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Research Article

Metabolic Engineering of *Escherichia coli* for Production of Aroma Compounds 2-phenylethanol and 2-phenylethylacetate

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Abstract

Microbial cell factories for production of fragrant and flavour molecules provide sustainable and attractive alternative for food and cosmetic industries. Majority of the bio-derived fragrant and flavour molecules are derived as either single compound or as mixture from flowers, fruits, seeds or even animals. Two such specific compounds that are used extensively in food and cosmetics industries are 2-phenylethanol (2-PE) and 2-phenylethylacetate (2-PEA) which give characteristic rose and jasmine aroma respectively. In this study, we describe a metabolic engineering approach for developing microbial cell factories for production of 2-PE and 2-PEA. *E. coli* DH5α produced 132.66 mg L^{-1} of 2-PE and 181.7 mg L^{-1} of 2-PEA. IPTG induced *E. coli* BL21 (DE3) culture produced 96.08 mg L^{-1} of 2-PE and 97.28 mg L^{-1} of 2-PEA in Luria – Bertani (LB) media. Engineering shikimate pathway can lead to production of many aromatic compounds. This study provides basis for *E. coli* as a platform for production of flavour and aroma compounds using synthetic biology tools.

Keywords: Metabolic Engineering; Aromatic Compounds; Gas Chromatography; Shikimate Pathway

Introduction

The global market size for flavors and fragrances was valued at USD 20.75 billion in 2018. This market is expected to grow at a CAGR of 4.7% till 2025 [1]. Increasing demand from application industries such as food, beverages, and cosmetics and toiletries is projected to drive this growth. Each flavour or fragrance is a property of a single compound or mixture of compounds. Solubility and volatility are properties that affect efficacy of different aromas [2]. Global warming is affecting the ability of plants to produce these compounds. Some compounds with high volatility get evaporated or are easily oxidised due to heat. Thus, it is imperative to create alternate sources for production of these compounds [3]. Some of the most commonly used aromas are of flowers such as rose, jasmine, lily, lavender, etc. Scent of rose has a traditional, aesthetic and medicinal value. Perfume industry today, relies on extraction of natural fragrance compounds from flowers or plant extract or production using chemical reactions. Chemical synthesis of these molecules is neither sustainable nor eco-friendly and not always suitable for applications due to impurities [4]. In addition, fluctuation in supply of raw materials and challenges in logistics management impact the quality and yield across various seasons. All of this add up to the health hazards that chemical manufacturing brings with it. Screening of compounds specifically responsible for fruit-like or rose like aroma has led to identification of 2-phenylethanol (2-PE) and its ester 2-phenylethylacetate (2-PEA) [5]. These compounds are currently produced by bromination of benzene in presence of

pyridine. This creates bromobenzene which is further reacted with magnesium metal to result in phenylmagnesium bromide. In a separate reaction, ethene is generated from ethanol by reacting with sulphuric acid at 150-170°C, which is further reacted with bromine to form 1,2-dibromoethane. Both phenylmagnesium bromide and 1,2-dibromoethane are converged in presence of ether and aqueous ammonium chloride to form 2-phenylbromoethane. Reaction of 2-phenylbromoethane in presence of ether and sodium hydroxide results in 2-PE and side product diethyl ether [6]. There is an increasing demand for 2-PE and similar other compounds and legal restrictions abstain the use of chemically synthesized aroma compounds as it can have potentially hazardous isomers and by products. This means use of naturally, biologically manufactured aroma and flavour compounds is a must [7]. 2-PE has anti-microbial properties and this adds to the value of product [8].

Biologically produced natural 2-PE is mainly extracted from rose petals using solvents. However lower yields make it very expensive and time consuming process. This process is also not very sustainable and yield of the active compounds greatly varies in every extraction processes. Moreover, global warming is causing many flowers to lose their aroma as the heat and climate changes, either oxidises the compounds or affects quality of plant material. Many yeast strains naturally produce small amounts 2-phenylethanol (2-PE) and 2-PEAc from the catabolism of amino acids via the Ehrlich pathway [9].

