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ORIGINAL ARTICLE

Genetic Engineering of *Escherichia coli* W for Linalool Production Using Beet Juice as the Sole Carbon Source

Matthew Mckillop[1] · Taufiq Nawaz[1] · Liping Gu[1] · Shah Fahad[1] · Ewumbua Monono[2] · Ruanbao Zhou[1]

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Abstract
Linalool is a high-value monoterpene alcohol widely used in the fragrance, flavor, pharmaceutical industries, as well as a component of jet fuel. However, conventional extraction from linalool-producing plants faces the challenges of costly, low productivity and sustainability. In this study, *Escherichia coli* W (ATCC 9637) was genetically engineered with plasmids pZR808, pZR1464 to enable linalool biosynthesis using beet juice-derived sucrose as the sole carbon source. The expression of linalool synthase (LinS) in genetically engineered *E. coli* W was confirmed by Western blot. GC-MS analysis confirmed linalool production. Continuous linalool production by *E. coli* W (pZR1464) accumulated to 141.6 µmols of linalool within 72 h. Among the engineered strains, the pZR1464 strain, carrying both the LinS gene and the DXP operon, achieved the highest linalool yield of 50 mg L^{-1}. Day^{-1}. $OD_{600}{}^{-1}$. Approximately 3–10 times greater yields than strain pZR808 bearing only the LinS. Sucrose utilization analysis indicated that *E. coli* consumed approximately 1.44 mmol of sucrose in the first 24 h, primarily for biomass production. Conversion efficiency of sucrose to linalool significantly improved over time. It increased from 27.28 µmols of linalool mol^{-1} sucrose (within 24 h) to 94.34 µmols of linalool mol^{-1} sucrose within 48 h, demonstrating metabolic adaptability. The engineered strain exhibited stable growth kinetics without adverse effects, positioning strain pZR1464 as a promising candidate for sustainable linalool production. Future selection of more powerful LinS genes combined with introduction of an exogenous mevalonate (MVA) pathway, could boost the yields by more than 300-fold, leading to commercially viable production of linalool using beet juice as the sole carbon source.

Keywords Terpene alcohol · Sugar beet application · Sucrose utilization · Synthetic biology

Matthew Mckillop and Taufiq Nawaz has equal contribution authorship.

Extended author information available on the last page of the article

Introduction

Linalool ($C_{10}H_{18}O$) is a naturallyoccurring monoterpene alcohol that has gained significant interest due to its diverse applications across various industries. Its uses range from the production of insecticide and insect repellents to the making of flavoring agents, brewing additives and fragrances, however, making it an essential ingredient in products designed to appeal to consumers [1]. More importantly, linalool (a long-chain alcohol) has an energy density of 40 mj kg^{-1}, heat of vaporization of 0.19 mj/kg, and octane of 102. These physicochemical properties make linalool as a precursor for the high-density fuel RJ-4 (a liquid rocket propellant used in missiles and a component of jet fuel) [2, 3].

Linalool, a monoterpene alcohol found naturally in many aromatic plants, it is naturally produced from the MEP pathway [4] where the universal isoprenoid intermediate geranyl diphosphate (GPP) is converted to linalool-by-linalool synthase (Fig.1) [5, 6]. Linalool is a highly sought-after compound in the flavor and fragrance industry. The biological activities of linalool and linalool-rich essential oils include antimicrobial, anti-inflammatory, anticancer, and antioxidant qualities. Additionally, multiple in vivo studies have verified the various effects of linalool on the central nervous system [7]. Beyond these conventional applications linalool holds significant potential in the pharmaceutical industry where ongoing research investigates its use in the synthesis of drug which could be further utilized in treating cancer as well as various serious health conditions [8]. This broad array of application highlights economic and industrial importance of linalool while its cost-effective production is crucial for meeting global demand and sustainability. Currently, the primary method of obtaining linalool involves extracting essential oils from the plants grown in tropical areas, particularly during the flourishing season when linalool is most concentrated in these plants [9, 10]. However, the reliance on seasonal tropical plants as linalool sources has some limitations

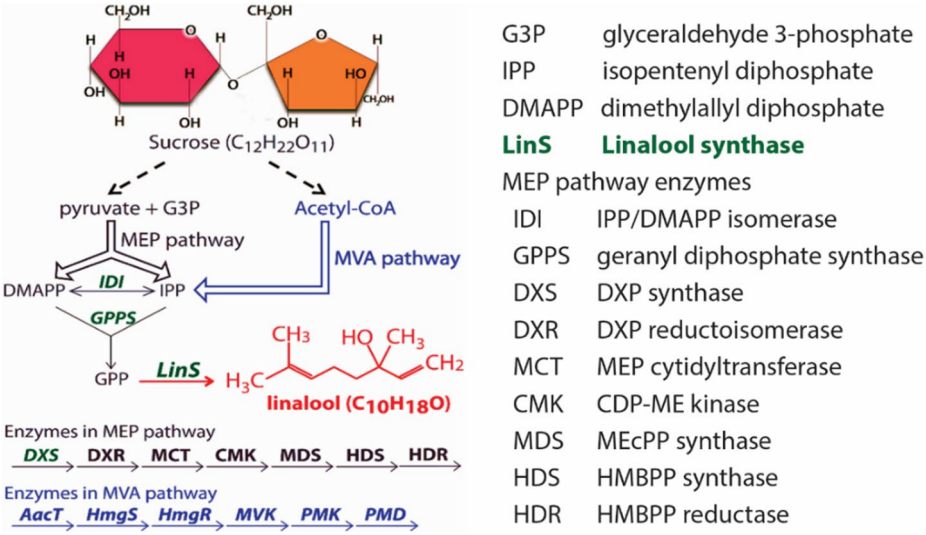

Fig. 1 Genetic engineering *E. coli* W to synthesize and secrete linalool using beet juice as the sole carbon source. The native methylerythritol phosphate (MEP) pathway shown in black. The genetically introduced exogenous MVA pathway (blue). Dashed arrows indicate steps of sucrose converting to pyruvate, G3P and acetyl-CoA. Enzymes are bold

