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ORIGINAL PAPER

Studies on the production of shikimic acid using the *aroK* knockout strain of *Bacillus megaterium*

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Abstract Shikimic acid has various pharmaceutical and industrial applications. It is the sole chemical building block for the antiviral drug oseltamivir (Tamiflu®) and one of the potent pharmaceutical intermediates with three chiral centres. Here we report a modified strain of *Bacillus megaterium* with aroK (shikimate kinase) knock out to block the aromatic biosynthetic pathway downstream of shikimic acid. Homologous recombination based gene disruption approach was used for generating aroK knock out mutant of *B. megaterium.* Shake flask cultivation showed shikimic acid yield of 2.98 g/L which is ~6 times more than the wild type (0.53 g/L). Furthermore, the shikimate kinase activity was assayed and it was 32 % of the wild type. Effect of various carbon sources on the production of shikimic acid was studied and fructose (4 %, w/v) was found to yield maximum shikimic acid (4.94 g/L). The kinetics of growth and shikimic acid production by *aroK* knockout mutant was studied in 10 L bioreactor and the yield of shikimic acid had increased to 6 g/L which is ~12 fold higher over the wild type. It is evident from the results that aroK gene disruption had an immense effect in enhancing the shikimic acid production.

Keywords Shikimic acid · Knockout · Shikimate kinase · *Bacillus megaterium* · Bioreactor

Introduction

Shikimic acid (SA), a hydroaromatic metabolite produced in the aromatic amino acid biosynthesis pathway, is an attractive building block for the synthesis of biologically active compounds due to its unique structure with three chiral centers (Kramer et al. 2003; Chandran et al. 2003; Adachi et al. 2006). SA is also the sole precursor (chiral template) for the chemical synthesis of the neuraminidase inhibitor, oseltamivir phosphate (Knop et al. 2001; Ghosh et al. 2012), known as Tamiflu (Clercq 2002), which has been clinically employed for the treatment of the avian virus, type H5N1, and A/H1N1 influenza infections (Russell et al. 2006).

In the early days, SA was mainly produced by chemical synthesis and by extraction from the fruits of *Illicium verum*, but these processes are expensive and unwieldy with low yield (Rawat et al. 2013a; Haslam 1993; Ghosh et al. 2012). During the pandemic of swine flu, the supply crisis and high cost were held responsible for the shortage of drug (Rawat et al. 2013b, 2013c). But since the last decade, an alternative cost-effective and sustainable fermentation-based production method has been developed using several modified *E.coli* strains and few other organisms (Ghosh et al. 2012; Kramer et al. 2003; Escalante et al. 2010; Frost et al. 2002; Knop et al. 2001). In *E. coli*, the shikimate pathway has been widely investigated and metabolically engineered for the biosynthesis of various useful products (Patnaik and Liao 1994; Patnaik et al. 1995; Koma et al. 2012; Kim et al. 2013). Shikimate pathway starts with the formation of 3-deoxy-D-arabino-

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heptulosonate-7-phosphate (DAHP) which is catalyzed by DAHP synthase isoenzmyes (aroF, aroG, aroH) by the coupling of glycolysis and pentose phosphate pathway (Fig. 1a). The resulting DAHP is further catalyzed through three enzymatic steps to form shikimate. In turn, shikimate is transformed to shikimate-3-phosphate by the shikimate kinase (*aroK* and aroL) (Fig. 1). Further, shikimate-3-phosphate is channeled to form the aromatic amino acids phenylalanine, tyrosine, tryptophan and other aromatic products through chorismic acid. Several modifications of central carbon metabolism (CCM) and SA pathway in *E. coli* have been executed to enhance the shikimic acid yield (Escalante et al. 2010; Frost et al. 2002; Knop et al. 2001; Chen et al. 2014; Ghosh et al. 2012).

The modifications of the SA pathway mainly include the partial or total blockage of the SA flux into subsequent conversions, which has been achieved by decreasing or completely blocking shikimate-3-phosphate (SHK-3P) synthesis by inactivating the aroL and *aroK* genes. To avoid possible feedback inhibition of 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHPS), the first step of the SA pathway, plasmid-coded feedback resistant (fbr) aroF or aroG genes have been expressed (Johansson and Lidén 2006; Zhou et al. 2010). The aroB and aroE genes, encoding the rate-limiting enzymes, 3-dehydroquinic acid (DHQ) synthase and shikimate dehydrogenase, respectively, have also been overexpressed to increase SA production (Krämer et al. 2003; Johansson et al. 2005). Combined effect of CCM modification and *aroK*/*aroL* knockout was studied to increase the SA production (Chen et al. 2012; Cui et al. 2014). Inactivation of *aroK* gene with deletion of aroL, ptsHIcrr and ydiB, and overexpression of tktA, glk, aroE and aroB genes generated an *E.coli* strain which produced 1.85 g/L shikimic acid (Chen et al. 2012). Based on the inactivation of ptsHIcrr, *aroK*, *aroL*, pykF and lacI genes and constitutive expression of selected genes from the pentose phosphate and aromatic pathways, a high yielding strain of *E. coli* was constructed with a yield of 43 g/L (Rodriguez et al. 2013). In recent years, strains other than *E. coli* are exploited to develop metabolically engineered organisms for higher SA titer. Simultaneous overexpression of genes for 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase (aroA) and SA dehydrogenase (aroD) in *Bacillus subtilis* was mentioned in literature which resulted in SA titer of 3.2 g/L (Liu et al. 2014). A modified *B. subtilis* strain with inactivation of pyruvate kinase and shikimate kinase was also reported to produce 4.67 g/L titer of shikimate (Licona-Cassani et al. 2014).

