogen (**Figure 5(H)**). In ammonium sulphate, almost all of the genes were not upregulated significantly, corresponding to the low concentration of IAA production in this nitrogen source (**Figure 2**).

Based on the optimization assay, the inorganic salt also influenced the production of IAA in UBCF_13 thus we observed its effect on the expression of IAA synthesis genes. The expression of *tyrB* was weakly upregulated in calcium carbonate by 1.4 folds (**Figure 6(A)**). However, the addition of each inorganic salt decreased the expression level of *ipdC* (**Figure 6(B)**). The addition of sole inorganic salt also showed a mild effect to influence the expression of *nthB*, *oxdA*, *puuC*, *nthA* and *DDC*. The expression of *puuC* was increased in magnesium sulphate and calcium carbonate addition by 1.5 folds. The maximum expression levels of *DDC* and *oxdA* were observed with calcium carbonate addition. In both nitrile hydratase genes, *nthA* and *nthB*, the expression levels were elevated with the addition of magnesium sulphate (**Figures 6(C)** and **6(G)**). Interestingly, the addition of magnesium sulphate also increased the expression level of *amiE* significantly by 4.8 folds compared to no inorganic salt (**Figure 6(H)**). Therefore, the addition of magnesium sulphate influenced the expression level of genes involved in IAM pathway.