in terms of cost and scalability. In the commercial sector particularly the perfume industry, synthetic production of linalool fulfils about 60 to 80% of the demand underscoring the high demand and potential for alternative production methods [11]. Under this scenario, the synthetic source is energy-intensive and costly, spurring the interest of producers in biologically based alternatives. A promising alternative involves the utilization of genetic engineering to produce linalool in microbial hosts [12]. In particular,*Escherichia coli*W is a well-suited bacteria due to its lab safety, fully sequenced genome, and capability to metabolize sucrose. Engineering this strain to convert sugar into linalool is a promising technique as it has several benefits like year-round production. Additionally using a crop-based carbon source is rich in fermentable sugars which could further enhance the economic feasibility of microbial linalool production [13]. This approach offers an innovative way to supply linalool for commercial and pharmaceutical products potentially reducing the reliance on synthetic methods and seasonal plant sources [14]. Among the plant extracts, Beet is a valuable carbon source as it contains a high concentration of sucrose along with other nutrients making it suitable for microbial fermentation [15]. Beet juice is reported as a renewable, cost-effective feedstock for biotechnological applications. Figure1 briefly demonstrates how the sucrose in beet juice is converted into one of the high-value chemicals, Linalool. First, the sucrose is up-taken by sucrose transporter (CscB) and then converted into glucose and fructose through the invertase (CscA) in *E. coli*W [16]. Subsequently glucose and fructose enter into glycolysis pathway [17] to produce pyruvate, G3P. These three carbon compounds enter*E.coli*W's native MEP pathway to produce geranyl diphosphate (GPP) that is then converted into linalool by genetically introduced plant linalool synthase (LinS) [5].

This study is to genetically engineer *E. coli* W (ATCC 9637) as a microbial factory to convert the low-cost sucrose sources such as beet juice into advanced biofuels or high-value chemicals through genetically engineered two isoprenoid pathways: MVA pathway and MEP pathway (Fig. 1). Currently, the beet juice is a far-underutilized feedstock except for extraction of sucrose (table sugar). The selected high-value chemical in this study is a fragrant, long-chain alcohol, linalool ($C_{10}H_{18}O$).

Materials and methods

Chemicals/Reagents/Plasmids/PCR Primers

The required chemicals Laura Brittny (LB), glycerol and calcium chloride ($CaCl_2$) and all other chemicals/regents were procured from the Sigma/Aldrich or Thermo Fisher Scientific-US. Liquid nitrogen was acquired from the local vendor.

The plasmids used for transformation including pZR1188 (Km^r, cloning vector) [18], pZR808 (*LinS*gene) [19] and pZR1464 (*LinS*+DXP operon) [20]. were constructed by our own laboratory. In addition, the required primers for performing colony PCR to verify the correct plasmid transformants. The primers listed in Table2 including ZR22, ZR45, ZR1670, ZR949 and T7 were synthesized at IDT (Integrated DNA Technologie).

The plasmids and the *E. coli* W strains are described in Table 1. The PCR primers used in this study were summarized in Table 2.

Table 1 Plasmids/E. coli W strains used in this study

Plasmid	**Relevant characteristic(s)**	**Source**
pZR1188	A dual promoter (Pnir/PpsbA1)-containing shutter vector for expression of foreign genes in both E. coli and cyanobacteria; Km^r (kanamycin resistance)	[18]
pZR808	Expression plasmid bearing His_6-LinS containing gene; Km^r	[19]
pRL1464	Three rate limiting enzyme genes (DXS, IDI and GPPS) were subcloned into pZR808 to produce pZR1464 (LinS + DXS + IDI + GPPS); Km^r	[20]
Strains		**Source**
E. coli W		ATCC
E. coli W (pZR1188)		This study
E. coli W (pZR808)		This study
E. coli W (pZR1464)		This study

Table 2 Oligo primers used in this study

Primers	Oligonucleotide sequence (5' to 3')	Purpose
ZR22	AGATCTTGAGTCAGCCCAATAAC	For screening the positive transformants of *E. coli* W bearing pZR1188
ZR45	CCTCGTAGAACTAGCAAAG	
ZR45	CCTCGTAGAACTAGCAAAG	For screening the positive transformants of *E. coli* W bearing pZR808
ZR1670	CCTCGTAGAACTAGCAAAG	
ZR949	GTGCAGACCGCCTTTCTG	For screening the positive transformants of *E. coli* W bearing pZR1464
T7	TTAATACGACTCACTATAGGG	

Production of *E. coli* W competent cells

The strain *E. coli* W was chosen for the transformation and genetic engineering due to its ability to survive in the mineralized water (BG-11), 2% sucrose and nitrogen. The strains were first grown on the LB-agar media and kept overnight in the incubator at 37 °C. A single colony was then transferred to 10 mL of the liquid LB media in another 125 mL conical flask and incubated under the aforementioned conditions. After that, the broth culture was 1 L broth in a 2.8 L wide mouth flask and incubated at 250 rpm till the optical density (OD_{600}) reached ~0.75 absorbance. Furthermore, the culture having optimized growth was distributed into four pre-chilled centrifuge tubes and placed in the ice-chamber for next 20 min followed by the centrifugation at 5000 x g at 4 °C for 10 min to make cell pellet. The resulting pellets were again resuspended in the 15 mL sterilized $CaCl_2$ (0.1 M) and transferred into 50 mL of a sterilized tube. The tubes were again placed in the ice chamber for 30 min and centrifuged as per the conditions mentioned above. In the last step, the pellets were further suspended into the microcentrifuge tubes having 9 mL $CaCl_2$ (0.1 M), glycerol (15%) and

previous aliquots (30 µL) followed by the flash frozen in the liquid nitrogen and stored in the −80 °C freezer till further use.

