Journal of Bioscience and Bioengineering
VOL. 127 No. 4, 458–464, 2019

www.elsevier.com/locate/jbiosc

Modulation of culture medium confers high-specificity production of isopentenol in *Bacillus subtilis*

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Received 17 February 2018; accepted 3 October 2018
Available online 26 October 2018

Enthusiasm for mining isoprenoid-based flavors, pharmaceuticals, and nutraceuticals from GRAS (Generally Regarded as Safe) status microbial hosts has increased in the past few years due to the limitations associated with their plant-based extraction and chemical synthesis. *Bacillus subtilis*, a well-known GRAS microbe, is a promising alternative due to its fast growth rate and the ability to metabolize complex carbon sources. The study focused on the high-specificity production of isopentenol in *B. subtilis* by modulating the culture medium. Media modulation led to a 2.5 folds improvement in isopentenol titer in the wild-type strain. In the recombinant strain, optimization of physico-chemical factors, coupled with overexpression of the nudF enzyme resulted in a maximum isopentenol titer of ~6 mg/L in a shake flask. The recombinant strain produced ~5 mg/L isoprenol (~80% of the total isopentenol production) and ~1.8 mg/L prenol (~65% of the total isopentenol production) by utilizing sorbitol and pyruvate as the carbon sources, respectively. Replacement of glucose with sorbitol and pyruvate reduced the production of the undesired metabolites and enhanced high-specificity production of isopentenol. Upon replacement of the carbon source with a low-cost substrate, a non-detoxified rice-straw hydrolysate, the engineered strain produced 2.19 mg/L isopentenol. This proof-of-concept study paves the path for the high-specificity production and cost-effective recovery of isopentenol from industrially competent microbial strains with engineered isoprenoid pathways.

[**Key words:** *Bacillus subtilis*; High-specificity; Isopentenol; Media modulation; Rice-straw hydrolysate]

Isopentenol (prenol and isoprenol) are hemiterpene-based unsaturated C5 alcohols and primary product of isoprenoid pathway (1). They are one of the simplest forms of isoprenoid which are used as intermediates for the synthesis of a wide range of aroma chemicals, pharmaceuticals (2). Minute fractions (1–10 ppm) of prenol are used as a flavoring agent and up to 0.1% in cosmetic products (3). Previously, we have demonstrated that isoprenoid-based compounds and plant extracts (containing isoprenoid as a major constituent) are potential candidates for the treatment of aging and age-related ailments (4–6). In a recent study, we have found that isopentenol conferred longevity and stress tolerance in lower eukaryote animal model, *Caenorhabditis elegans* (Phulara et al., manuscript submitted). Isopentenols are found in trace amounts in fruits ranging from 8 μg/kg to 450 mg/kg (7–10), where they act as important intermediates in various biosynthetic pathways (10). The low-level expression in plants limits their large-scale application. Moreover, seasonal production, soil and land requirements and difficulties in harvesting and extraction also limit their applicability. Industrially, isopentenol are synthesized by petroleum-derived isobutene and formaldehyde which, however, is an environmental and health concern due to the hazardous nature of these chemicals.

To overcome the challenges associated with the chemical synthesis and plant-based extractions, microbial production of isopentenol has been extensively studied in past few years (2,11–14). Strain improvement strategies have enabled researchers to explore microbial hosts for the non-natural production of isoprenoid-based compounds (15). Due to its fast growth rate and easy genetic manipulations, *Escherichia coli* is the most widely explored microorganism for the microbial production of isopentenol (11–14). However, the non-GRAS (Generally Regarded as Safe) status of *E. coli* restricts its application for the commercial production of isoprenoid-based flavors, pharmaceuticals and their precursors (16).

Bacillus subtilis, a spore-forming gram-positive bacterium can be a potential alternate due to its GRAS status and rapid growth rate. Its ability to metabolize vast array of the substrate ranging from glucose to economical carbon feed stocks (17) and competence to withstand adverse culture conditions (like a change in the pH and temperature during fermentation) provide additional advantages. Most importantly, it is amongst the highest isoprene producing bacteria (18,19) and has been reported to produce ~18-fold higher isoprene than *E. coli* (19). Further, various phosphatases and pyrophosphatases including ADP-ribose pyrophosphatase (nudF) have shown potential to convert prenyl precursors, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) into isoprenol and prenol, respectively, in engineered *E. coli* (11,12,20)

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1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2018.10.002