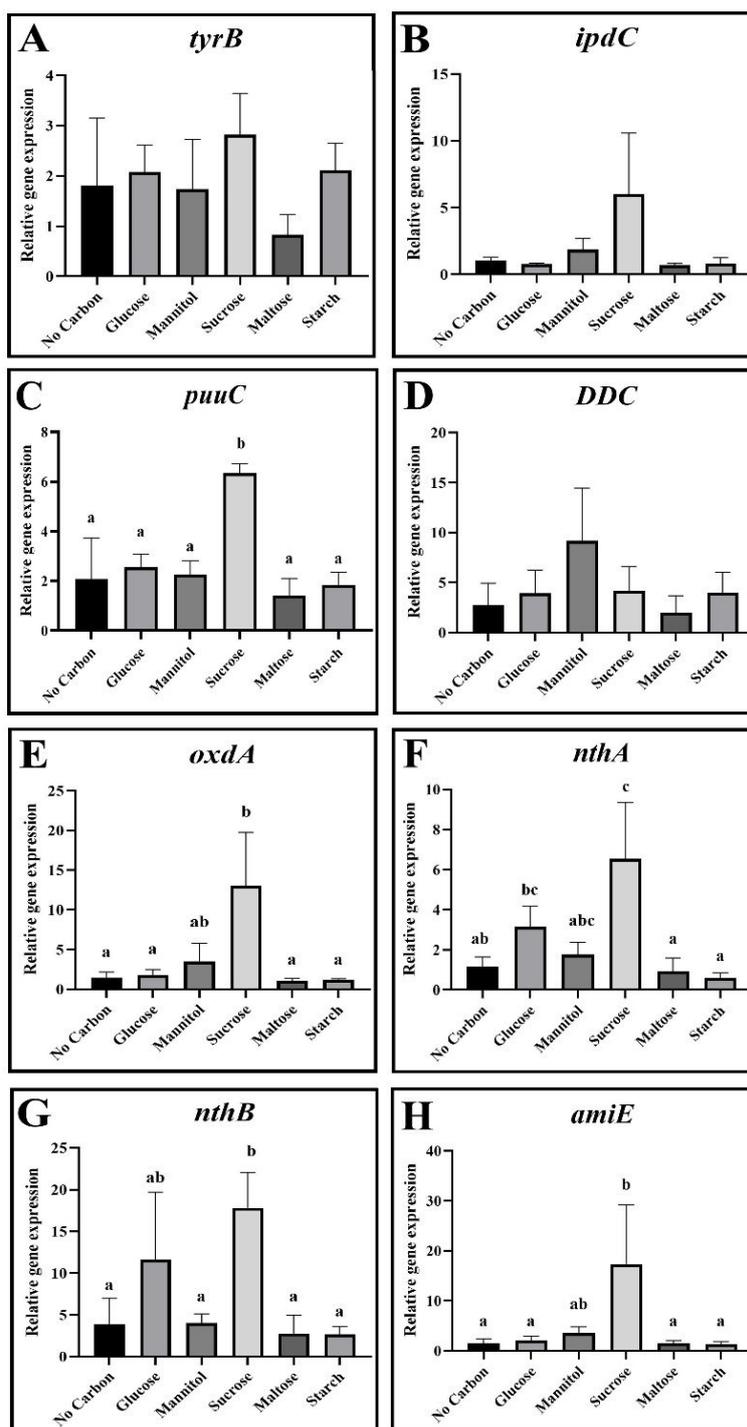


Figure 4 The effect of carbon source on the relative expression of IAA synthesis genes in UBCF_13. (A) The expression level of *tyrB*; (B) The expression level of *ipdC*; (C) The expression level of *puuC*; (D) The expression level of *DDC*; (E) The expression level of *oxdA*; (F) The expression level of *nthA*; (G) The expression level of *nthB*; (H) The expression level of *amiE*. Values were means of 3 replicates \pm SD. Lowercase letters represent significant differences among treatments by DMRT ($\alpha = 0.05$ %).

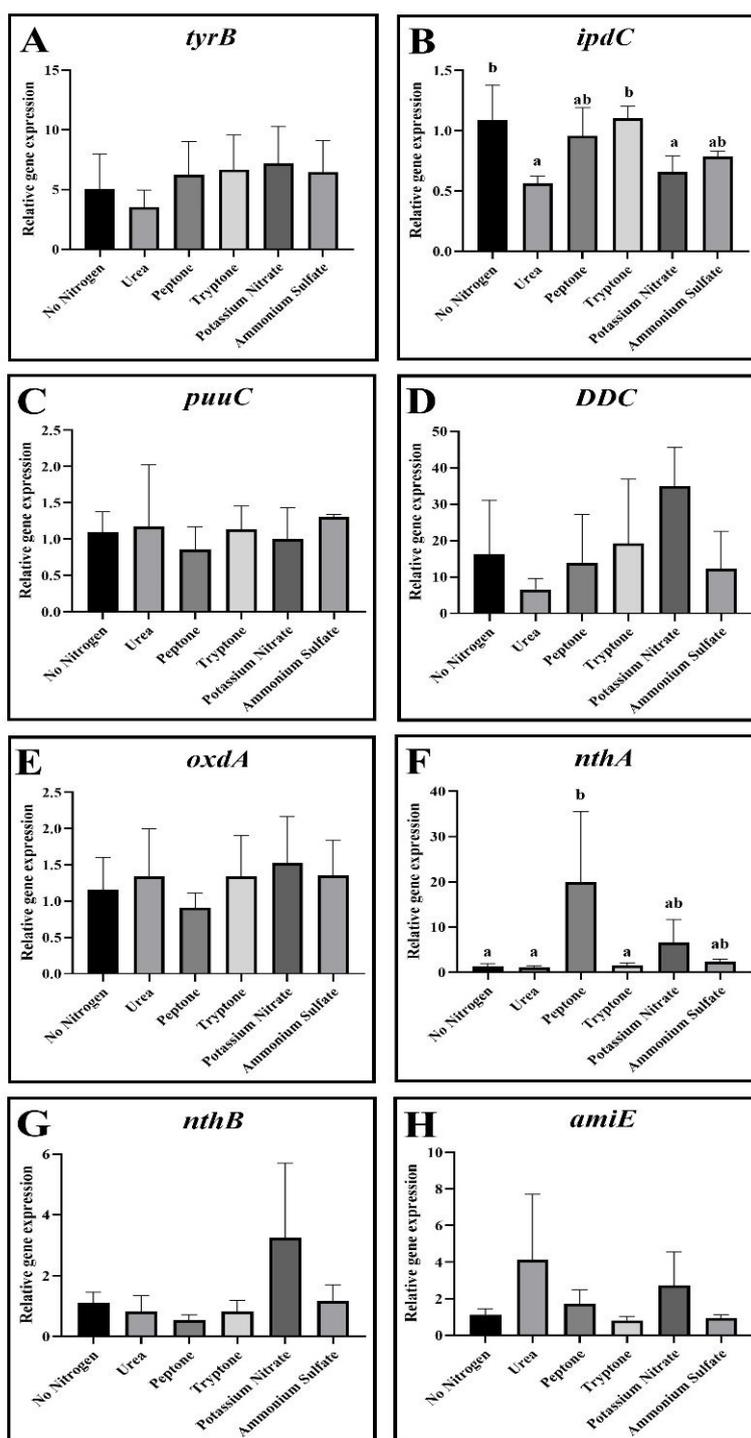


Figure 5 qRT-PCR analysis of the relative expression of IAA synthesis genes in different nitrogen sources. (A) *tyrB*; (B) *ipdC*; (C) *puuC*; (D) *DDC*; (E) *oxdA*; (F) *nthA*; (G) *nthB* and (H) *amiE*. Values were means of 3 replicates \pm SD. Lowercase letters represent significant differences among treatments by DMRT ($\alpha = 0.05$ %).