2-PE can be produced biochemically in microorganisms via the Ehrlich metabolic pathway using L-phenylalanine as a precursor. Another route is the Shikimate pathway, which governs the de novo synthesis of L-phenylalanine. Once L-phenylalanine in the media is scarce, aromatic amino acids are produced from glucose via D-erythrose-4-phosphate. 2-PE production from yeasts is fairly low, usually in the 10–100 mg L^{-1} range, via de novo synthesis [10,11]. Conversion of L-phenylalanine to 2-PE is made possible through a series of enzymes that are specific to their respective substrates. These enzymes are aromatic amino acid transferase (AAAT), phenylpyruvate decarboxylase (PPDC), phenylaldehyde reductase (PAR) and alcohol acetyltransferase (ACT) [12]. Like *E. coli* and yeasts, cyanobacteria can also be genetically engineered to produce aromatic compounds [13].

During this study, we constructed a plasmid that drives expression of all the four genes to produce 2-PE and 2-PEA in *Escherichia coli*. Expression of multiple genes in a cascade can be challenging as the synchrony needs to be maintained. For this we use lac operon as a model. *AAAT* gene was expressed using *T7* promoter and lac operator. Gaps between genes *lacZ*, *lacY* and *lacA* were utilized as spaces between genes *PPDC*, *PAR* and *ACT*, thus creating a construct similar to lac operon. Expression of *PPDC*, *PAR* and *ACT* genes was carried out using lac promo ter and lac operator. We estimate the production of these compounds in *E. coli* and evaluate future scope.

Material and Methods

In-situ plasmid construction and synthesis

Potential gene candidates for each of enzymes *AAAT, PPDC, PAR* and *ACT* for conversion of respective substrates were evaluated using resources from National Center for Biotechnology Information (NCBI) and UniProt. San1: *ARO8* (*AAAT*), San2: 2 *KDC* (*PPDC*), San4: *ATF1* (*ACT*) from Saccharomyces cerevisiae and San3: YjgB (PAR) from *E. coli* and were identified. Each gene was codon optimized for expression in *E. coli* using Optimizer [14]. The expression assembly was created such that *T7* promoter drives the expression of all four genes in synchronisation and for this, spaces between the genes were filled with lac operon as base. Space between San1 and San2 was filled with nucleotides between *LacZ* and *LacI*, space between San2 and San3 was filled with nucleotides between *LacZ* and *LacY* and space between San3 and San4 was filled with nucleotides between *LacY* and *LacA*. Stop codon was added after San4 gene followed by cleaving site for XhoI. As pET28a$^+$ based T7 promoter system would be used, XbaI cloning site was added along with a 41 bp sequence ahead of the San1 gene. Thus XhoI and XbaI restriction would enable integration of entire four gene cascade into the pET28a+ expression plasmid. A 11,477 bp expression plasmid for synthesis of 2-PE and 2-PEA labelled as pet28a$^+$San was developed.

Strains and culture conditions

Escherichia coli strains Dh5α (genotype F$^-$*endA1glnV44 thi-1 recA1relA1 gyrA96deoR nupGpurB20* φ80d*lacZ*ΔM15Δ (*lacZYA-argF*) U169, hsdR17($r_K^-m_K^+$), λ$^-$ [15] and BL21(DE3) (genotype fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5) [16] were used during this study. Dh5α was used for cloning as well as to check for expression, as the spacing model was Lac operon based and there was a chance that expression would occur. Calcium chloride based chemically competent cells were used for transformation of plasmids using heat shock method followed by streaking on Luria

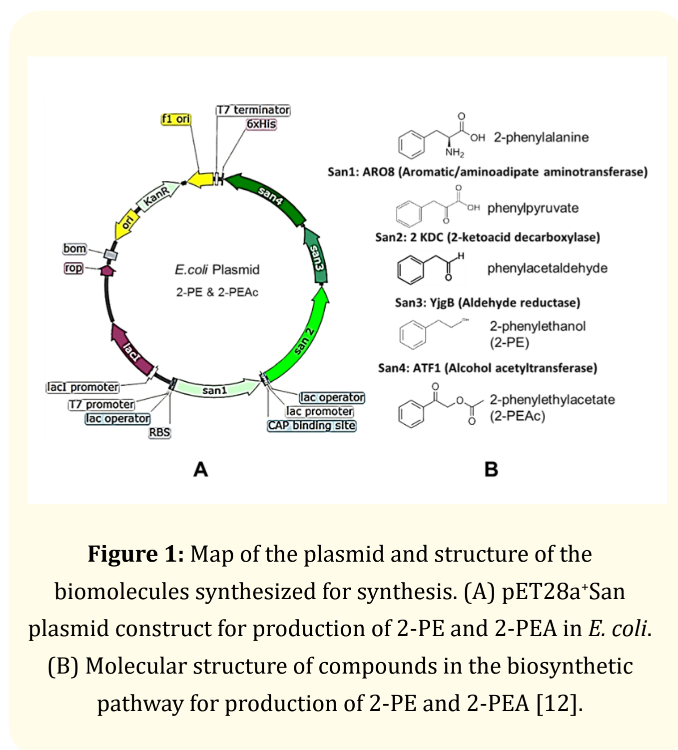

Figure 1: Map of the plasmid and structure of the biomolecules synthesized for synthesis. (A) pET28a+San plasmid construct for production of 2-PE and 2-PEA in *E. coli*. (B) Molecular structure of compounds in the biosynthetic pathway for production of 2-PE and 2-PEA [12].