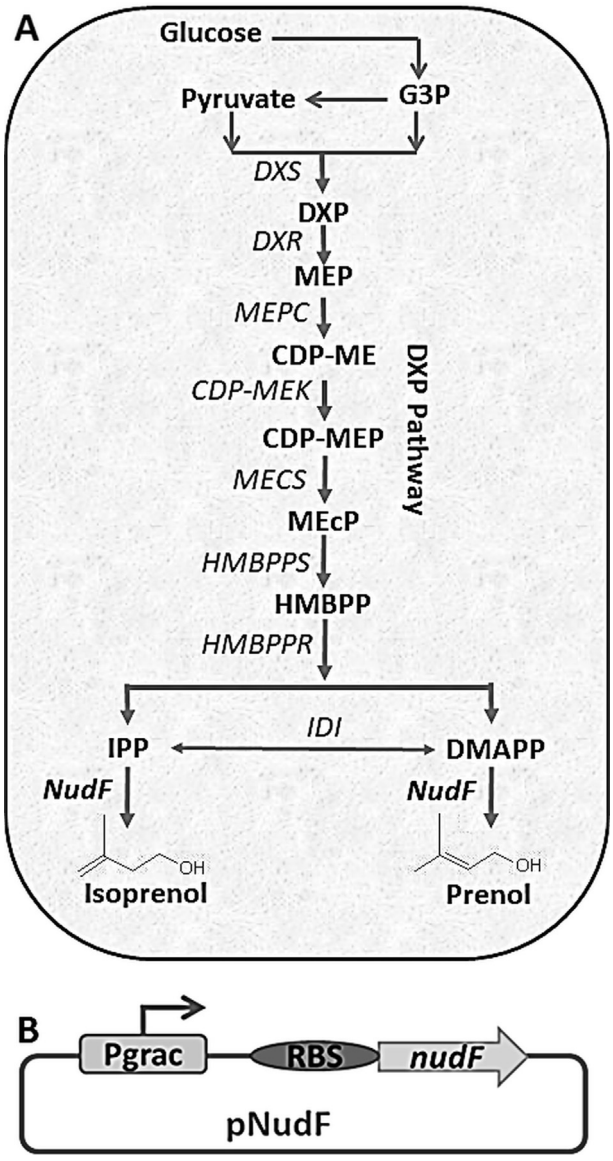

FIG. 1. Isopentenol biosynthesis and overexpression system. (A) Isopentenol biosynthesis in engineered *B. subtilis*. G3P, glyceraldehyde-3-phosphate; DXP, deoxyxylulose-5-phosphate; DXS, DXP synthase; DXR, DXP reductoisomerase; MEP, 2-*C*-methylerythritol-4-phosphate; MEPC, MEP cytidyltransferase; CDP-ME, 4-(cytidine-5′-diphospho)-2-*C*-methylerythritol; CDP-MEK, CDP-ME kinase; CDP-MEP, 2-phospho-4-(cytidine-5′-diphospho)-2-*C*-methylerythritol; MEcP, 2-*C*-methylerythritol-2,4-cyclodiphosphate; MECS, MEcP synthase; HMBPP, hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate; HMBPPS, HMBPP synthase, HMBPPR, HMBPP reductase; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; IDI, isoprenyl diphosphate isomerase; NudF, ADP ribose pyrophosphatase. (B) Illustration of the expression system pNudF. *nudF* was expressed under the control of IPTG inducible promotor, P_{grac}. RBS, ribosomal binding site.

(Fig. 1A). Encouraged by these findings, the present study has explored isopentenol production from the wild-type and recombinant (overexpressing nudF enzyme) *B. subtilis* strains. It is well-known that modulation of medium composition and growth conditions increases metabolites titer from microbes (16,21). Therefore, the study focused on the improvement of isopentenol production in both wild-type and engineered strain through media modulation. Furthermore, the recovery of microbial metabolites can be simplified if the production of fermentation by-products can be reduced. Production of single isoprenol has been achieved through genetic modulations (11); however, the effect of media modulation on the high-specificity production of isopentenol has not been investigated yet. The present study demonstrates the effect of carbon-sources on the high-specificity production of isopentenol from engineered *B. subtilis.* Finally, the possibility of a cost-effective isopentenol production was also explored in the engineered strain using a non-detoxified rice straw hydrolysate.

MATERIALS AND METHODS

Bacterial strains and plasmid *B. subtilis* strain MTCC121 (ATCC6051) was obtained from Microbial Type Culture Collection and Gene Bank (MTCC), CSIR-Institute of Microbial Technology, Chandigarh, India. For sub-cloning, plasmid isolation for sequencing and other related experiments *E. coli* strain NEB10β (New England BioLabs, Ipswich, MA, USA) was used. *E. coli*–*B. subtilis* shuttle vector pHT01 (MoBiTec, Göttingen, Germany) having an IPTG-inducible strong promoter, P_{grac}, was used to construct expression vectors in *B. subtilis*.

Vector construction and cloning The *nudF* gene was amplified using *B. subtilis* genomic DNA by employing appropriate primers (Table 1). To construct plasmid pNudF (Fig. 1B), *nudF* gene from *B. subtilis* was amplified from primers pHT-NudF (F) and pHT-NudF (R) designed with NEBuilder software from New England BioLabs. The plasmid, pHT01, was digested with XbaI and the amplified gene fragment was ligated to the digested plasmid by using NEBuilder HiFi DNA assembly master mix (New England BioLabs).