Here we report a modified strain of *Bacillus megaterium* with *aroK* (shikimate kinase) knockout for the enhanced titer of shikimic acid. Using *B. megaterium* for this type of study is advantageous over the other non *E. coli* organisms, as it is a more stable system (Suarez et al. 2012). It has been used as a new model to study the effect of pathway modifications on shikimic acid yield. Homologous recombination based gene disruption has been used to generate this Δ*aroK* strain of *B. megaterium* (Fig. 1b). This is the first report of *aroK* knockout in *B. megaterium* to engineer the shikimate pathway for the production of shikimic acid.

Materials and methods

Bacterial strains, plasmids and reagents

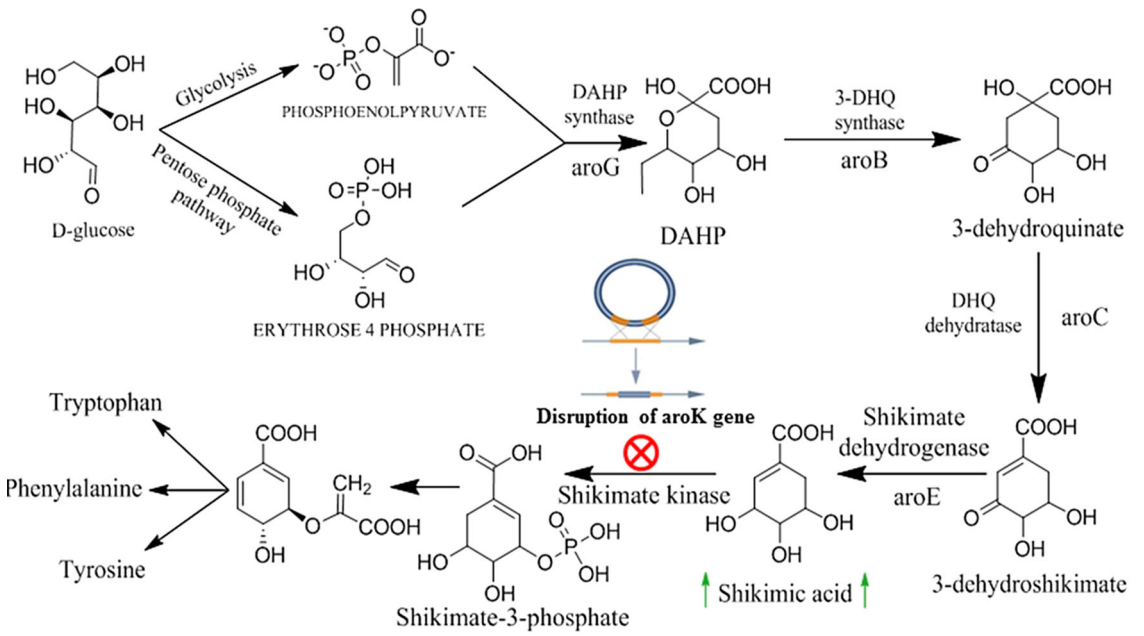
The wild type strain *B. megaterium* MTCC428, procured from Microbial Type Culture Collection Center, Institute of Microbial Technology, Chandigarh, India was used for generating the knockout strain. The *E. coli* strain Top10 (gifted by the Department of Biotechnology, NIPER, India) was used for plasmid maintenance. Plasmid vector pBR325 (MobiTec, Germany) was used as template for generating the Cm^R gene cassette and shuttle vector pWH1520 (MobiTec, Germany) was used for generating the final construct. All the restriction enzymes, T4DNA ligase and other PCR related reagents were purchased from Fermentas (Thermo Scientific, Germany). Primers were obtained from IDT (USA).

Growth medium

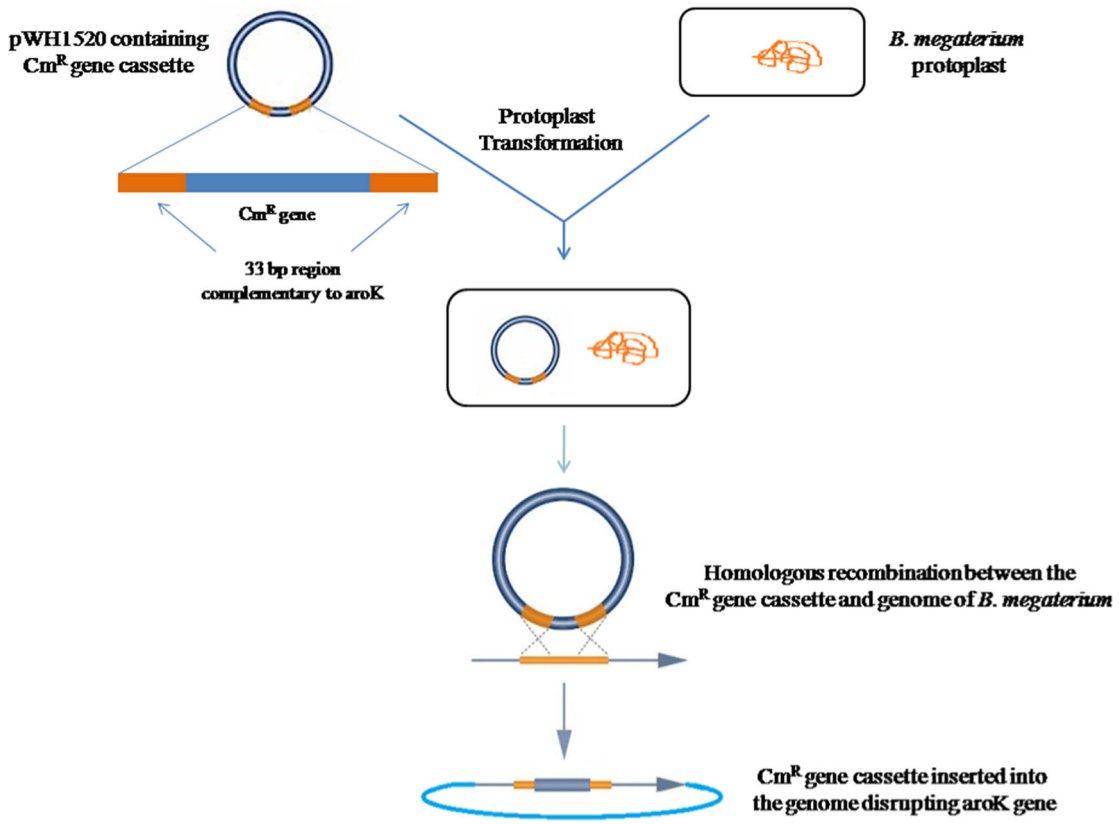
E. coli Top10 and wild type *B. megaterium* were cultured in Luria-Bertani (LB) medium which was procured from Hi-Media (Mumbai, India). Antibiotics, aromatic amino acids and carbon sources were purchased from Hi-Media (Mumbai, India). For selection of transformants, ampicillin (100 μg/mL, for *E. coli*), tetracycline (10 μg/mL, for *B. megaterium*) and chloramphenicol (5 μg/mL) were used.