Plasmid Transformation

The control plasmid pZR1188 (Km^r) was transformed into *E. coli*W by following the heat sock standard transformation technique [18]. The transformation of pZR1188, pZR808, pZR1464 were verified by colony polymerase chain reaction (cPCR) using primers ZR45 and ZR22, ZR45 and ZR1670; T7 and ZR949, respectively.

Medium Preparation

A concentrated beet juice extracted from the *Beta vulgaris*L. (pH 6.3, 60% solid fraction, 60% sucrose v/v) was prepared by Dr. Monono at North Dakota State University [21, 22]. The concentrated beet juice as the sole stock media was further diluted about 20 times using BG11 medium [23] supplied with NH_4Cl (20 mM) and Km50 (50 µg mL^{-1}) to attain a 3.0% sucrose. This solution was prepared by mixing 100 mL of concentrated beet juice with 1470 mL of BG-11 solution in a 2 L volumetric flask and adjusting the pH up to 7.1. This mixture was further subdivided into four bottles and centrifuged at 12,000 x g at 22 °C for 10 min. After that 7.5 mL of HEPES buffer (1 M) + 0.35 mL Km50 (50 mg mL^{-1}) and 1.625 mL NH_4Cl (5 M) were added as per the standard protocol to every flask containing 350 mL media.

Inoculation of *E. coli* W Bearing a Plasmid for Linalool Production

The *E. coli* W bearing the plasmid pZR808, pZR1188 and pZR1464 recovered from the −80 °C and incubated in the separate flask having the solution of BG-11 + NH_4Cl (20 mM) + sucrose-containing beet juice (3.0% sucrose) + Km50 (50 µg mL^{-1}) to prepare a seed culture. After adding the reagents, the culture was incubated for 8 h to attain an optimized growth rate. Subsequently, 1 mL seed culture added to a 250 mL flask containing 100 mL of beet Juice (pre-aliquoted) media in the rubber-capped aeration tubes. 1 mL culture from each flask was collected to measure the OD_{600}. The fresh culture was again incubated followed by aeration at 100 mL min^{-1}.

Linalool Capture

Linalool was captured through the collection columns having Supelpak-2SV beads, each column 1.5” glass tube equipped with autoclaved wool plugs. The linalool’s capture followed the method for capturing limonene/farnesene [24, 18]. The beads weighing 0.11 g were added to each column tube which were connected to aerated flask by 0.75” rubber tubbing and each flask was assigned a number. The quantification of captured linalool beads was eluted with a pentane solution (5 µg mL^{-1} tetracosane) as an internal standard.

Gas Chromatography-Mass Spectrometry (GC-MS) To Confirm Linalool Production

Linalool and tetracosane were detected using an Agilent GC 7890 A, MS 5975 C with an HP-MS column (30 m×250 μm×0.25 μm) and a flame ionization detector (FID). A 1μL sample was injected with 99.99% pure H_2 as a carrier gas. The temperature was initially set to 60 °C for 2 min then increased by 20 °C min^{-1} to 300 °C and held for 2 min resulting in a total 16-minute run time. Linalool had a retention time (RT) about 5.16 min, while the peak of tetracosane showed at 12.1 min. The quantification was done as per the standard calibration curve of linalool and tetracosane.

High-Performance Liquid Chromatography To Measure Sucrose Consumption

The concentration of sucrose was measured on the Agilent 1220 infinity-II HPLC machine equipped with a refractive index detector (RID) coupled with sugar Pak I-column (Waters™ Technologies corporation). The solution of ethylenediaminetetraacetic acid (EDTA) was run as a mobile phase with a flow rate of 0.5 mL min^{-1}. The cultures of *E. coli* W pZR1464 were centrifuged, the supernatant was collected, filtered and the concentration of sucrose was analyzed through HPLC. The standard sucrose calibration showing the 8 min as retention time which was implemented for the quantification of sucrose. The sucrose consummation by *E. coli* W bearing pZR1188, and/or pZR1464 was also measured using the ZORBAX carbohydrate column on the same HPLC machine. The mobile phase flow rate was 1.4 mL min^{-1} having 75% of the acetonitrile in double distilled water. The retention time of sucrose in this column was 4.7 min which was confirmed by a standard calibration curve.

Generation of Growth Curve

The growth phases of *E. coli* W bearing pZR1188 (control) and *E. coli* W bearing pZR1464 (experimental strain) were compared. Every strain under experimentation was initially inoculated by frozen stock (−80 °C) into a separate 100 mL beet juice media for seed culture growth and seed culture was incubated for another 8 h as per the standard method mentioned in the previous sections to ensure both strains reached the optimized growth phase. After the end of incubation time, 1 mL of culture was transferred into three separate flasks each having 100 mL of media and sealed with cotton-inserted plugs of cotton and aluminum foil. The OD_{600} was measured by taking 1 mL from each flask at the time of inoculation. The flasks were incubated and OD_{600} was measured four hours after attaining the stationary phase.

SDS-PAGE and Western Blotting

Equivalent amounts of *E. coli* cells from different cultures were collected from 1.0 mL of culture (OD_{600}=1.0–1.2.0.2) by centrifugation (12,000 × g). Unless otherwise specified, extracts were prepared by resuspending cells in 50 μL ddH_2O, then adding 50 μL of 2× SDS-PAGE sample buffer [50 mM Tris·HCl, pH 6.8, 4% SDS, 20% (vol/vol) glycerol, 200 mM DTT, 0.03% bromophenol blue] and boiling for 3 min. After centrifugation (12,000

×g), 10 µL of supernatant was loaded onto a mini-protean TGX precast gels (4–20%, Bio-Rad) for separation of proteins. The two SDS-PAGE gels were run in the SDS-PAGE (1X-buffer) at the constant current of 40 mA for 90 min. Western blot analysis was followed the method described previously [25, 26]. Antibodies to pentahistidine (Qiagen, Valencia, CA) were used at 1:5,000 dilution.