The newly constructed plasmid, pNudF, was transformed to *E. coli* NEB10β cells using Gene Pulser Xcell electroporation system (Bio-Rad, Hercules, CA, USA) as per its instruction manual and pre-set electroporation program for *E. coli*. The positive clones having pNudF plasmid were screened onto 50 mg/L ampicillin-containing LB agar plates (Sigma–Aldrich, St. Louis, MO, USA). Plasmid DNA was purified from the positive clones by GeneElute plasmid mini prep kit (Sigma–Aldrich) and the cloned sequence was verified by gene sequencing. *B. subtilis* MTCC121 cells were transformed with pHT01 and sequence verified pNudF plasmid by electroporation as described by Sun et al. (22) with minor modifications. Briefly, the overnight grown culture of *B. subtilis* was diluted to an OD_{600} of 0.05 into fresh LB medium containing 0.5 M sorbitol. The culture was then incubated in a shaker incubator at 37°C. When the culture attained an OD_{600} between 0.80 and 1.00, the medium was supplemented with 2% glycine and 0.03% Tween 20. The cells were allowed to grow for another hour and then kept on ice bath for 20 min prior to harvesting at 5000 × *g* for 10 min at 4°C. The harvested cells were washed thrice with ice-cold electroporation medium (0.5 M sorbitol, 0.5 M mannitol, and 10 % v/v glycerol) and re-suspended in 1/50 (v/v) electroporation medium. Competent cells were transferred to pre-chilled 1.5 ml microfuge tubes in 80 μL aliquots and stored at −80°C for further use. A 1 μL aliquot of 100 ng/μL plasmid DNA was mixed with the competent cells and kept on ice for 20 min. Cells were then transferred to a pre-chilled electroporation cuvette (2-mm electrode gap, Bio-Rad) and shocked by a single electric pulse (2000 V, 200 Ω, 5 ms) generated by Gene Pulser Xcell electroporation system (Bio-Rad). Immediately after electroporation, a 920 μl of recovery medium (LB containing 0.5 M sorbitol and 0.38 M mannitol) was added to the transformed cells and allowed to grow for 4 h at

TABLE 1. List of primers used in this study.

Primer	Orientation	Sequence	Reference
Cloning			
pHT-NudF	F	aattaaaggaggaaggatccATGAAATCATTAGAAGAAAAAACAATTG	This work
pHT-NudF	R	ctgccccggggacgtcgactctagagtTCATTTTTGTGCTTGGAG	This work
RT-PCR			
16S	F	ACGGTCGCAAGACTGAAACT	17
16S	R	TAAGGTTCTTCGCGTTGCTT	17
NudF	F	TGAGCGGACGATCGTTGAAA	49
NudF	R	CAAGTTCCCGAAGTGCCGTA	49

F, forward primer. R, reverse primer.

37°C in a shaking incubator. To select the positive clones, 100 µl aliquots from the cell suspension were spread onto LB agar plates containing 10 mg/L chloramphenicol (Sigma–Aldrich). Recombinant *B. subtilis* cells bearing pNudF and pHT01 plasmids were designated as BS-nudF and BS-pHT, respectively.

Transcription analysis by quantitative real-time PCR An overnight grown LB starter culture of *B. subtilis* was diluted to an OD_{600} of 0.05 into fresh LB medium containing 10 mg/L chloramphenicol. The culture was incubated at 37°C in a shaking incubator at 200 rpm until it attained an OD_{600} of 0.8–1.0. The cells were then induced with 1 mM IPTG (Sigma–Aldrich) and incubated at 25°C in a shaking incubator. After a 4-h-incubation, RNA was isolated from the cultures using Zymo RNA mini prep. kit and cDNA was synthesized from purified mRNA using NEB cDNA synthesis kit (New England BioLabs). The primers used for the RT-PCR are listed in Table 1. For gene expression analysis, amplification steps were performed using a CFX Connect real-time PCR detection system (Bio-Rad) with CFX Manager Software. The relative quantification of the overexpressed genes was analyzed using the comparative C_T ($\Delta\Delta$ C_T) method (23).

SDS-PAGE electrophoresis analysis For protein expression, BS-nudF cells were induced with 1 mM IPTG as described above and then incubated at 25°C for 6 h in a shaking incubator at 130 rpm. The cells were harvested at 10,000 × *g* for 10 min at 4°C and the harvested cells were washed thrice with lysis buffer (1xPBS containing 15% sucrose and 0.2 % lysozyme). The cells were re-suspended in an equal volume of lysis buffer and disrupted by an ultra-sonicator (33 ± 3 kHz, 200 W). The cell lysate thus obtained was centrifuged at 13,000 × *g* for 30 min at 4°C to remove insoluble material. The crude protein extract was then analyzed by SDS–PAGE using 10% (w/v) acrylamide gel.