Construction of antibiotic resistance gene cassette

In homologous recombination based knockout mutant generation, construction of antibiotic resistance gene cassette is the crucial part. This cassette consists of any antibiotic resistance gene with two flanking regions having sequence homology with the target gene. The length of the flanking regions plays an important role and success rate of recombination also depends on it. Systematic gene inactivation using specialized vector was reported in *B. subtilis* with flanking region of 17 bp, homologous to the target gene (Vagner et al. 1998). In another report, the effect of flanking region length was studied using 10–70 bp homologous sequence of target gene (Yu et al. 2000). It was seen that flanking region of 30-50 bp was sufficient for successful recombination (Yu et al. 2000) and with 30 bp



(a)



(b)

Fig. 1 **a** Shikimate pathway showing the aroK gene targeting, **b** generation of ΔaroK strain using homologous recombination mediated gene disruption

flanking region, 10^4 recombinants with Cm^R gene cassette was obtained. Here, chloramphenicol resistance (Cm^R) gene cassette was generated using PCR, with primers having 30 bp homologous sequence of *aroK* gene as the left and right flanking regions. Cm^R gene was amplified from plasmid pBR325, maintained in *E. coli* Top10 cells.

PCR amplification of Cm^R gene cassette

Cm^R gene cassette was amplified from pBR325 vector as template. The reverse and forward primers were designed in such a way that it contains 33 bp homology sequence of *aroK* gene.

Sequence of primer

SpeI
Forward primer: 5'GATCTACTAGTGATTTTGTCGATATAGACCAAGAAATTGAAA
AAATGGAGAAAA AAATCACTGGATAT 3'

KpnI
Reverse primer: 5'GATCTGGTACCGAGGTTTCATCTTTGACTGTTAAACCTATAACTTTA
CGCCCCGCCCTGCCACTCATC 3'

The final amplicon will have the Cm^R gene with two flanking region at both end, complementary to the *aroK* gene. The PCR reactions were performed in Eppendorf Thermocycler. The PCR protocol comprised with an initial denaturation step (95 °C, 5 min), followed by 30 cycles consisted of a denaturation step at 95 °C for 1 min, an annealing step at 55 °C for 1 min, and an elongation step at 72 °C for 1 min. The PCR products were separated by electrophoresis using 0.8 % (w/v) agarose gel and DNA bands were stained with ethidium bromide. The desired band observed on gel was cut and collected in a micro-centrifuge tube. The PCR product (Cm^R gene cassette) was extracted from gel slice using gel extraction kit (Thermo, India) and kept at −20 °C for future use.

Cloning of Cm^R gene cassette into vector pWH1520

The Cm^R gene cassette and the vector pWH1520 were digested with *SpeI* and *KpnI* (Fast digest, Fermentas, 37 °C, 4 h). Double digested vector was given CIAP (Calf intestinal alkaline phosphatase) treatment to prevent self ligation. After digestion, the double digested vector and insert was extracted from agarose gel using Gel extraction kit. ATP-dependent T4 DNA ligase was used for ligation in 1:5, vector:insert ratio (16 °C, 16 h). The ligation mixture was transformed into competent *E. coli* Top10 cells and the transformants were checked on LB-ampicillin plate (Sambrook et al. 1989). To confirm the successful cloning of Cm^R gene cassette into pWH1520, the clones were double digested with *KpnI* and *SpeI* (Fast digest, 37 °C, 4 h). Further, the vector-construct was transformed into *B. megaterium* protoplasts following the method reported by Biedendieck et al. (2011). After transformation, the protoplasts were plated on Tet-LB-agar plate and incubated at 37 °C to check for positive clones.

Detection of *aroK* knockout mutant of *B. megaterium*

PCR based detection

Colonies were taken from the Tet-LB agar plates and grown in 20 mL Cm-LB medium (5 µg/mL chloramphenicol) at 37 °C for 24 h. Cells were harvested by centrifugation (at 10,000×*g*) and genomic DNA was isolated using the genomic DNA isolation kit (Macherey-Nagel). PCR amplification was carried out using forward and reverse primer of chloramphenicol resistance gene and genomic DNA as the template following the same reaction condition as mentioned previously.