Coomassie Bright Blue Staining

The above SDS-PAGE gel was stained with the Coomassie Brilliant Blue R-250 by placing the gel in a glass box with 15 mL of a staining solution. The box was kept on a shaker at 45 rpm at 22 °C for overnight. The gel underwent the destaining for 1–2 h. After the completion of destaining, the gel picture was captured/imaged by the scanner (Epson GT-1500).

Results

cPCR Verification of Plasmid Transformation

The verification of plasmid transformation into *E. coli* W was confirmed through colony PCR (cPCR) using specific primers tailored to each plasmid. cPCR for plasmid pZR1188 was performed using the primers ZR45 and ZR22. The gel electrophoresis results displayed an expected 625 bp band in lanes 1–5 and 7–9 (Fig. 2A), confirming the successful transformation of pZR11878 into these colonies. Similarly, for pZR808 that contained the *LinS*, The cPCR was conducted using primers ZR45 and 1670. The cPCR result showed a expected 1073 bp band in lanes 1 and 2 (Fig. 2B). For pZR1464 that contained the *LinS* and the DXP operon was verified by cPCR with primers ZR949 and the T7 promoter primer. The cPCR produced an expected 2665 bp band in lanes 1–5 and 7–9 (Fig. 2C). The attained results confirm the proper integration and amplification of the plasmid within the transformed colonies. The expected cPCR bands confirmed that pZR808, pZR1188, pZR1464 were successfully transformed into *E. coli* W. Therefore, *E. coli* W(pZR808), *E. coli* W(pZR1188), *E. coli* W(pZR1464) were created (Table 1 strains).

Expression of Linalool Synthase (LinS)

The successful expression of the *LinS* protein in *E. coli* W(pZR1464) was confirmed by Western blot analysis. The expected 68.8 kDa (68764 Da) band of H6-LinS were detected in lanes 24 h, 48 h, and 72 h (Fig. 3A). This demonstrates that the *LinS* protein was effectively synthesized in the transformed *E. coli* cells carrying the pZR1464 plasmid. The absence of corresponding bands in the control strain *E. coli* W(pZR1188) lacking the LinS gene validates the specificity of the *LinS* gene expression. The pZR1464 strains consistently expressed the *LinS* protein throughout the experimental period (24 h to 72 h) with no reduction in the expression levels over time. This stability was evident in the consistent band intensity observed in lane 72 h of Fig. 3A which matched the intensities seen in lanes 24 h and 48 h. The observed consistency indicates the stable and robust expression of the *LinS* protein in *E. coli* W(pZR1464) strain. The accuracy assurance was confirmed by assessing the protein expression, protein loading across all the lanes was confirmed to be consistent.

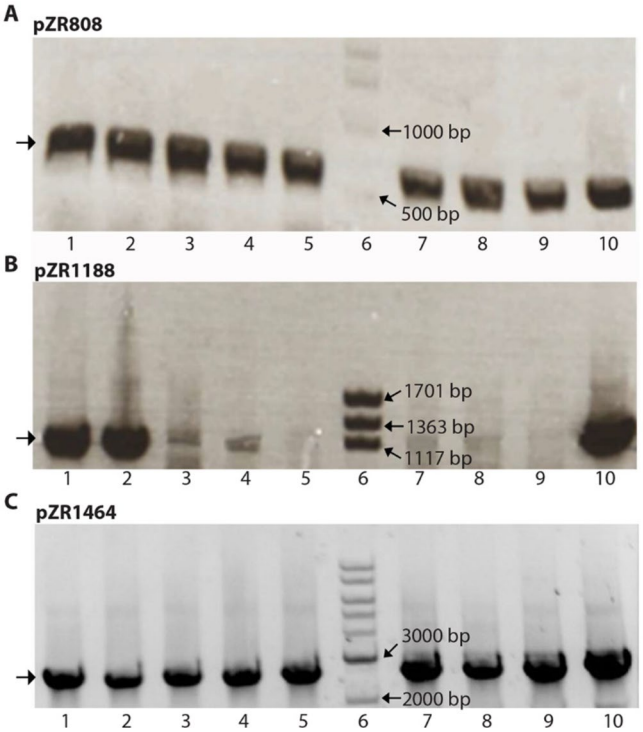

Fig. 2 Colony PCR (cPCR) to verify the colony successfully transformed with the corresponding plasmid. (**A**) cPCR of *E. coli* W colonies bearing pZR1188. Arrowed expected band of 625 bp was detected in all selected colonies (Lanes 1–5,7–9) as well as the positive control (Lane 10). DNA ladder used was NEB 1 kb DNA ladder (Lane 6). (**B**) cPCR of *E. coli* W colonies bearing pZR808. Two positive colonies (Lanes 1 and 2) showed the expected band of 1073bp as shown in the positive control (Lane 10). DNA ladder used was homemade (Lane 6). (C) cPCR of *E. coli* W colonies bearing pZR1464. Expected band of 2665 bp was detected in all selected colonies (Lanes 1–5,7–9) as well as the positive control (Lane 10). DNA Ladder used was NEB 1 kb DNA ladder (Lane 6)

This was verified by the Coomassie-stained gel analysis (Fig. 3B). This step was crucial for validating that the observed variations in the Western blot signals were due to differences in *LinS* expression rather than discrepancies in samples loading. The combined evidence from the Western blot and Coomassie-stained gel analysis underscores the effective and stable expression of the *LinS* protein in the pZR1464 strain.