Growth curve assay Growth curve assay was conducted as described by Xue and Ahring (17) with minor modifications. Briefly, the overnight grown starter cultures of BS-nudF and BS-pHT were diluted into a fresh LB medium containing 10 mg/L chloramphenicol to an OD_{600} of 0.05. A 150 µL aliquot from each of the diluted culture was transferred to microtiter plate in triplicate and allowed to grow at 30°C in a rotatory shaker incubator at 180 rpm. When the cells attained an OD_{600} of about 0.15–0.2, the recombinant strain was induced with 1 mM IPTG and OD_{600} was measured at 30-min-intervals.

Media and growth conditions *B. subtilis* and *E. coli* strains were grown at 37°C in LB medium. For plasmid harboring *E. coli* and *B. subtilis* strains, ampicillin (50 mg/L) and chloramphenicol (10 mg/L) were added to the culture medium, respectively. Initial production medium consisted of LB, M9 salts (8.5 g/L $Na_2HPO_4 \cdot 2H_2O$, 3.0 g/L KH_2PO_4, 1.0 g/L NH_4Cl), 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.3 g/L ferric ammonium citrate, trace metal mix (1.0 mg/L $MnCl_2 \cdot 4H_2O$, 1.7 mg/L $ZnCl_2$, 0.43 mg/L $CuCl_2 \cdot 2H_2O$, 0.60 mg/L$CoCl_2 \cdot 6H_2O$, 0.60 mg/L $Na_2MoO_4 \cdot 2H_2O$), 10 mg/L chloramphenicol and 100 mM glucose (24).

For isopentenol production, the desired production medium was inoculated with 2% overnight grown starter culture and incubated at 37°C in a shaking incubator at 200 rpm. The pH of the production medium was adjusted to 7.0 with acetic acid (60.05 g/mol; 99.8%, Merck Life Science Pvt. Ltd., Bengaluru, Karnataka, India) prior to inoculation. On attaining an OD_{600} of 0.8–1.0, the culture was induced with 1 mM IPTG. The culture was then incubated in a shaking incubator (130 rpm) at 25°C so that the nascent overexpressed protein can be correctly folded in its functional conformation due to a reduced growth rate under these conditions (25–27). After 48 h of continuous incubation, isopentenol production was observed.

Modulation of production medium To enhance isopentenol production, the medium composition was optimized by investigating the effect of pH and carbon source. Because 200 mM concentration of K_2HPO_4 was found to be effective in enhancing the production of isoprenoid-based metabolites in *B. subtilis* (16), the effect of this concentration was investigated on the medium pH during isopentenol production in this study.

For further improving isopentenol production, the effect of carbon source was also studied by replacing glucose with sorbitol, fructose, mannitol, sodium pyruvate, sucrose, and glycerol in the production medium wherever required.

In-situ extraction of isopentenol Isopentenols are volatile compounds that tend to evaporate during their production process (13). Therefore, in-situ extraction of isopentenol was executed in order to minimize the evaporative losses. A 20% oleyl alcohol overlay was added to the production medium that acted as a blanket over the optimized growth medium.

Preparation of mild acid saccharified rice straw hydrolysate To explore the possibility of the production of isopentenol from an economical carbon source mild acid saccharified rice straw hydrolysate was used. Prior to saccharification, the biomass was delignified using microwave assisted alkali mediated pretreatment as described by Gupta and Parkhey (28). Mild acid saccharification of the pretreated biomass was carried out as described by Diwan et al. (29). Non-detoxified, pooled hydrolysate (from two subsequent saccharifications) containing approximately 40 g/L reducing sugars (29) was used as feed for the production of isopentenol. An equal ratio of LB and pooled hydrolysate was used to formulate production medium and pH was adjusted to seven with 2.0 M K_2HPO_4. The production was carried out as mentioned in the previous section.

Analysis of isopentenol by GC–FID Samples were prepared by ethyl acetate extraction method. The cultures were spiked with 20 mg/L 3-methyl-1-butanol prior to solvent extraction. An equal volume of cell culture and ethyl acetate was mixed vigorously for 15 min and then centrifuged at 13,000 × *g* for 3 min. The upper ethyl acetate layer was analyzed by GC (Agilent, 7890 A) equipped with flame ionization detector (FID) and an Elite-Wax (PerkinElmer) capillary column (30 m × 0.25 mm × 0.25 mm) as described by Zheng et al. (11). The initial oven temperature was 50°C for 1 min, 100°C with a gradient of 5°C/min. Finally, the temperature was raised to 200°C at a rate of 25°C/min and held for 5 min. Nitrogen was used as the carrier gas. The injector and detector temperatures were 230°C and 250°C, respectively. For the culture containing 20% oleyl alcohol overlay, the total volume was extracted with ethyl acetate after 48 h incubation.