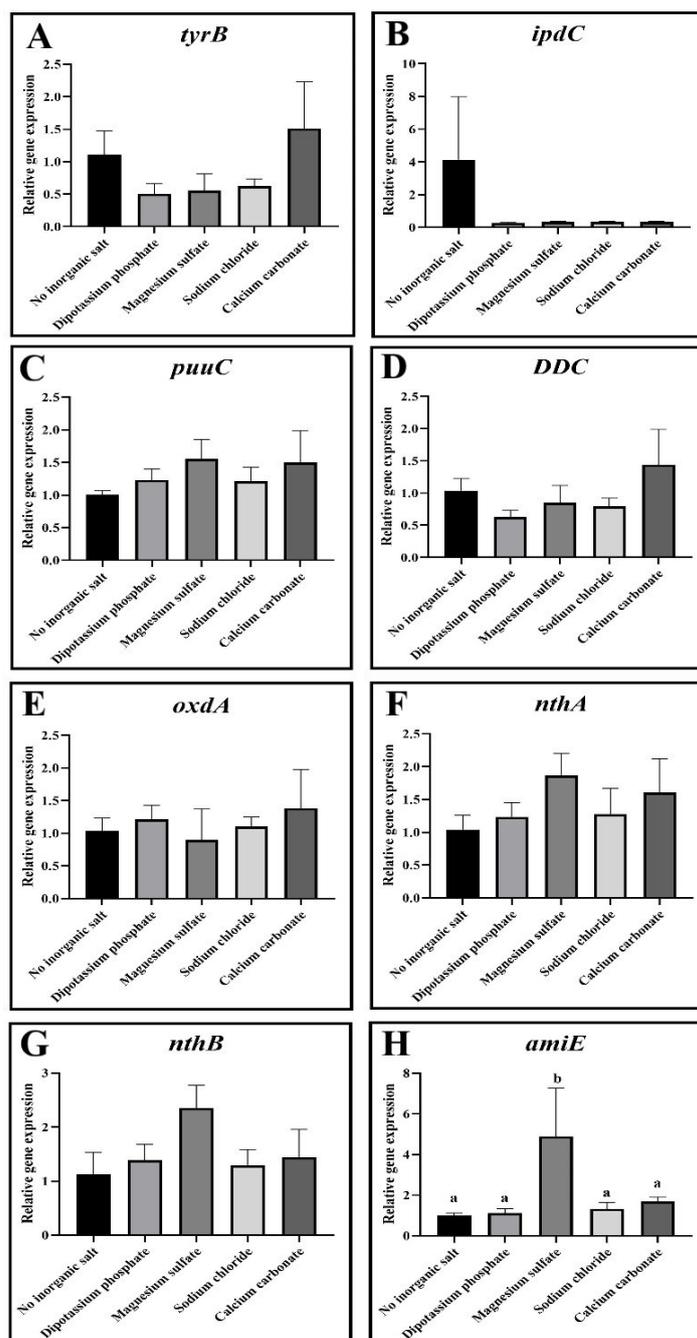


Figure 6 The relative expression of IAA biosynthesis genes in various sole inorganic salt sources. Values were means of 3 replicates \pm SD. Lowercase letters represent significant differences among treatments by DMRT ($\alpha = 0.05$ %).

Since each compound on medium optimization impacted the production of IAA by UBCF_13, using qRT-PCR, we evaluated the expression of the IAA-producing genes. Several studies found that the medium component affected the expression of genes associated with bacterial metabolite production. For instance, various carbon sources influenced the expression of lactate synthesis genes in *Streptococcus bovis* S1 [64,65]. Nitrogen source also impacted the expression of [FeFe]-hydrogenases genes regarded to hydrogen production of *Clostridium butyricum* CWBI1009 [66]. Moreover, the expression of rhamnolipid production genes has been influenced by various combinations of carbon and nitrogen sources [35]. The optimized medium culture also positively correlated with the expression of riboflavin synthesis genes in *Lactobacillus plantarum* CRL 725 and the expression of WL gum synthesis genes in *Sphingomonas* sp. WG [67,68].