GC-MS Identification of Linalool Production

Figure 4 illustrates the GC-MS identification of linalool production by two genetically engineered *E. coli* W strains. The two strains were analyzed to compare their linalool production capacity, while the chromatogram of the control *E. coli* W(pZR1188) (Fig. 4A) which showed no detectable chromatogram at the retention time of 5.15 min indicating no linalool production. This confirmed that linalool synthesis requires the presence of the linalool synthase gene (*LinS*) and supporting metabolic pathways. The chromatogram for *E. coli* W(pZR808) that contains only the *LinS* gene from *Picea abies* (Fig. 4B) displayed a peak at

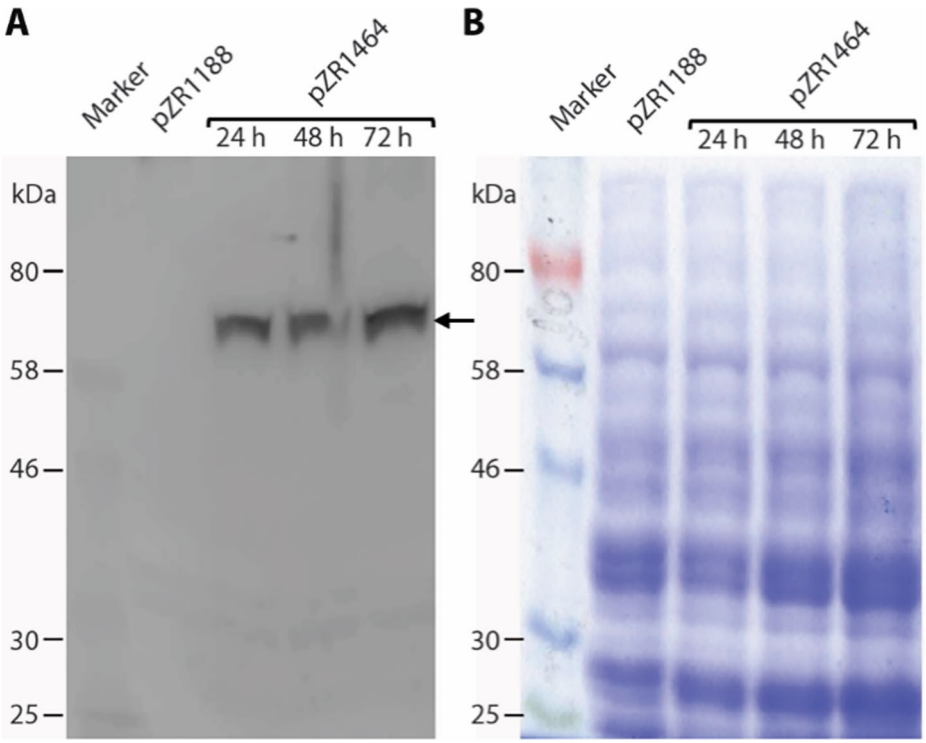

Fig. 3 Expression of linalool synthase (LinS) in *E. coli* W bearing pZR1464 was measured during 72 h by Western blot. (**A**) Detection of linalool synthase (LinS) in *E. coli* W bearing pZR1464 (LinS + DXP operon) in comparison with vector control pZR1188. (**B**) Coomassie Bright Blue R-250 staining of the loaded proteins to confirm a same amount of total protein loading. Marker: NEB Colorplus Prestained Protein Ladder, lanes 24 h, 48 h, 72 h were the total cell lysates from pZR1464 (24 h), pZR1464 (48 h), pZR1464 (72 h). Anti-pentaHistidine antibody (Qiagen) was used for the Western Blot detection of six-Histidine-tagged linalool synthase (H_6-LinS)

5.15 min (black arrow). This small but substantial peak suggests that the *LinS* gene enables linalool production as its standalone expression results in low metabolite levels likely due to the limitations in the availability of precursors or insufficient flux through the native MEP pathway. In contrast, the chromatogram of the *E. coli* W(pZR1464) containing both the DXP operon and *LinS* gene revealed a significant bigger peak at 5.15 min (Fig. 4C). This finding demonstrates the enhanced production of linalool due to the overexpression of rate-limiting enzymes (DXS, IDI and GPPS in Fig. 1) in the MEP-pathway provided by the DXP operon. The higher flux of carbon through the MEP pathway in pZR1464 strain is likely to boost the biosynthesis of the linalool precursors significantly increasing its production compared to the pZR808 strain. The validation of the identified peak in pZR1464 the standard of linalool 5 µg mL^{-1} was also analyzed by GC-MS which confirmed the retention time of linalool as 5.157 min (Fig. 4D). Furthermore, the confirmation was also achieved through mass spectrometric analysis of the peaks. The mass spectrum of the peak at 5.157 min from the pZR1464 strain (Fig. 4C) showed a fragmentation pattern identical to that of the linalool standard (Fig. 4D). The observed match unequivocally identifies the metabolite as linalool and verifies its production by the engineered *E. coli* W strains. In addition to this internal