Statistical analysis All the experiments were performed in three replicates for each test condition and the average values were presented. Error bars represent the standard errors of the mean.

RESULTS AND DISCUSSION

Isopentenol production in *B. subtilis* MTCC121 Like most of the prokaryotes, *B. subtilis* synthesizes isoprenoid-based metabolites from DXP pathway (Fig. 1A) and overexpression of the DXP pathway enzymes has shown to increase the titers of isoprenoid-based compounds in *B. subtilis* (16,17,30). Although, *B. subtilis* is reportedly one of the highest producers of isoprene; however, its isopentenol-producing ability has not been studied yet. The present study exploited the ability of wild-type *B. subtilis* to produce isopentenol without any genetic modulation and observed ~0.3 mg/L isoprenol titer from wild-type MTCC-121 strain (Fig. 2A). An endogenous enzyme nudF might be responsible for this natural production of isoprenol that has already shown potential to convert excess prenyl precursors into isopentenol in engineered *E. coli* (11,12,20). No detectable level of

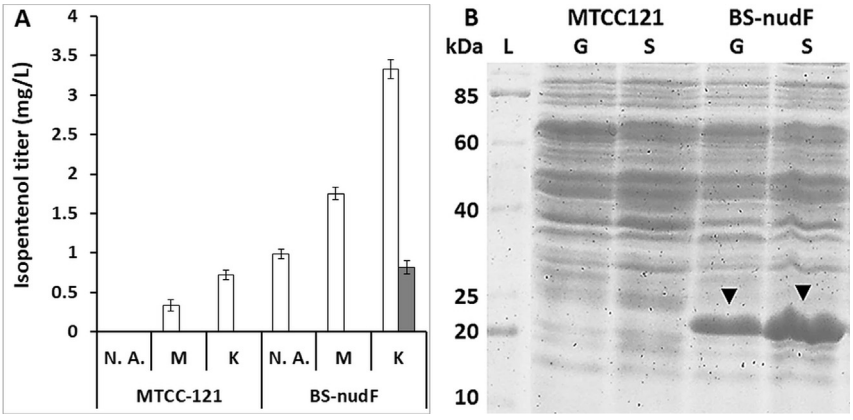

FIG. 2. Isopentenol production and protein expression analysis of wild-type (MTCC121) and BS-nudF. (A) Isopentenol production by MTCC121 and BS-nudF. Holllow bars represent isoprenol production and closed bar represents prenol production; N.A., no addition; M, M9 slats; K, K_2HPO_4. (B) SDS-PAGE analysis of soluble intracellular protein fractions of strains grown in glucose (G) and sorbitol (S) supplemented medium. L, protein ladder.

prenol was observed in the culture medium. This could be due to the inherent low level of DMAPP as the DXP pathway generally produces IPP and DMAPP in the ratio of 5:1 (31). Another reason for the non-detectable levels of prenol might be the conversion of DMAPP into isoprene in *B. subtilis* by an endogenous enzyme having isoprene synthase activity (32).

nudF* overexpression increased isopentenol production in *B. subtilis The *B. subtilis* nudF enzyme has shown potential to convert excess prenyl precursors into isopentenol in engineered *E. coli* (11,12,20). However, it has not yet been reported whether overexpression of *nudF* can increase the production of isopentenol in *B. subtilis* without any genetic modification in the DXP pathway. To test the possibility, the endogenous *nudF* gene was overexpressed in wild-type *B. subtilis* MTCC-121 (Fig. 1B). To confirm the overexpression of *nudF* in the newly constructed BS-nudF strain, mRNA levels of the *nudF* gene were analyzed by real-time RT-PCR. The real-time analysis data revealed a significant increase in the expression of *nudF* in BS-nudF compared to the control strain (BS-pHT) harboring vector backbone only. Overexpression of the nudF protein was also examined by SDS-PAGE analysis of crude protein extract of BS-nudF. A band corresponding to the size of nudF (20 kDa) was observed in SDS-PAGE gel (Fig. 2B) indicating the overexpression of nudF protein in BS-nudF strain.

The overexpression of *nudF* led to a ~6-fold increase in the isoprenol production over the control strain. The BS-nudF produced ~1.8 mg/L isoprenol in initial production medium containing 100 mM glucose (Fig. 2A). However, overexpression of *nudF* failed to improve prenol titer in the recombinant strain. This might be due to the low level of DMAPP inside the cellular pool, as discussed in the previous section. It was also observed that the overexpression of *nudF* did not impose any negative effect on the growth of BS-nudF. There was no significant difference between the growth curves of BS-nudF and BS-pHT (Fig. S2).