Shikimate kinase assay

The *aroK* knockout strain was grown in 50 mL Tet-LB medium at 37 °C for 24 h. Cells were harvested by centrifugation at 7000×*g* for 10 min and resuspended in lysis buffer (50 mM Tris, 0.4 mM dithiothreitol and 1.2 mM PMSF). Further, the cells were disrupted using a French Press at 600 psi and the homogenate was centrifuged (15 min, 4 °C, 10,000×*g*). The supernatant was collected and used for the assay. To estimate the shikimate kinase activity of crude extract, method reported in literature was followed (Pittard and Wallace 1996). The reaction mixture contained 4 µmol ATP, 1 µmol SA, 10 µmol NaF, 5 µmol $MgCl_2$, 25 µmol Tris-HCl buffer (pH 9.0) and cell extract with 0.1–1.0 mg protein in a total volume of 1.0 mL. The appropriate substrate, enzyme and ATP blanks were used in the assay. Shikimate kinase activity was determined by measuring the disappearance of SA (Gaitonde and Gordon 1958). To the reaction mixture, 0.5 mL periodic acid (1 %, w/v) was added, mixed thoroughly and incubated at 37 °C for 3 h. The solution was

made alkaline by the addition of 0.5 mL 1 N NaOH and further incubated at 37 °C for 10 min. Finally, 0.3 mL glycine (1 M) was added as color stabilizer. The optical density of the yellow color formed was measured at 380 nm. One unit of shikimate kinase was defined as μmol of SA disappeared per minute per μg of protein (Chen et al. 2012).

SDS-PAGE analysis

Protein sample of the *aroK* knockout strain was checked for expression profile. Wild type strain was used as control for comparison of protein profile. SDS-PAGE of 18 % was used for the analysis following standard protocol (Sambrook et al. 1989). Standard protein ladder (broad range, 6.5–200 kDa) was used as the marker. Post run, the gel was stained with Coomassie blue and destained following the standard protocols to check for disappearance of shikimate kinase (~20 kDa) band.

HPLC analysis of the culture broth

Shikimic acid concentration in the culture broth was determined by reversed-phase HPLC [Waters Alliance e2695 series and Alltech OA-2000 organic acid column (100 × 6.5 mm, 6.5 μm; Grace Davison Discovery Science, USA] maintained at 30 °C. The mobile phase was 0.005 N H_2SO_4 with a flow rate of 0.3 mL/min. Shikimic acid was detected at 254 nm with a photodiode array detector and quantified using a standard curve (Ghosh and Banerjee 2015).

Growth and production of shikimic acid by *aroK* knockout mutant of *B. megaterium*

To study the effect of *aroK* knockout on the growth and shikimic acid yield, the Δ*aroK* strain was grown in LB-Tet medium for 72 h at 37 °C (200 rpm) at shake flask level. Samples were taken at regular time interval and dry cell weight was checked to study the growth pattern. The samples were analyzed by reversed-phase HPLC to estimate the shikimic acid concentration. As per the literature reports, blocking the aromatic amino acid synthesis pathway by *aroK* knockout results in less growth. To overcome this effect, aromatic amino acids [tryptophan 20 μg/mL; tyrosine (5 μg/mL); and phenyl alanine (10 μg/mL)] were added in the medium.

Determination of specific growth yield (μ), growth yield coefficient $Y_{(X/S)}$, specific substrate utilization rate (q_S) and specific product formation rate (q_p)

Growth and substrate utilization has a direct relationship for any organism. Here, the specific growth rate (μ) and cellmass yield coefficient ($Y_{X/S}$) has been determined separately and both the values have been used to determine the specific substrate utilization rate (q_S).

The specific growth rate was calculated from the following formula:

$$\frac{dx}{dt} = \mu \cdot X \quad \text{or} \quad \mu = \frac{1}{t}\ln(X_1/X_2)$$

where X_1 = Initial cellmass concentration; X_2 = Final cellmass concentration; t = Time of growth.

The specific substrate utilization rate (q_S) is determined to show the substrate utilization profile of the strain. To determine q_S and q_p, different glucose concentrations (0.5, 1, 1.5, 2, 2.5, 3 %, w/v) were used and both the growth and residual glucose concentration were measured using the standard procedures. The slope obtained from the graph between $X_m - X_0$ (final dry cell weight) and $S_0 - S$ (substrate utilized) is the cellmass yield co-efficient, $Y_{(X/S)}$.

$$X_m - X_0 = Y_{(X/S)}(S_0 - S)$$

where, X_m = final cellmass; X_0 = initial cellmass; S_0 = initial substrate concentration; S = residual substrate concentration.

$$\text{The growth yield coefficient} = Y_{(X/S)} = \frac{dx}{ds} = X_m - X_0/(S_0 - S)$$

The substrate utilization coefficient (q_S) is calculated from the formula given below:

$$Y_{(X/S)} = \mu/qs$$

therefore, $q_S = \mu / Y_{(X/S)}$

The specific product formation rate is determined to estimate the product formation rate with respect to cellmass growth. The product formed was analyzed by HPLC at different time intervals. The concentration of shikimic acid was calculated from standard curve. The slope obtained from the graph between $P - P_0$ (product formed) and $S_0 - S$ (substrate utilized) is the product yield coefficient ($Y_{P/S}$). The specific product formation rate (q_p) is calculated from the formula given below:

$$Y_{(P/S)} = \frac{1/x \cdot \frac{dP}{dt}}{1/x \cdot \frac{dS}{dt}} = \frac{q_P}{q_S}$$

$$q_P = Y_{(P/S)} \cdot q_S$$

This was calculated for wild type and as well as Δ*aroK* strains of *B. megaterium*. A comparative study between these strains was carried out to show the effect of metabolic engineering on the growth profile and product formation rate.