Bertani (LB) agar plates with kanamycin at 50 µg mL^{-1} as selection marker [17]. These plates were incubated at 37°C for 16 hours. As a negative control, pET28a+ without the gene insert was used. Recombinant colonies of *E. coli* for both Dh5α and BL21 (DE3) strains were streaked on separate LB agar plates with kanamycin.

Restriction digestion for confirmation of transformants

Five colonies of each BL21 (DE3) with pET28a+, BL21 with pet28a+San, DH5α with pET28a+ and DH5α with pet28a+San were picked and grown in 5 mL LB media with kanamycin 50 µg/mL^{-1} concentration for 8 hours at 37°C. Restriction digestion was performed using double digestion with *XhoI* and *XbaI* and single digestion with *XhoI* to confirm plasmid insertion. Starter culture of each of the colonies was maintained or regenerated every time as required.

Growth, induction and chromatography for estimation of 2-PE, 2-PEA productivity chromatography)

10 mL starter cultures of confirmed transformants were inoculated in 100 mL LB media in a 250 mL capacity flask under sterile conditions. The cells were allowed to grow at 37°C under shaking conditions at 200 rpm and reach OD of 0.5 at 600 nm followed by induction with Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 1mM concentration. Cell cultures were further shake incubated at 20°C overnight (12-18 hours) to allow expression and also to reduce evaporation if any. 15 mL of each of the cell cultures were centrifuged at 15,000 rpm for 10 minutes to separate biomass from spent broth. Obtained cell pellets were transferred to 2 mL vials and re-suspended in 500 µL of distilled water. Glass beads were added to this suspension and using a bead beater, cells were lysed [17]. Following cell lysis, 500 µL of chloroform was added to the suspension and the mixture was vortexed and kept steady to allow phase separation. 2-PE and 2-PEA being more chloroform soluble are transferred to this phase. Chloroform phase was collected in a separate vial and 1 µL of this phase was used for analysis using Gas Chromatography Mass Spectroscopy (GCMS). Similarly, spent broth was also subjected to chloroform extraction to identify if 2-PE and 2-PEA are released by cells in the media. Standard compounds for 2-PE (1533250) and 2-PEA (290580) were procured from Sigma Aldrich. Calibration and standard graph were generated using standards. GCMS column and conditions were same as used by Guo., *et al*. 2017. All experiments were conducted in triplicates.

Results and Discussion

Restriction Digestion for confirmation of transformants

The genetic construct has been codon optimized for *E. coli* expression and is based on Lac operon assembly. *DE3* designation in BL21(DE3) means that strains contain the λDE3 pro-phage that carries the gene for *T7* RNA polymerase under control of the lacUV5 promoter. Lactose or its derivative (IPTG) is required to induce expression of the *T7* RNA polymerase in order to express recombinant genes cloned downstream of a *T7* promoter [18]. pET28a+ plasmid usually does not show any expression through *T7* promoter in *E. coli* Dh5α as this strain lacks *T7* RNA polymerase. However, in this assembly, there are two lac operators and two lac promoters.

Restriction digestion was performed to identify transformation of *E. coli* strains and as shown in figure 2. Double digestion using *XhoI* and *XbaI* enzymes was observed on agarose gel following electrophoresis. The plasmid pET28a+San was rightly cut into two DNA bands one of 6285 bp and the other of 5192 bp as observed