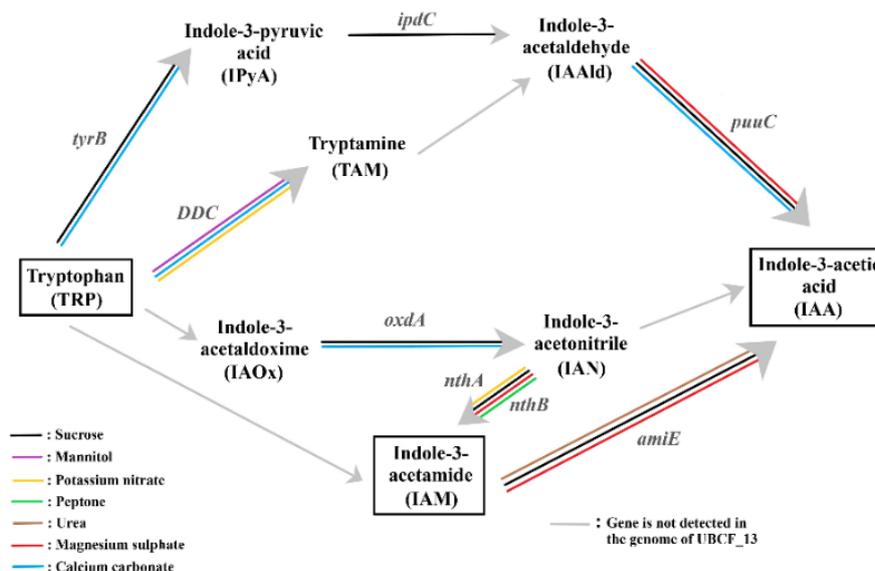


Figure 7 The effect of medium optimization component on the relative expression of IAA synthesis genes in UBCF_13. Each color line represents the upregulation of gene expression from each medium component. The box line shows metabolite detected in optimized medium culture.

An overview of each compound that most influenced the expression of the genes involved in IAA production in UBCF_13 is described in **Figure 7**. The genes related to the indole-3-pyruvate (IPyA) pathway consist of *tyrB*, *ipdC* and *puuC*. The expression of *tyrB* was increased by sucrose and calcium carbonate. The *tyrB* gene encodes aromatic aminotransferase, which converts TRP to IPyA [69]. Additionally, environmental factors such as low oxygen levels have been reported to increase *tyrB* expression [70]. The expression of *ipdC* was influenced only by sucrose (**Figure 7**). *ipdC* is the critical gene in the IPyA pathway that encodes indole-3-pyruvate decarboxylase. The expression of this gene could be influenced by environmental factors such as biogas-supplemented culture [71]. In *puuC*, the expression was increased by sucrose, calcium carbonate and magnesium sulphate (**Figure 7**). This gene encodes aldehyde dehydrogenase which converts IPyA to IAA [37]. Li *et al.* [72] reported that the addition of *Caenorhabditis elegans* extracts to the medium culture of *Arthrobacter pascens* ZZ21 increased the expression of *puuC* and other aldehyde dehydrogenase genes. In the TAM pathway, the expression of *DDC* was upregulated by mannitol, potassium nitrate and calcium carbonate (**Figure 7**). *DDC* or *TDC* encodes L-tryptophan decarboxylase, responsible for converting TRP to TAMTRY [73]. Tullio *et al.* [74] reported that the addition of apigenin in the medium culture of *Rhizobium tropici* CIAT 899 increased the expression level of the *TDC* gene. The expression of *oxdA*, involved in the indoleacetaldoxime (IAOx) pathway, was influenced by sucrose and the addition of calcium carbonate (**Figure 7**). A study by Buezo *et al.* [75] revealed that various nitrogen sources, especially in ammonium addition gave the highest level expression of the *oxd* gene. On the other hand, in this study, different nitrogen sources did not significantly upregulate *oxdA* expression in UBCF_13 (**Figure 5**). The *oxd* gene encodes indoleacetaldoxime dehydratase which converts IAOx to IAN [76]. The expression of *nthA* and *nthB* was upregulated maximum in sucrose and magnesium sulphate whereas, on nitrogen source, *nthA* and *nthB* were upregulated maximally in peptone and potassium nitrate, respectively (**Figures 4 - 6**). Liu *et al.* [77] also observed *nthA* upregulation with the addition of soybean root exudates in the *Bradyrhizobium diazoefficiens* culture medium. The genes *nthA* and *nthB* encode nitrile hydratase, crucial in converting IAN into IAM and connecting the IAN pathway to the IAM pathway [78]. *amiE*, the last gene associated with the IAM pathway, exhibited increased expression levels in sucrose, urea and magnesium sulfate (**Figure 7**). *amiE* encodes aliphatic amidase that converts IAM to IAA [79]. The expression of *amiE* regarding the effect of the medium component was still unknown. However, the addition of tryptophan in medium culture upregulated the *amiE* expression in *Pseudarthrobacter* sp. NIBRBAC000502770 and *Serratia plymuthica* UBCF_13 [26,30].