Effect of pH stabilizer on isopentenol production in wild-type and engineered strain The pH of the production medium plays an important role in the production of microbial metabolites and controlled pH has shown significant improvement in the production of isoprenoid-based metabolites in *B. subtilis* (16). It was observed that the pH of the glucose-supplemented production medium reduced in the absence of pH regulators (Fig. 3A). Further, the addition of pH stabilizers such as M9 salts and di-potassium phosphate (K_2HPO_4) attenuated the decline of the medium pH and improved isopentenol production in both wild-type and recombinant strains.

The main components of M9 are Na_2HPO_4 and KH_2PO_4, which have high buffering capacities and maintain the pH to nearby neutral (33). This might be a possible reason for the attenuation of the decline in the medium pH during isopentenol production in the M9-supplemented medium. Higher concentrations of phosphates such as K_2HPO_4 and KH_2PO_4 in fermentation medium have shown enhanced titers of isoprenoid-based metabolites from engineered microbes (16,34). Zhang et al. (34) have optimized the concentration of KH_2PO_4 for an enhanced production of lycopene and amorphadiene in engineered *E. coli*. It was demonstrated that a 200 mM concentration of KH_2PO_4 is optimum for the production of both the isoprenoid-based metabolites in *E. coli* (34). Later, in another work from the same laboratory, phosphate concentration was optimized for an enhanced production of amorphadiene in *B. subtilis* by utilizing various concentrations of K_2HPO_4 (16). Similar to the findings with KH_2PO_4, the 200 mM concentration of K_2HPO_4 was found to be optimum for the production of amorphadiene in *B. subtilis* (16). In line with the previous reports, our study also observed that the addition of 200 mM K_2HPO_4 stabilized the medium pH more effectively and controlled it to near neutral (Fig. 3A). It was found that 200 mM K_2HPO_4 increased the total isopentenol production by ~2.4-fold over the M9 supplementation in BS-nudF strain (Fig. 2A). The BS-nudF strain also produced ~0.82 mg/L prenol in 200 mM K_2HPO_4 containing production medium; however, it was not detected in production medium containing M9 salts as pH stabilizer (Fig. 2A, Table S1). Consistent with the previous findings, K_2HPO_4 supplementation is beneficial for isopentenol production in *B. subtilis*. The wild-type strain grown in the absence of pH regulators did not produce isopentenol (Table S1) indicating the vital role of pH in isoprenoid production.

Sorbitol supplementation increased isopentenol titer An overexpression of recombinant proteins is often associated with accumulation of insoluble protein aggregates (inclusion bodies) having little or no activity (26,35,36). Use of osmolyte such as sorbitol has shown to improve the solubility of recombinant proteins thereby increasing the yield of microbial metabolites (21,35,37). To observe the effect of sorbitol on the solubility of nudF protein and on the production of isopentenol in BS-nudF, the recombinant strain was grown in production medium supplemented with 500 mM sorbitol. The SDS-PAGE analysis of crude protein extract of the cells grown in the presence of 500 mM sorbitol showed an increased yield of nudF protein (Fig. 2B). Consistent with the previous findings, the improved protein expression enhanced isopentenol titer in recombinant strain and BS-nudF produced ~5.3 mg/L isopentenol in 500 mM sorbitol-supplemented medium.

Optimization of sorbitol concentration enhanced high-specificity production of isopentenol To optimize the isopentenol production, BS-nudF was grown in a production medium with different concentration (250 mM, 100 mM, and 50 mM) of sorbitol and 200 mM K_2HPO_4. It was noticed that BS-nudF produced a maximum isopentenol titer of 6.05 mg/L in production medium supplemented with 100 mM sorbitol

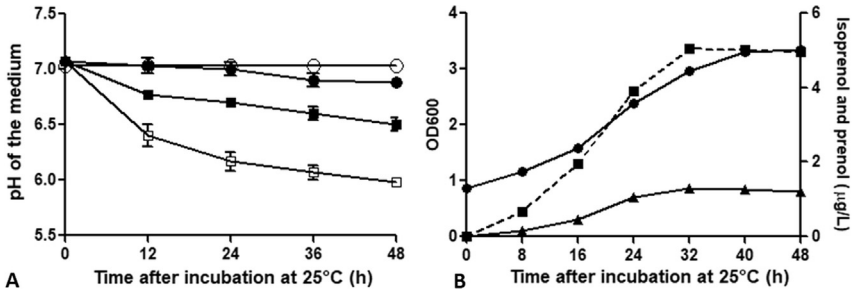

FIG. 3. Time course of the change in medium pH, biomass formation and isopentenol production by BS-nudF. (A) Effect of M9 and K_2HPO_4 on the medium pH during growth in 100 mM glucose supplemented medium; open circles represent un-inoculated medium (blank), open squares represent no addition; closed squares and closed circles represent M9 and K_2HPO_4 addition, respectively. (B) Time course of biomass formation and isopentenol production in 100 mM sorbitol supplemented medium; circles represent OD_{600}, squares represent isopentenol production and triangles represent prenol production.