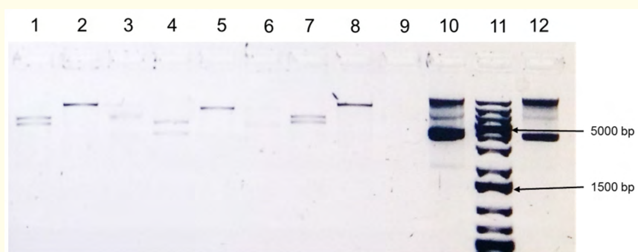

Figure 2: Agarose gel electrophoresis of the restricted plasmids harboring the synthetic genes for PE and PEA. Lane 1,4: pET28a+San from transformed *E. coli* BL21(DE3) double digest with *XbaI* and *XhoI*, Lane 2,5: pET28a+San from transformed BL21(DE3) single digest with *XhoI*; Lane3,6: pET28a+ vector double digest wth *XbaI* and *XhoI*; Lane 7: pET28a+San from transformed *E. coli* DH5α double digest with *XbaI* and *XhoI*; Lane 8 pET28a+San from transformed *E. coli* DH5α single digest with *XhoI*. Lane 9: empty; Lane 10: pET28a+San undigested; Lane 11: Thermo Scientific GeneRuler 1 kb Plus DNA Ladder; Lane12: pET28a+ vector undigested.

in lanes 1 and 4 for BL21DE3 strain and in lane 7 for DH5α. Single digestion was also performed using *XhoI* enzyme and the plasmid pET28a+San linearized to yield a DNA band of 11477 bp. At the same time, pET28a+ vector was also processed using single digestion as positive control. Both the undigested plasmids were loaded on to the gel to observe super coiled, open circular and linear forms (lane10 and 12).

Expression and Chromatography assay

Binding of IPTG to *T7* repressor results in expression of San1 *ARO8* (*AAAT*) gene through the lac operator present within the construct. Further, lac promoter and operator present downstream of san1 drives a polycistronic expression of San2, San3 and San4 to yield *KDC* (*PPDC*), *YjgB* (*PAR*) and *ATF1* (*ACT*) respectively. This expression system is analogous with the native lac operon expression system in *E. coli* providing a stable expression without imposing stress on the organism. Chloroform extract was analysed using GCMS and the obtained chromatogram is shown in figure 3. Interestingly, it was observed the transformed induced Dh5α strain produced 132 ± 24 mg L^{-1} of 2-PE and 181 ± 12 mg L^{-1} of 2-PEA, whereas induced BL21(DE3) produced 96 ± 5 mg L^{-1} of 2-PE and 97 ± 5 mg L^{-1} of 2-PEA. The variation in productivity suggests, the *T7* RNA polymerase may have put additional pressure for over expression of the four proteins required for synthesis of 2-PE and 2-PEA. This in turn would have led to deficiency in the pool of L-phenylalanine. Component analysis of LB media suggests, approximately 490 mg of L-phenylalanine from the casein hydrolysate and 100 mg from yeast extract, providing a pool of approximately 600 mg of L-phenylalanine per litre. If we consider a total of 313 mg L^{-1} of targeted products, we have a conversion rate of 52 % for DH5α strain. Interestingly, addition of 450 mg of crude L-phenylalanine in media did lead to increase in 2-PE and 2-PEA produced by both the strains.

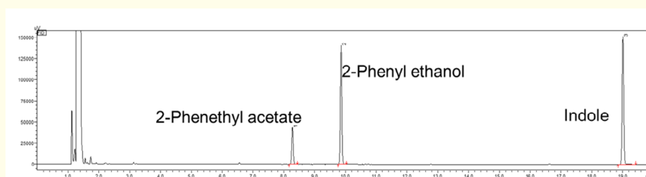

Figure 3: Gas Chromatography Mass Spectroscopy Chromatogram for Chloroform extract from *E. coli* strain indicating retention time for 2-PE, 2-PEA and Indole.(x-axis: retention time, y-axis: abundance).

Strain	L-phenylalanine	2-PE (mg L^{-1})	2-PEA (mg L^{-1})	Conversion (%)
DH5α	600	132.66 ± 24.22	181.70 ± 12.47	52 ± 6
BL21DE3	600	96.08 ± 5.22	97.28 ± 5.61	32 ± 2

Table 1

Comparative assay of *E. coli* cell factories

Guo., *et al*. followed a heterologous gene expression approach where genes ARO8, KDC, YjgB and ATF1 were amplified from *S. cerevisiae* and introduced to *E. coli* MG1655 strain to yield multiple strains, however none of their genes were codon optimized for *E.*