Several components optimized in the medium have been shown to upregulate gene expression levels in the IAM pathway, indicating that UBCF_13 may employ this pathway for IAA production. Based on genome mining, the IPyA pathway was found to be the only pathway for IAA biosynthesis in *Serratia plymuthica* G3, *Serratia marcescens* UENF-22GI, *Serratia* sp. ZM and *Serratia marcescens* S217 [5,80-

82]. In addition to the IPyA pathway, the TAM pathway has been identified as the only IAA biosynthesis pathway in *Serratia fonticola* GS2 [83]. The genomes of other *Serratia* strains also had genes associated with multiple IAA biosynthetic pathways. For example, the *Serratia marcescens* RSC-14 genome had genes associated with the IPyA, IAM and IAN pathways [84]. *Serratia sp. M24T3* also possesses genes associated with several IAA biosynthetic pathways, including the TAM, IAM and IAN pathways [21].

Identification and quantification of indole compound in optimized medium culture by HPLC

HPLC analysis was employed to further investigate the presence of indole compounds in the optimized medium. Tryptophan (TRP), IAA and IAM were detected in all optimized medium cultures (Table 1). The concentration of TRP was significantly high in yeast extract sucrose with complete inorganic salt (sucrose) and yeast sucrose with calcium carbonate only (calcium carbonate). The IAM concentration was not significantly different among the optimized medium. In sucrose, no nitrogen and calcium carbonate showed the IAM concentration was lower than the TRP and IAA concentration. The highest IAA concentration was found in yeast extract sucrose with the complete inorganic salt medium. A similar result was observed in the colorimetric assay in which the highest IAA concentration was detected in sucrose (Figures 1 and 2). The IAA concentration of calcium carbonate was 3 times higher than magnesium sulphate in HPLC analysis. However, in the colorimetric assay, the IAA concentration of calcium carbonate is 50 % lower than magnesium sulphate (Figure 3).

Table 1 Indole quantification by HPLC analysis.

	TRP (µg/mL)	IAM (µg/mL)	IAA (µg/mL)
Sucrose	191.68 ^c	102.56	429.79 ^c
No Nitrogen	138.79 ^{ab}	72.66	346.21 ^{bc}
Magnesium sulphate	62.98 ^a	81.97	58.04 ^a
Calcium carbonate	198.81 ^c	90.88	199.45 ^{ab}

Data represents mean concentrations from 3 biological replicates and different lowercase letters indicate significant differences between treatments based on DMRT (alpha = 0.05 %).

Overall, the significant upregulation of expression levels observed in genes associated with the IAM pathway (*nthA*, *nthB* and *amiE*) corresponds to the presence of IAM as the only intermediate detected in the extract of the optimized medium culture. Additionally, TRP and IAA were consistently detected in all extracts from the optimized medium culture (Table 1) (Figure 7). Similar findings by Yusfi *et al.* [26] also reported the presence of IAM in UBCF_13 extracts following time culture optimization. Therefore, the IAM pathway might be applied in UBCF_13 to synthesize IAA. Further investigations are warranted to analyze transcriptomic and metabolomic data related to the IAA biosynthesis pathway of UBCF_13. Transcriptomic and metabolomic studies can be conducted using LC-MS and RNA-seq techniques. Both methodologies offer insights into differentially accumulated metabolites (DAMs) and differentially expressed genes (DEGs), thus elucidating the primary pathway, gene regulation and alterations at the genotype and phenotype levels in response to treatment [85,86]. Evaluating other metabolites in the IAA biosynthesis pathways using LC-MS also provides additional insights into the flux of auxin biosynthesis.

Conclusions

In conclusion, in this study, it was found that sucrose and yeast extract with complete inorganic salt (dipotassium phosphate, magnesium sulphate, sodium chloride and calcium carbonate) as the optimal medium for IAA production by UBCF_13. Expression levels of *amiE*, *nthA*, *oxdA*, *tyrB*, *nthB*, *puuC* and *ipdC* were also increased when sucrose served as the carbon source. Magnesium sulphate and calcium carbonate played significant roles in IAA production, as evidenced by colorimetric and HPLC assays. This observation aligned with the upregulation of the genes *tyrB*, *puuC*, *DDC* and *oxdA* in media containing calcium carbonate and the upregulation of the genes *puuC*, *nthA*, *nthB* and *amiE* in media containing magnesium sulphate.

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