Optimization of carbon source for the growth and production of shikimic acid using *aroK* knockout mutant of *B. megaterium*

To study the effect of carbon source on the growth and shikimic acid production, using *aroK* knockout mutant of *B. megaterium*, various carbon sources (glucose, maltose, fructose, lactose, sucrose, starch etc.) were used. Each carbon source was added in the concentration of 1 % (w/v) in LB medium. After inoculation, the flasks were kept in an incubator shaker at 37 °C (200 rpm) for 72 h. Samples were taken at different time interval to measure the growth of cellmass and residual sugar concentration. Shikimic acid concentration was determined by reversed-phase HPLC. Further, the concentration of the optimized carbon source was increased from 1 to 5 % (w/v) and samples were analyzed accordingly.

Reactor studies

A 14 L bioreactor (Bioflo 310, New Brunswick) with a working volume of 10 L was used for the growth and production of shikimic acid by the knockout strain of *B. megaterium*. LB medium with the optimized carbon source was used. The starter culture was grown in the presence of tetracycline (10 µg/mL) for the selective growth of the knockout strain. The reactor was aerated at 0.5 VVM and the dissolved oxygen concentration was maintained above 30 % saturation by agitation at 200–500 rpm. Antifoam (polypropylene glycol) was added manually when needed. Samples were withdrawn at a regular time interval and processed accordingly. Cells were separated by centrifugation, residual glucose was measured by DNS method and shikimic acid was extracted and estimated by the standard method (Bochkov et al. 2012; Ghosh and Banerjee 2015).

Results

Cloning of Cm^R resistance gene cassette

The vector construct, isolated from the successful transformants, was given double digestion with *KpnI* and *SpeI* (Fast digest, 37 °C, 4 h). Distinct band of insert i.e. Cm^R gene cassette (~0.8 kb) and digested vector was observed on gel indicating the successful insertion and formation of the desired vector construct for recombination (See Figure S1 in supporting information). Further, the vector construct was transformed into the protoplasts of *B. megaterium and* clear colonies on LB-Tet-agar plate confirmed the successful transformation.

Detection of homologous recombination in transformed *B. megaterium*

PCR based detection

The mutant strain was grown in chloramphenicol-LB medium to check for insertion of the Cm^R gene into the genome. As the Cm^R gene is under the control of xylose inducible promoter, so in the absence of xylose induction, the strain can only confer chloramphenicol resistance if the Cm^R gene gets integrated into the genome of *B. megaterium*. PCR reaction was carried out using the genomic DNA isolated from the cells, grown in Cm-LB medium. After the PCR reaction, distinct band (~0.8 kb) was observed (Figure S1) in gel (0.8 %, *w/v*) confirming the integration of Cm^R gene into the genome of *B. megaterium*.

Shikimate kinase assay

To compare the shikimate kinase activity between the wild type and the knockout strain, shikimate kinase was assayed following the protocol previously discussed. The supernatant of the cell lysate was used for the assay. The shikimate kinase activity in the *aroK* knockout strain has decreased to 0.05 U/µg and it was 35 % of the wild type (0.14 U/µg).

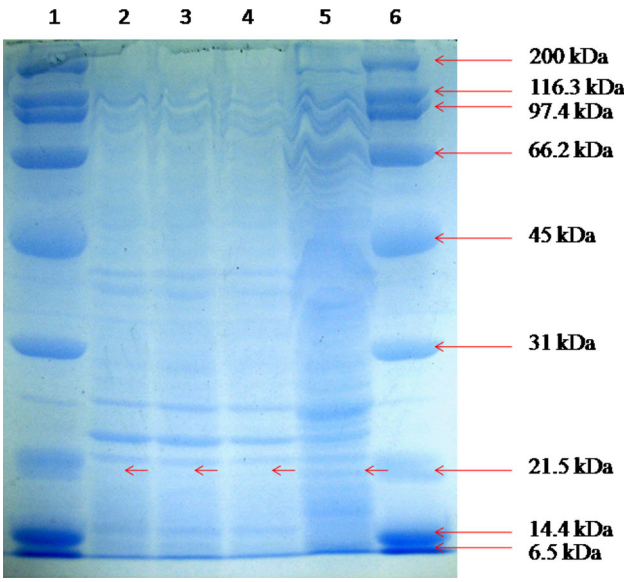


Fig. 2 SDS PAGE (18 %) showing the comparison between the wild type and ΔaroK strain of *Bacillus megaterium* with distinct difference in the expression profile. *Lane 1* and *6* Protein marker (6.5 kDa to 200 kDa); *Lane 2, 3*, and *4* ΔaroK strain; *Lane 5* wild type. (The *red arrow* indicating the loss of band in ΔaroK strain with reference to the wild type). (Color figure online)

Protein expression based detection

Protein expression profile of both *aroK* knockout and wild type strain was studied to check for *aroK* expression. Samples were prepared following the protocol mentioned in materials and methods section and loaded into gel (SDS-PAGE 18 %) for analysis. Post run, a loss of band around 20 kDa region (Fig. 2) was observed in the gel which is similar to that of shikimate kinase reported in literature (Cheng et al. 2005). It supports the inference of PCR-based detection of recombination based insertion of Cm^R-gene cassette into the genome within *aroK*.