coli. Irrespective of their methodology, they inserted the genes in same pET28a⁺ expression system and obtained expression in *E. coli* MG1655, a *T7* RNA polymerase deficient strain. They observed 45.9 mg L^{-1} of 2-PE and 53.7 mg L^{-1} of 2-PEA. 65% L-phenylalanine was channelled towards 2-PE and 2-PEA production when added in pure form to LB media. Same group also tried genetic engineering by overexpressing 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase *aroGfbr* and chorismate mutase/prephenate dehydratase *pheAfbr* for increasing phenylpyruvate production in *E. coli.* This would allow more glucose to be channelled towards phenylpyruvate production. This approach almost doubled the total production of targeted compounds 2-PE and 2-PEA. During this study, indole which is the waste product for aromatic amino acids was formed. Indole production was almost observed in pET28a⁺ which indicates it is a general product and not resultant of 2-PE or 2-PEA. The net concentration of indole was almost as much as that of 2-PE. Indole at higher concentrations provide a fruity smell, however at lower concentrations it gives an unpleasant fecal smell. Engineering *E. coli* where indole synthesis can be regulated would create opportunity for further increasing productivity of 2-PE and 2-PEA from L-phenylalanine. Recent studies show enhanced production of 2-PE (8.4-9.1 g/L) from glucose or glycerol by coupling of an E. coli expressing the L-phenylalanine biosynthesis pathway for L-phenylalanine production with expressing enzyme cascades of L-phenylalanine to 2-PE [19].

Conclusion

SHIKIFACTORY100 [20] is 8 million euros project by the European Union that focuses on optimizing Shikimate pathway for production of 100 high-added value compounds. This pathway is present bacteria, fungi and plants to synthesize aromatic amino acids phenylalanine, tryptophan and tyrosine. These aromatic amino acids serve as branch points in biosynthesis and metabolism of many valuable compounds. The project aims to establish synthetic biology route in *Saccharomyces cerevisiae* and *Escherichia coli*. Short chain aldehydes such as vanillin, veratraldehyde, and heliotropin along with alcohols, nor-isoprenoids that are made from degradation of terpenoids, and terpenoids themselves like 2-PE and 2-PEA are responsible for characteristic flavors and aromas [21]. While compounds may be produced, it is important that they are perceived rightly by the olfactory transduction mechanism in humans, and this makes it all the more challenging [22]. Furuya., *et al*. [21] using an immobilized process demonstrated vanillin production from ferulic acidto yield vanillin in concentrations between 365 and 578 mg L^{-1} within 24-h of biotransformation cycles. The enzymes used in this process were ferulic acid decarboxylase (Fdc) and coenzyme independ*ent* oxygenase (Cso2). Fungi such as *Yarrowia lipolytica, Kluyveromyces marxianus, Kluyveromyces lactis have been used to produce 2-PE in the range of 0.2 to 1.3 g* L^{-1} *in a shake flask. Diacetyl which provides a buttery aroma has been produced in hosts like Lactobacillus casei*, *Lactococcus lactis*, *Enterobacter aerogenes*, *Candida glabrata* at concentrations ranging from 0.36 g L^{-1} to 4.7 g L^{-1}. Limonene provides orange like citric aroma and is used in many perfumes has been produced in *E. coli* ranging from 0.4 g L^{-1} to 2.7 g L^{-1}. Vanillin has also been produced in *E. coli* and *S.cerevisiae* at titres of 120 mg L^{-1} and 500 mg L^{-1} respectively [23].

The current study uses a novel approach to utilize innate gene expression module in *E. coli* in the form of lac operon. While doing so, it was clear that best promoters such as *T7* DNA polymerase, for gene expression, which result in high protein or enzyme yields, may not be the ideal promoters when the main goal is to biosynthesize a product utilising the activity of these enzymes. Discovering native genetic elements that would catalyse biosynthesis by multifold is thus crucial for synthetic biology projects. Although *E. coli* and yeasts have been studied for a long time, with their genomes sequenced and information on genetic available, one has to realize these organisms require extrinsic nutrients such as peptone, sugars, etc. These nutrients are sourced from animals or plants and thus make the whole production process secondary. Development of synthetic biology tools for cyanobacteria and microalgae will hold key to overcome these limitations. Cyanobacteria and microalgae are capable of producing their own sugars, amino acids, lipids and secondary metabolites through photosynthesis, thus making them independent of any other resource. The scope of producing aromatic compounds in cyanobacteria has recently been studied by Żyszka-Haberecht., *et al*. 2019. Producing aromatic compounds using synthetic biology in microalgae and cyanobacteria has the potential to make the process carbon neutral if not negative and thus is worth pursuing [24,25].

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Declaration of Competing Interest

The authors declare they have no conflict of interest.