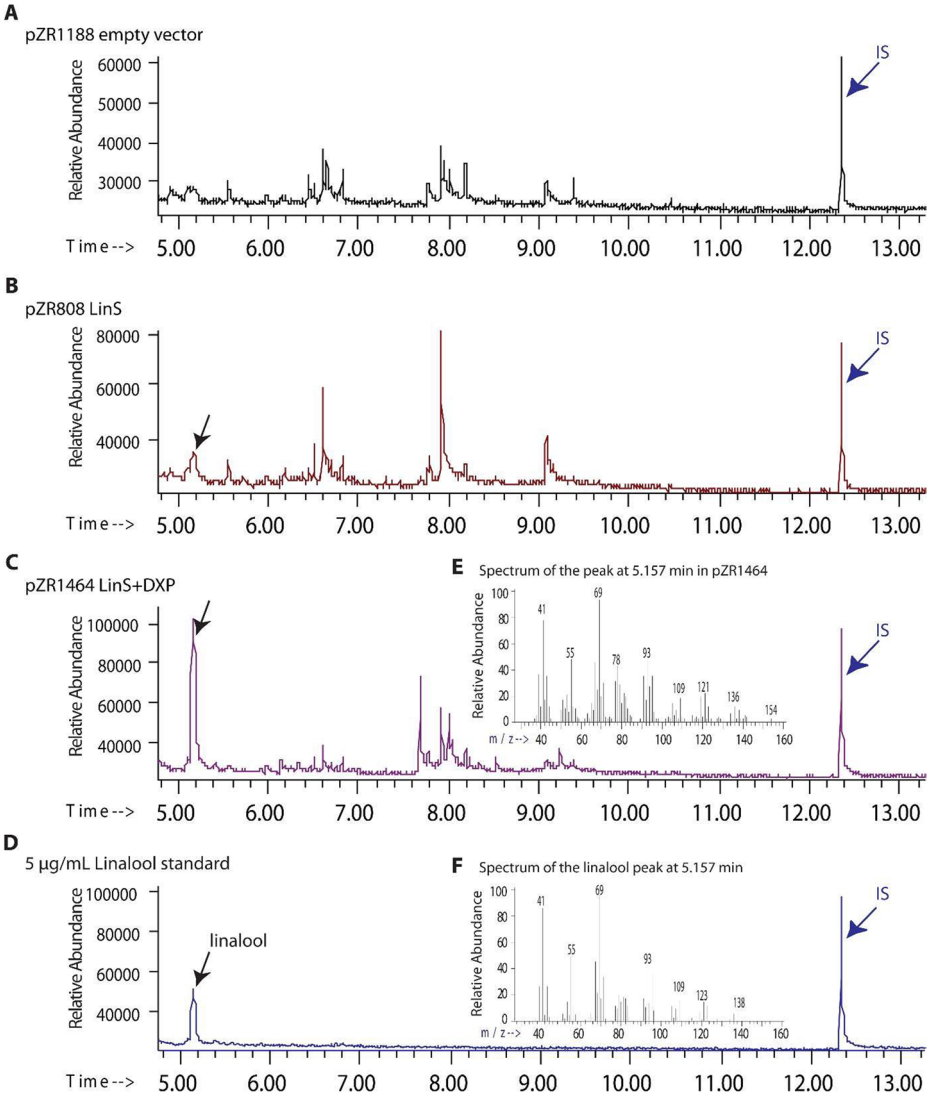

Fig. 4 Identification of linalool produced by *E. coli* W bearing linalool synthase gene and/or DXP operon. **A.** GC-MS chromatograph of the volatile metabolites captured by 2SV resin from control *E. coli* W bearing pZR1188, no linalool was detected; **B**. GC-MS chromatograph of the volatile metabolites captured by 2SV resin from *E. coli* W bearing pZR808; **C.** GC-MS chromatograph of the volatile metabolites captured by 2SV resin from *E. coli* W bearing pZR1464. A peak at the retention time of 5.157 min (black arrowed) found in *E. coli* W matches the linalool standard (**D**). The insert graphs of E and F were mass spectra of the 5.167 min peaks displaying the fragmentation pattern for linalool. Five µg mL^{-1} tetracosane serves as an internal standard (IS, blue arrow)

standard of tetracosane was included in all the chromatograms to ensure the retention time alignment and reliable quantification of linalool production. The analysis reveals the superiority of the pZR1464 strain over the pZR808 strain in the linalool production demonstrating the crucial role of metabolic pathway optimization for achieving high yields.

Linalool Production

The production of linalool was primarily investigated in the strain *E. coli* W(pZR1464) due to the significant lower production of linalool chromatogram in the *E. coli* W(pZR808) (Fig. 4B vs. C). A total two experimental setups were employed to evaluate linalool production i.e., a short-term study conducted over 24 h with the quantity taken during the exponential growth phase of seven hours and a longer-term study spanning 72 h to capture the production dynamics across different growth phases. In the 72-hour experiment, linalool production was initiated during the optimum growth stage of bacterial culture yielding 22.50 ± 6.10 µmols during 0 h to 7 h (Fig. 5A). The attained production indicates that the biosynthetic pathway was actively engaged during the high metabolic activity of the cells. Linalool synthesis continued during the stationary phase demonstrating the strain's ability to sustain production even when culture growth slowed down. Despite the persistence of linalool production over the three days, the productivity of linalool (µmoles/h) exhibited a declining trend (from 3.21 µmol/h to 1.97 µmol/h, Table S1) over time (7 h to 72 h) (Fig. 5A) possibly due to the depletion of metabolic precursors or a reduction in sucrose concentration during prolonged culture periods. Therefore, use of higher concentrations of sucrose (e.g. 6% to 16%) may boost the linalool productivity during the prolonged production periods. The detailed analysis of sucrose conversion efficiency revealed the highest productivity during 25 h to 48 h interval reached 94.34 µmols of linalool produced per mole of sucrose consumed. This peak efficiency (25 h to 48 h) significantly outperformed the conversion rates (27.28 µmols of linalool/mole of sucrose) observed during the 0–24 h followed by 49–72 h intervals (68.22 µmols of linalool/mole of sucrose) (Fig. 5B and Table S1 & S2). The current data suggest that the optimal linalool production coincided with the late exponential to early stationary growth phases when metabolic activity and resource availability were balanced.

Sucrose Utilization

The engineered *E. coli* W(pZR1464) demonstrated the capacity for sucrose utilization with a significant reduction in the sucrose concentration observed during the initial 24 h

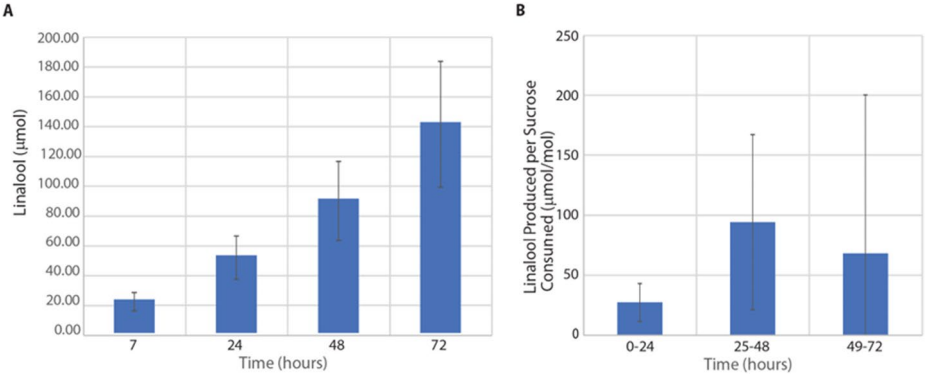

Fig. 5 (**A**) The combination chart representing the production of linalool by engineered *E. coli* W (pZR1464) using beet juice; (**B**) The production of Linalool (per mole of sucrose consumed) by *E. coli* W(pZR1464)