(Fig. 3B), where isoprenol accounted for over 80% of the total production (Fig. 4A). In *E. coli*, increase in the isoprenoid production has been achieved by channeling glucose metabolic flux to either pentose phosphate (PP) pathway or Enter–Doudoroff (ED) pathway (12,38). Both the studies utilized genetic modulation to shift carbon flux towards PP and ED pathway for increasing the precursor (pyruvate and G3P) and co-factor (NADPH) supply towards DXP pathway. However, the effect of the media modulation on the carbon flux has not been studied yet for the enhanced production of isoprenoid-based compounds.

There was a considerable reduction in the undesired metabolites peaks in ethyl acetate extract of sorbitol-containing medium compared to glucose-containing medium (Fig. S3, Table S2). Moreover, a major peak between isoprenol and prenol was completely absent in sorbitol-containing medium, while it was prominent in glucose-supplemented medium (Fig. S3, Table S2). This suggested that sorbitol supplementation enhanced carbon efflux towards DXP pathway. It is well-known that after uptake, sorbitol is converted to fructose by sorbitol dehydrogenase in *B. subtilis* (39,40). Fructose is then phosphorylated either to fructose-1-phosphate (F1P) by the phosphotransferase system or to fructose-6-phosphate (F6P) by fructokinase. The F1P and F6P are then converted to fructose-1,6-biphosphate (FBP) by F1P kinase or by F6P kinase, respectively. Finally, FBP is converted to G3P, a direct precursor to the DXP pathway, by FBP aldolase (39,41). This might be a possible reason for the high-specificity production of isopentenol. This high-specificity production can be utilized to simplify the recovery of isopentenol from the fermentation broth.

Encouraged by the findings with sorbitol, 100 mM fructose was also evaluated for the production of isopentenol from BS-nudF. It was found that fructose supplementation delayed the growth of recombinant strains at 37°C. This finding is also supported by the studies in which *B. subtilis* grown in the presence of fructose has shown a reduced growth than glucose supplementation (42,43). Further, lowering the incubation temperature to 25°C after induction with 1 mM IPTG delayed the growth and reduced the biomass of BS-nudF strain in 100 mM fructose-supplemented production medium (Fig. S4). Fructose metabolism is a complex process in *B. subtilis* and which is also evident from the previous studies (39,44,45).

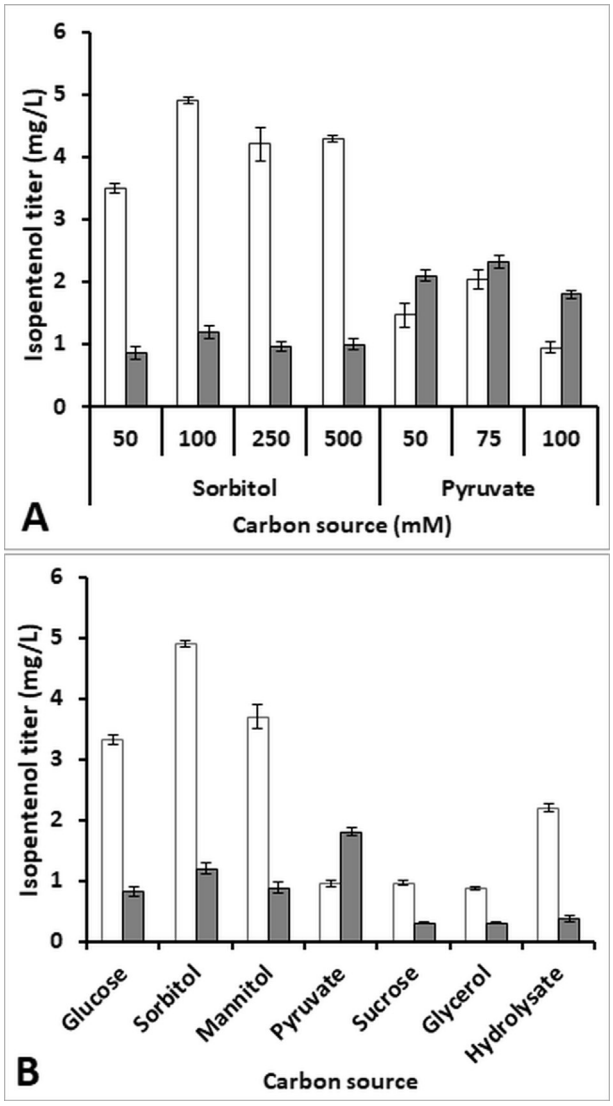

FIG. 4. Isopentenol production by BS-nudF. (A) Isopentenol production in sorbitol- and pyruvate-supplemented medium at different concentrations of sorbitol and pyruvate. (B) Isopentenol production in 100 mM concentrations of different C-sources. Open bars represent isoprenol production and closed bars represent prenol production.