Shikimic acid production using the *aroK* knockout strain of *B. megaterium*

The slowing down of growth due to the knocking out of *aroK* (shikimate kinase) gene, blocking the aromatic amino acid biosynthesis was previously reported by Davis in 1952. Aromatic amino acids, tryptophan (20 µg/mL), tyrosine (5 µg/mL) and phenyl-alanine (10 µg/mL) were added in the medium to support the growth. Samples were taken at different time intervals and cellmass growth and shikimic acid yield were analyzed accordingly. It is evident from Fig. 3 that the growth has been reduced significantly in the Δ*aroK* strain compared to the wild type, though the shikimic acid yield of the knockout strain has been increased 6 times over the wild type strain producing 2.98 g/L.

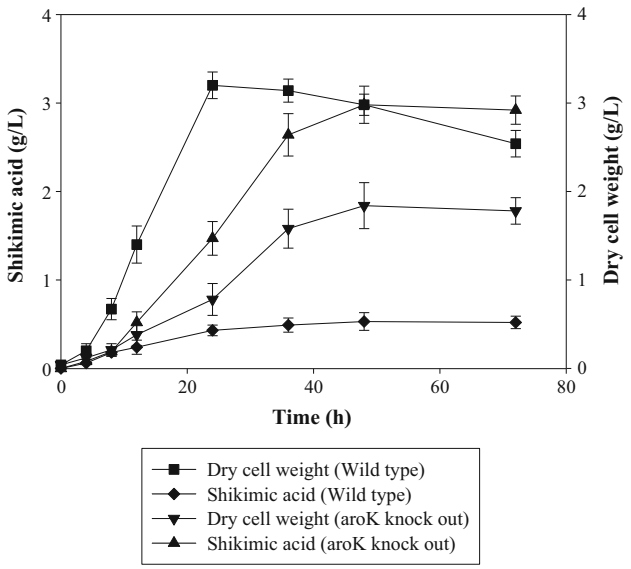


Fig. 3 Comparison between wild type and ΔaroK strain of *B. megaterium* for growth and shikimic acid production

Relationship of specific growth, substrate utilization (q_S) and product formation (q_P) rates of *aroK* knockout strain of *B. megaterium*

The specific growth rate (µ) was calculated to be 0.275 h^{-1} from the graph (See Figure S2 in supporting information). The cellmass yield co-efficient ($Y_{X/S}$) was found to be 0.337. The specific substrate utilization rate (q_S) was found to be ($Y_{X/S} = \mu/q_S$, therefore, $q_S = \mu/Y_{X/S}$, $q_S = 0.275/0.337$) 0.81 h^{-1}. The specific substrate utilization rate of knockout strain (0.81 h^{-1}) is higher than that of the wild type (0.69 h^{-1}). This shows that the *aroK* knockout has positive effect on the organism in terms of substrate utilization, to channel more substrate towards growth and metabolism. The specific product formation rate (q_P) shows the capability of the strain for utilizing the substrate to channelize it to product. The product yield co-efficient ($Y_{P/S}$) of 0.457 was determined from the graph (Figure S2). The specific product formation rate (q_p) was determined to be ($Y_{(P/S)} = q_p/q_S$, therefore, $q_p = Y_{(P/S)} \times q_S$, $q_p = 0.457 \times 0.81$) 0.37 h^{-1}. The specific product formation rate of knockout strain (0.37 h^{-1}) is higher than that of wild type (0.05 h^{-1}) which is ~7 fold enhancement in the Δ*aroK* strain. It shows that the *aroK* knockout has significant effect in enhancing the shikimic acid yield as it channelled more substrate towards the product formation.

Effect of carbon source for the production of shikimic acid using *aroK* knockout strain of *B. megaterium*

Different carbon sources (1 %, w/v each) such as glucose, maltose, sucrose, fructose, soluble starch, and lactose were used to study their effect on the yield of shikimic acid. Total residual sugar content was estimated by DNS method. The maximum production of shikimic acid (3.8 g/L) was achieved with 1 % fructose as carbon source. Hence, fructose has been selected for further optimization experiments.

Table 1 Cellmass growth and shikimic acid yield with different carbon sources by the knock out strain of *B. megaterium*

Carbon source (10 g/L)	DCW (g/L)	Shikimic acid (g/L)	$Y_{P/X}$
Glucose	1.52	2.94	1.93
Lactose	1.49	1.42	0.95
Maltose	1.63	0.86	0.52
Starch	1.63	3.14	1.92
Sucrose	1.43	2.97	2.07
Fructose	1.42	3.8	2.67