of growth. In the early phase, nearly 15 mM sucrose was consumed indicating an active metabolic activity coinciding with the exponential growth phase, the rate of sucrose consumption is likely to reflect the metabolic demands of the strain which support both cell proliferation and the biosynthesis of linalool (Fig. 6A, Table S3). After the initial 24 h, the sucrose utilization exhibited a steady decline with each interval. This trend is likely due to the reduced metabolic activity of the cells as they transitioned from the exponential to the stationary phase coupled with the accumulation of metabolic byproducts or a potential shift in resource allocation towards maintenance energy rather than growth and secondary metabolite production. The decreasing sucrose consumption over time underscores the dynamic relationship between substrate availability, bacterial growth, and metabolic output. Both *E. coli* W(pZR1464) strain and the control strain *E. coli* W(pZR1188) exhibited the ability to consume sucrose from beet juice. After normalizing for initial sucrose concentrations, the *E. coli* W(pZR1464) exhibited approximately 2.8 times more sucrose consumption compared to the control *E. coli* W(pZR1188) (Fig. 6B and Table S4). The improved sucrose consumption suggests that the metabolic engineering introduced in pZR1464 effectively redirected resources towards linalool biosynthesis, highlighting the metabolic versatility of the strain and the success of the genetic modifications in promoting higher substrate uptake.

The findings of the current study emphasize the significance of sucrose as a carbon source driving both growth and linalool production in the pZR1464 strain. However, the steady decline in sucrose utilization over time also indicates the potential areas for optimization such as fine-tuning the metabolic pathways to maintain higher substrate uptake rates or exploring alternative carbon sources to sustain production during extended cultivation periods. These insights provide valuable direction for further improving the efficiency of this bioengineered system.

Growth Curve

The growth profile of genetically engineered *E. coli* W strain having the plasmid pZR1464 and pZR1188 was found nearly identical throughout the trial indicating that the introduction of linalool synthase (*LinS*) gene and DXP operon in pZR1464 did not adversely affect the bacterial growth dynamics. Both strains demonstrated their most rapid growth within the first 8 h corresponding to the exponential phase where the cells actively utilized available

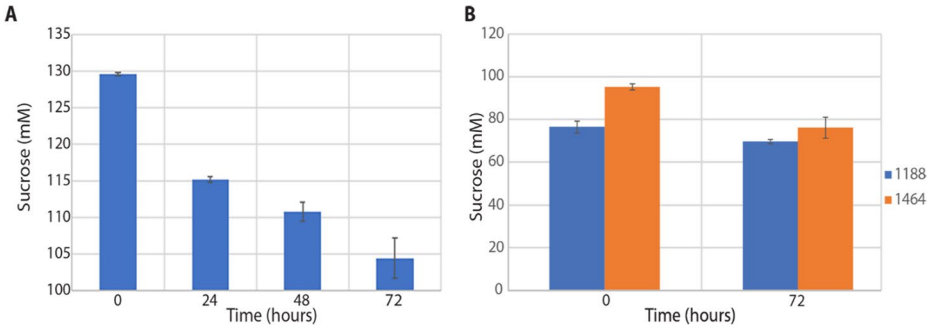

Fig. 6 (**A**) The conversion of sucrose (mM) during linalool production by *E. coli* W (pZR1464). (**B**) The comparison of sucrose concentration in the linalool producing *E. coli* W (pZR1464) and *E. coli* W (pZR1188) at 0- and 72-hours interval

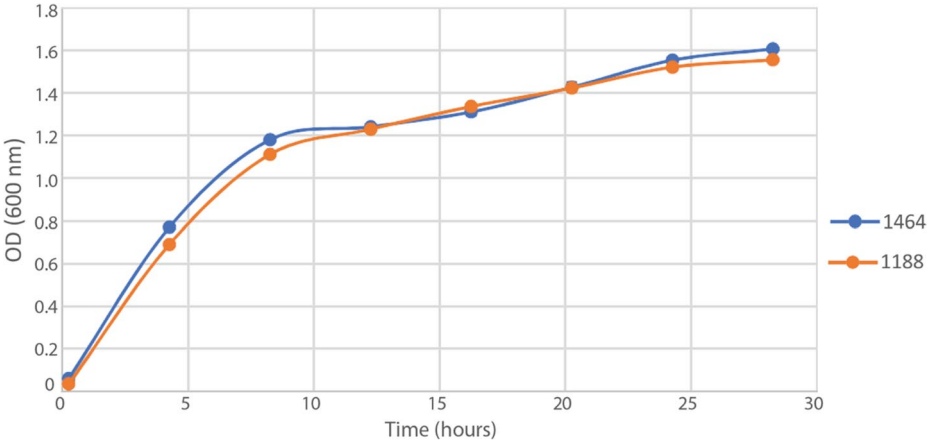

Fig. 7 The growth curve of *E. coli* W(pZR1464) vs. E. coli W (pZR1188) at the interval of every 4 h till 28 h incubation period

sucrose and nutrients to build biomass. During this phase, the specific growth rate was at its peak as seen by the steep slope of the growth curve (Fig. 7). After 8 h the growth rate slowly transitioning into a steady, gradual increase as the cultures approached the stationary phase. This gradual declaration is typical as nutrients begin to deplete, and metabolic byproducts accumulate. Both strains reached the stationary phase between 24 and 28 h characterized by the plateauing of the growth curves (Fig. 7 and Table S5). This phase indicates a balance between cell division and cell death often resulting from resource limitation and the buildup of inhibitory compounds in the medium.