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Bibliography

1. Grand View Research. Flavors and Fragrances Market Size, Share and Trends Analysis Report By Product (Natural, Aroma), By Application (Flavors, Fragrances), By Region, And Segment Forecasts, 2019 - 2025.
2. Lukin I., *et al*. "Techniques for the recovery of volatile aroma compounds from biochemical broth: A review". *Flavour and Fragrance Journal* 33.3 (2018): 203-216.
3. Holopainen JK., *et al*. "Climate change effects on secondary compounds of forest trees in the northern hemisphere". *Frontiers in Plant Science* 9 (2018): 1445.
4. Dingsheng L and Sangwon S. "Health risks of chemicals in consumer products: A review". *Environment International* 123 (2019): 580-587.
5. Chreptowicz K., *et al*. "Production of natural 2-phenylethanol: From biotransformation to purified product". *Food and Bioproducts Processing* 100 (2018): 275-281.
6. Unuigboje A and Emenike B. "Convergent preparation of 2-phenylethanol". *African Journal of Pure and Applied Chemistry* 5.6 (2011): 123-126.
7. Zander K and Hamm U. "Consumer preferences for additional ethical attributes of organic food". *Food Quality Prefer* 21 (2010): 495-503.
8. Muller WEG., *et al*. "Biological Activity of 2-Phenylethanol and Its Derivatives". *Archiv fur die gesamte Virusforschung* 40 (1973): 205-214.
9. Chantasuban T., *et al*. "Elevated production of the aromatic fragrance molecule, 2-phenylethanol, using Metschnikowia pulcherrima through both de novo and ex novo conversion in batch and continuous modes". *Journal of Chemical Technology and Biotechnology* 93 (2018): 2118-2130.
10. Herrmann KM. "The Shikimate pathway as an entry to aromatic secondary metabolism". *Plant Physiology* 107 (1995): 7-12.
11. Mir R., *et al*. "The Shikimate pathway: review of amino acid sequence, function and three-dimensional structures of the enzymes". *Critical Review on Microbiology* 41 (2015): 172-189.
12. Guo D., *et al*. "Metabolic engineering of Escherichia coli for production of 2-phenylethylacetate from L-phenylalanine". *Microbiology Open* 6.4 (2017): e00486.
13. Żyszka-Haberecht B., *et al*. "Metabolic relation of cyanobacteria to aromatic compounds". *Applied Microbiology and Biotechnology* 103 (2019): 1167-1178.
14. Puigbo P., *et al*. "OPTIMIZER: A web server for optimizing the codon usage of DNA sequences". *Nucleic Acids Research* 35 (2007): W126-W131.
15. Meselson M and Yuan R. "DNA Restriction Enzyme from E. coli". *Nature* 217 (1968): 1110-1114.
16. Studier FW., *et al*. "Use of T7 RNA polymerase to direct expression of cloned genes". *Methods Enzymology* 185 (1990): 60-89.
17. Green M and Sambrook J. "Molecular Cloning: A Laboratory Manual". 4th ed., 189-191. Cold Spring Harbor: Cold Spring Harbor Laboratory Press (2012).
18. Studier FW and Moffatt BA. "Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes". *Journal of Molecular Biology* 189.1 (1986): 113-130.
19. Balaji SS., *et al*. "Production of Natural 2-Phenylethanol from Glucose or Glycerol with Coupled Escherichia coli Strains Expressing l-Phenylalanine Biosynthesis Pathway and Artificial Biocascades". *ACS Sustainable Chemistry and Engineering* 7.14 (2019): 12231-12239
20. shikifactory100.eu

21. Furuya T., *et al*. "Biotechnological production of vanillin using immobilized enzymes". *Journal Biotechnology* 243 (2017): 25-28.

22. Vilela A., *et al*. "Beverage and Food Fragrance Biotechnology, Novel Applications, Sensory and Sensor Techniques: An Overview". *Foods* 8 (2019): 643.

23. Braga A., *et al*. "Generation of Flavors and Fragrances Through Biotransformation and De Novo Synthesis". *Food and Bioprocess Technology* 11 (2018): 2217-2228.

24. Guo D., *et al*. "Metabolic engineering of Escherichia coli for production of 2-phenylethanol and 2-phenylethylacetate from glucose". *Journal of Agricultural and Food Chemistry* 66.23 (2018): 5886-5891.

25. Kang Z., *et al*. "Metabolic Engineering of Escherichia coli for Production of 2-phenylethanol from Renewable Glucose". *Applied Biochemistry and Biotechnology* 172 (2014).

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