Pyruvate improved prenol production In light of the above findings, isopentenol production utilizing other carbon sources such as pyruvate, sucrose, glycerol, and mannitol was also investigated. Amongst them, 100 mM of mannitol supplementation resulted in quantitatively similar isopentenol production as obtained with 100 mM glucose supplementation from BS-nudF (Fig. 4A), while the other three carbon sources failed to improve the total isopentenol production in BS-nudF at 100 mM concentration (Fig. 4B). Surprisingly, an increase in prenol titer in 100 mM pyruvate supplemented medium was observed (Fig. 4A). In 100 mM pyruvate containing medium, prenol accounted for over 65% of the total produced isopentenol with ~2.2 folds higher prenol titer than that was achieved from the glucose-supplemented medium (Fig. 4A, Table S1). Eventually, production ratio of prenol and isoprenol was observed by using different concentrations of pyruvate in the production medium. It was interesting to note that the ratio of isoprenol and prenol varied with the pyruvate concentration in production medium. An almost equal ratio of isoprenol and prenol was produced in 75 mM pyruvate containing medium (Fig. 4A). It has been shown that pyruvate supplementation could enhance the production of ATP and NADPH in *Bacillus megaterium* (46). In agreement with the previous reports, enhanced level of cofactors might be favorable for the biosynthesis of the isopentenol in the pyruvate-supplemented medium. Further, more elaborated studies would be needed to analyze the pyruvate mediated effects.

Similar to the sorbitol supplementation, the GC chromatogram of the ethyl acetate extract of pyruvate-supplemented medium showed lower metabolite peaks and the major peak between isoprenol and prenol was also absent (Fig. S3, Table S2). This suggests that pyruvate supplementation also improves carbon flux towards isopentenol production.

Agro-waste as a carbon source for isopentenol production To harness the complex carbon metabolizing ability of *B. subtilis*, isopentenol production from BS-nudF was studied using rice-straw hydrolysate (29) as a carbon source. It was found that BS-nudF produced ~2.6 mg/L isopentenol from non-detoxified rice-straw hydrolysate (Fig. 4B). As the rice-straw hydrolysate explored in the present study was minimally treated, non-detoxified and hence economically synthesized (29), the study demonstrates a more cost-effective approach for isopentenol production. The long-approved, GRAS status of *B. subtilis* implies that the biomass can be reutilized further without any regulatory restrictions (17). Therefore, metabolic engineering tools in coordination with bioprocess techniques can

be valuable to enhance the low-cost production of isopentenol from lignocellulosic biomass using *B. subtilis* as a host for prospective commercial production.

Oleyl alcohol overlay enhanced isopentenol production It has been reported that isopentenol are volatile compounds and hence prone to evaporative losses during their production (13). Hydrophobic overlays such as decane, dodecane, and oleyl alcohols are used to prevent such evaporative losses during the production of isoprenoid-based compounds (13,16,47). However, for C-4 and C-5 alcohols oleyl alcohol overlay has been most widely used (13,48). In the present study, 20% oleyl alcohol overlay was used for the in-situ extraction of isopentenol in 100 mM sorbitol-supplemented medium. Expectedly, oleyl alcohol overlay led to an additional 1.7 fold increase in the isopentenol accumulation in 100 mM sorbitol supplemented medium. A production titer of ~10.6 mg/L isopentenol was observed in the production medium containing 100 mM sorbitol, 200 mM K_2HPO_4 and a 20% oleyl alcohol overlay (Table S1).

The present proof-of-concept study mainly focused on media modulation rather than genetic modulation of the DXP pathway of a host organism for the optimization of isopentenol production. To the best of our knowledge, the report highlights the highest metabolite production of any hemiterpene-based metabolite in *B. subtilis*, to date. Moreover, the high-specificity production of isopentenol can simplify product recovery from the fermentation broth. Further, quantitative improvement in isopentenol production can be obtained by more focused studies on the development of robust genetic tools for fine-tuning multiple gene expression in *B. subtilis*. It is noteworthy that the recombinant strain produced isopentenol from a low-cost substrate in similar order of magnitude that was obtained from the synthetic medium.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2018.10.002.

ACKNOWLEDGMENTS

Authors heartily acknowledge Director CSIR-Indian Institute of Toxicology Research, Lucknow, Uttar Pradesh, India and Director, National Institute of Technology, Raipur, Chhattisgarh, India for providing platform to conduct the research work. Authors are also thankful to Mr. Pramod Kumar Srivastava, Technical Assistant, Analytical Chemistry Division, CSIR-Indian Institute of Toxicology Research, Lucknow, Uttar Pradesh, India for conducting GC analysis of the samples. Authors also show their sincere gratitude to Dr. Vikrant Singh Rajput, Scientist, Biosafety Support Unit, Regional Centre for Biotechnology, Department of Biotechnology, New Delhi, India for critical assessment and suggestions on the manuscript.

The authors mutually declare that they have no conflict of interest. This study does not contain any studies involving human or animal subjects.

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