Interestingly the similar growth curves of the pZR1464 and pZR1188 strains suggest that the additional metabolic burden imposed by the *LinS* gene expression and DXP operon overexpression in pZR1464 did not compromise cell growth or viability. This observation is critical for industrial applications as it implies that the linalool production by the engineered strain can proceed without significantly hindering bacterial growth performance. Despite their similar growth kinetics, the pZR1464 strain consumed more sucrose during the experiment (Fig. 4b) reflecting the additional metabolic demand for linalool synthesis. This increased carbon flux did not delay the onset of the stationary-phase or reduce maximum OD_{600} achieved by the pZR1464 strain in comparison with control pZR1188 strain.

Discussion

The engineered strain equipped with *LinS*gene demonstrates significant efficiency for sustainable production of linalool by using the sucrose as a sole source of carbon which was rich in the Beet Juice [27]. The pZR1464 strain was genetically engineered as the superior producer among the tested strains while performing much better than pZR808 strain in the linalool synthesize. This increased the productivity which was attributed to the pZR1464 plasmid that not only bears the*LinS* gene of *Picea abies* but also genetically introduced a DXP operon. The DXP operon facilitates the overexpression of three key rate-limiting enzymes (DXS, IDI, GPPS) in the methylerythritol phosphate (MEP-pathway) critical for

isoprenoid biosynthesis. The pZR1464 strain achieved a remarkable 3–10-fold more linalool production (Fig. 4. C vs. B) in comparison with the pZR808 strain that contains only *LinS*. This improvement highlights the importance of metabolic engineering strategies that target upstream pathway bottlenecks to augment downstream product synthesis. Despite consuming a higher total carbon input than the control strain pZR1188 in contrast the pZR1464 strain exhibited no detrimental effects on its growth rate or progression to the stationary phase emphasizing its applicability to industrial scale. In addition, during the 24-hour experiment pZR1464 strain utilized approximately 1.44 mmol of sucrose primarily for biomass production which is a typical observation during the exponential growth phase when cells prioritize energy and resource allocation for replication and cellular maintenance. This led to a relatively low initial sucrose-to-linalool conversion efficacy (27.18 ± 15.84 µmol linalool per mol of sucrose). However, by the 48-hour mark, the efficacy improved by 3 to 4 fold as the cells transitioned to a stationary phase where the metabolic fluxes often shift towards secondary metabolite production including terpenoids line linalool [28]. In addition, the decline in the linalool production efficiency after 48 h was not associated with decreased-*LinS*expression as protein levels remained consistent throughout the 72 h of experimentation. The results suggest that other factors such as substrate limitation, accumulation of inhibitory byproducts or metabolic stress may play a role in the diminished production rates observed during the prolonged culture periods [29]. The optimization of incubation conditions including nutrient replenishment and controlled product recovery could address these limitations and sustain higher yields over the extended durations. Despite the promising findings, the linalool production in*E. coli*remains suboptimal compared to natural plant systems. In addition, the challenge can be overcome by integrating nonnative pathways like MVA pathway which is predominantly utilized by plants for terpenoid synthesis offers a promising avenue. The MVA pathway has been shown to provide a more direct and efficient route for isoprenoid precursor generation complementing the MEP pathway in microbial systems [30]. Additionally, co-transformation of*E.coli* with plasmids containing MVA pathway genes and alternative linalool synthase such as those derived from *Streptomyces clavuligers*has the potential to amplify production yields by up to 300 fold as demonstrated in previous studies [31].

Conclusion

The finding of the current study concluded that the genetical modification of *E. coli* W particularly those transformed the pZR1464 combined expression of *LinS* and DXP operon showed that the pZR1464 strain achieved a 3 to 10-fold increase (Fig. 4C vs. B) in the linalool production as compared with pZR808 strain which bearing only the gene of *LinS*. Despite the higher carbon utilization by the pZR1464 strain, there is no adverse effect on stationery and growth phase that was observed which highlights the efficiency of the system. Initial sucrose to linalool conversion efficiency was modest but improved significantly after 48 h correlating with optimized metabolic activity during the second and third days of incubation. These insights provide a foundation for enhancing the linalool biosynthesis through further pathway optimization including the integration of exotic metabolic pathway like MVA pathway. The observed improvement could pave the way for economically viable and renewable production of linalool at an industrial scale.

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Author Contributions Conceptualization: R.Z. (Ruanbao Zhou), L.G. (Liping Gu); Methodology: L. G. & R. Z T.N. (Taufiq Nawaz); Investigation, L.G, M.M, T.N. Resources: Ewumbua Monono (E.M) and R. Z; Data curation, R.Z. & L. G; original draft writing: M.M. T.N. (Taufiq Nawaz); Review and editing, R.Z. L.G. S.F. (Shah Fahad); Supervision: L.G, R.Z; Project administration, L.G, R.Z; Funding acquisition, L.G & R.Z. All authors have read and agreed to the published version of the manuscript.

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Data Availability All data generated or analyzed during this study are included in this article and its supplementary materials.

Declarations

Ethical Approval Not applicable. This research did not involve human participants, animals, or sensitive data requiring ethical approval.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Competing interests The authors declare that they have no conflict of interest.

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Authors and Affiliations

Matthew Mckillop[1] · Taufiq Nawaz[1] · Liping Gu[1] · Shah Fahad[1] · Ewumbua Monono[2] · Ruanbao Zhou[1]

✉ Liping Gu
lipid.glycoprotein@gmail.com

✉ Ruanbao Zhou
ruanbao.zhou@sdstate.edu

Taufiq Nawaz
taufiq.nawaz@jacks.sdstate.edu

Shah Fahad
shah_fahad80@yahoo.com

Ewumbua Monono
ewumbua.monono@ndsu.edu

[1] Biology and Microbiology, South Dakota State University, Brookings, SD 57007, USA

[2] Agricultural and Biosystems Engineering, North Dakota State University, Fargo, ND 58102, USA