$Y_{P/X}$ = Product yield with respect to cellmass

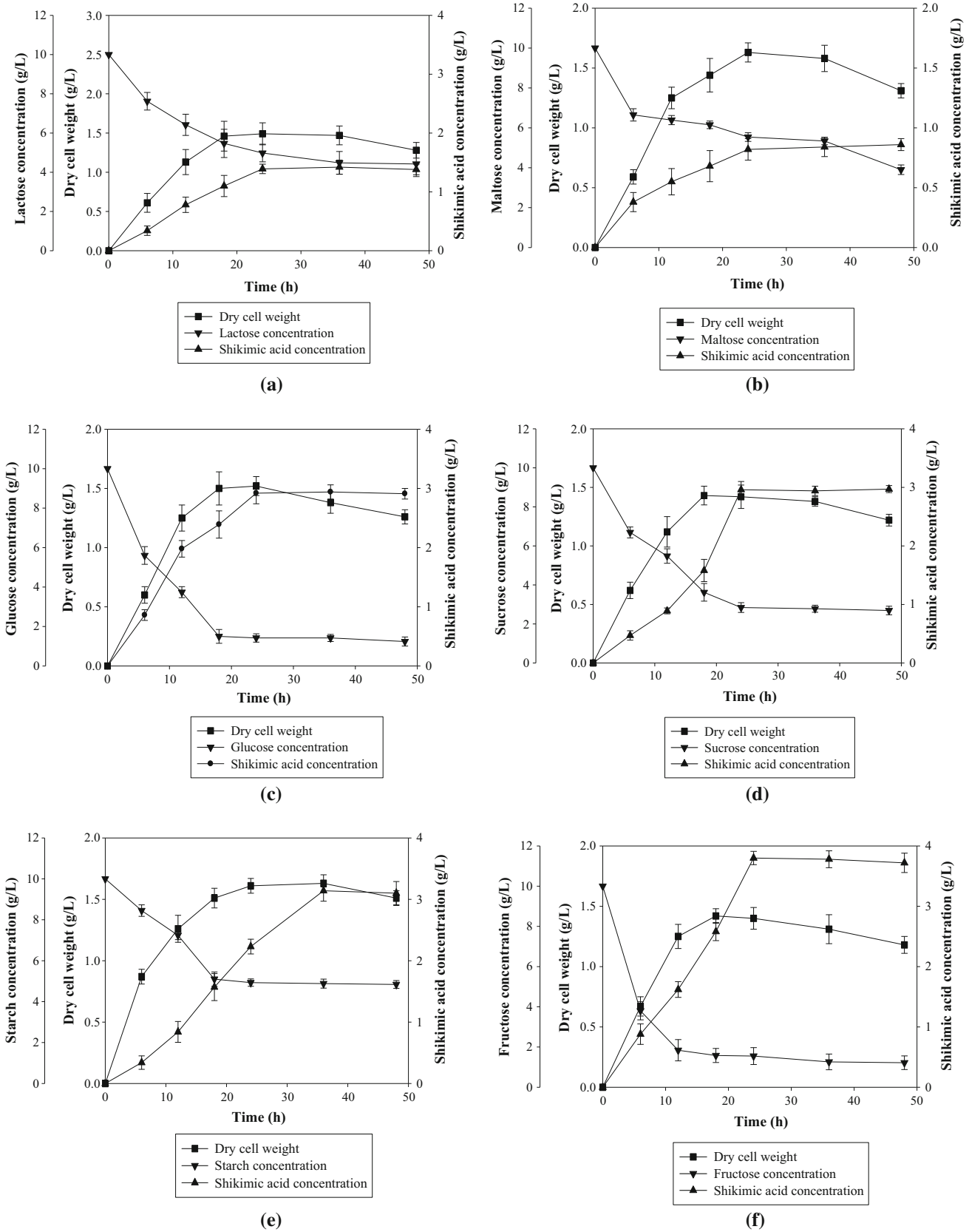


Fig. 4 Effect of carbon sources on the yield of shikimic acid using ΔaroK strain of *B. megaterium* **a** lactose, **b** maltose, **c** glucose, **d** sucrose, **e** starch, **f** fructose

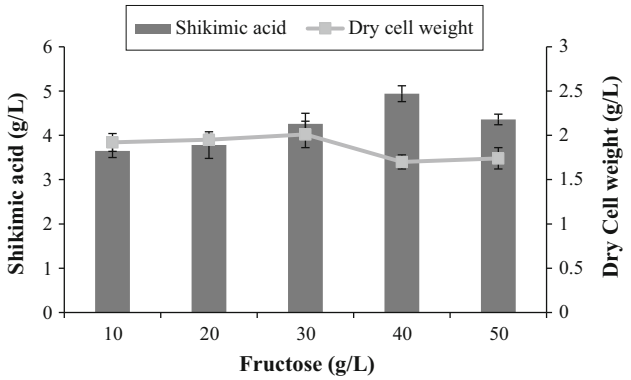


Fig. 5 Effect of fructose concentrations on the production of shikimic acid using aroK knock out strain of *B. megaterium*

The growth of cellmass and the shikimic acid production with various carbon sources are shown in Table 1. The shikimic acid yield with various carbon sources with respect to cellmass was then calculated. Although glucose is a highly metabolizable carbon source, the highest shikimic acid yield (2.67 g/g_{DCW}) was observed with fructose. Substrate channeling towards the product formation was more when fructose was used as a carbon source. This data indicates that the shikimic acid yield is not linked with the growth, as there are instances where even the growth was higher in other carbon sources compared to fructose; shikimic acid yield was more with fructose. Further, fructose concentration was increased in the medium from 10 to 50 g/L. It is evident from the Fig. 4 that the shikimic acid yield is increased with the increasing fructose concentration. Highest shikimic acid yield of 4.94 g/L was obtained with 40 g/L fructose in the medium (Fig. 5).

Production of shikimic acid using the Δ*aroK* strain in laboratory scale fermenter

For scaling up the shikimic acid production, batch fermentation in LB medium (with 4 % fructose) was performed with the Δ*aroK* strain of *B. megaterium* in a 14 L fermenter. It is evident from Fig. 6 that fructose concentration started decreasing from the very beginning upto 48 h and the residual fructose concentration was found to be 2.88 g/L at the end of fermentation. The maximum shikimic acid concentration of 6.02 g/L was obtained after 36 h, as analyzed and quantified by reversed-phase HPLC. This shows enhancement in shikimic acid yield over the previously reported aroK knockout strain of *Bacillus* sp. (Licona-Cassani et al. 2014) and makes it a better process in terms of substrate utilization. The cellmass concentration started increasing from the very beginning and a maximum of 4.03 g/L cellmass was obtained at 40 h. DO concentration showed the downward trend initially along the course of growth and reached almost zero at the end of fermentation. Finally, it started increasing with the cease of growth and reached to the saturation level at the end of fermentation. Agitation rate was increased upto 500 rpm starting with an initial mixing rate of 200 rpm. In order to keep the DO level high, agitation rate was continuously increased during the course of fermentation. The initial pH of the fermentation medium was 7.0 and during the course of growth, the pH of the broth was on the increasing side and it reached upto pH 8.61 at 32 h. The shikimic acid from the fermenter broth was isolated by using amberlite (IRA-400) anion exchange resin and further characterized by HPLC (See Figure S4, S5 in supporting information)

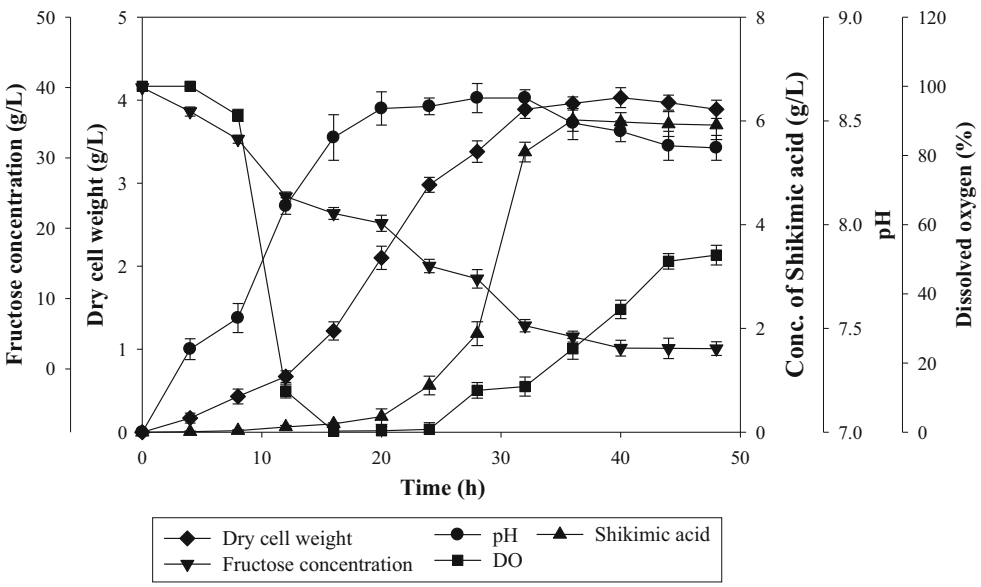


Fig. 6 Course of growth and shikimic acid production in a 14 L bioreactor using the ΔaroK strain of *B. megaterium*

and mass spectrometry (See Figure S6 in supporting information).

Discussion

In this work, we demonstrate the enhanced production of shikimic acid by *aroK* knockout strain of *B. megaterium.* Though metabolic engineering of shikimic acid pathway is not new (Kramer et al. 2003; Escalante et al. 2010; Frost et al. 2002; Knop et al. 2001), to best of our knowledge we are the first to report *aroK* knockout mutant in *B. megaterium* system for the production of shikimic acid. Homologous recombination based gene disruption is used to generate the Δ*aroK* strain of *B. megaterium*. The antibiotic resistance gene cassette with 33 bp homologous region of target gene *aroK* at both the flanking region has been used for the recombination. The vector-construct with the Cm^R gene cassette was transformed into *B. megaterium* and recombination occurred between the Cm^R gene cassette (with 33 bp homologous flanking region to aroK gene) and the *B. megaterium* genome. In this process, the Cm^R gene got integrated into the genome disrupting the *aroK* gene. The insertion of Cm^R gene into the genome was further confirmed by PCR based detection, shikimate kinase assay and by comparing the expression profile of shikimate kinase with the wild type. Shikimate kinase activity in the knock out strain was assayed and it was found to be 32 % of the wild type indicating the disruption of aroK gene. On confirmation, the mutant strain was used for the production of shikimic acid. At shake flask level, shikimic acid titer was 2.98 g/L which is ~6 fold higher over the wild type (0.53 g/L). This shikimic acid titre is better over the previous report by Chen et al. 2012 in *E. coli* system (1.85 g/L). Various biochemical engineering parameters were analyzed and compared with the wild type. It was found that the specific substrate utilization rate (q_S) and specific product formation rate (q_p) both has enhanced markedly in the knock out strain over the wild type. Effect of various carbon sources on the shikimic acid yield by the *aroK* knockout strain was studied. Among all the carbon sources studied, fructose (4 %, *w/v*) was found to be the most effective one for shikimic acid production with Δ*aroK* strain. Further, it has been scaled up to 10 L bioreactor and shikimic acid titre of 6 g/L was achieved showing significant enhancement. It shows the importance of blocking the pathway downstream of shikimic acid by knocking out the *aroK* gene. The aroK knock out strain showed less formation of side products as compared to the similar knock out strains reported in other microorganisms (Chen et al. 2012; Liu et al. 2014; Licona-Cassani et al. 2014). It suggests that by generating aroK knock out mutant in *B. megaterium*, one can have better metabolic control than other microbial platforms used for shikimic acid production. An advanced strain of industrial potential may be developed for shikimic acid production by combining the aroK knock out with other pathway modifications.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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