Novel Research in Microbiology Journal (2022), 6(6): 1821-1835
Research Article

(Print) (ISSN 2537-0286)
(Online) (ISSN 2537-0294)
https://nrmj.journals.ekb.eg/
DOI: 10.21608/nrmj.2022.276738

Mevalonate pathway analysis of *Saccharomyces cerevisiae* during bioisoprene synthesis

Simiat O. Jimoh*; Semirah A. Sanusi; Farouq A. Olaogun; Ramot B. Badmos-Oladapo; Kifayat O. Asafa-Adedimeji

Department of Biological Sciences (Microbiology Programme), Fountain University, Osogbo, Nigeria

*Corresponding author E-mail: olanike771@gmail.com

Received: 28 November, 2022; Accepted: 27 December, 2022; Published online: 28 December, 2022

Abstract

Isoprene, synthesized through two complementary biosynthetic routes known as the mevalonate (MVA) pathway and the deoxy-xylulose phosphate pathway, is a valuable monomer that is used for rubber and several other chemical industries. Despite the recent interest in the industrial and biomedical applications of isoprene and its derivatives, the complexity of controlling its chemical synthesis due to the formation of greenhouse gases is a significant problem. To overcome the productivity and yield challenges, in addition to generating environmental and economic benefits, this study aimed to focus on the direct fermentation of cellulosic materials into bioisoprene. In this study, bioisoprene was synthesized via a biotransformation process through enzymatic hydrolysis of cassava peel using *Aspergillus niger* 11JK and *Saccharomyces cerevisiae* 19KB strain. The mevalonate (MVA) pathway (synthetic route) exploited during bioisoprene production by *S. cerevisiae* 19KB strain was investigated using the hydrolyzed cassava peel broth. The obtained crude extract was analyzed for bioisoprene yield and enzymatic activities using Gas chromatography. Furthermore, results of the size exclusion chromatography revealed the presence of polysaccharide hydrolyzing enzymes (e.g., amylase and cellulase), and mevalonate pathway enzymes, including isoprene synthase, mevalonate-5-diphosphate decarboxylase, and isopentyl phosphate kinase, in addition to isoprene, mevalonic acid (MVA), and its isomer dimethylallyl diphosphate (DMAPP). Based on the results obtained in this study, bioisoprene synthesis via direct fermentation of cheap and abundant carbon sources such as cassava peel using the *S. cerevisiae* 19KB strain will overcome the high production costs and low yield challenges of bioisoprene, thus generating significant environmental and economic benefits.

Keywords: Mevalonate, Isoprene synthase, Isopentyl phosphate kinase, Isoprene, Amylase, Cellulase

1. Introduction

Isoprene (2-methyl-1,3 butadiene) is a critical organic chemical that is utilized in the production of synthetic rubber (cis-polyisoprene), which is used in the manufacture of tire; lube, glue, and several other functional elastomers, including latex gloves, baseballs, and footwear (Morais *et al*., 2015). Isoprene is also a valuable substrate that is used in the manufacturing of jet fuel, isoprenoid medications, and perfumes (Wang *et al*., 2016). The previous recent study reported by Batten *et al*., (2021) revealed that polyisoprene is a highly valued organic polymer, which is found naturally in rubber trees and in some flora such as *Guayule* and *Parthenium argentatum*, used in the production of rubber products. According to Isikgor and Becer, (2015), the current polyisoprene production techniques rely on the availability of rubber plantations and\ or synthetic procedures, which are used for the conversion of low molecular weight hydrocarbon byproducts of naphtha cracking. However, the polyisoprene polymer is mostly obtained through the manufacture of the natural rubber (*Hevea brasiliensis*) from the mono-crop plantations, which devastates the indigenous habitats completely (Batten *et al*., 2021).

Recently, rubber tree plantations have caused massive deforestation in Southeast Asia, because this area generates the bulk of the world's largest tree-based polyisoprene. In addition, the expansion in the new manufacturing necessitates the land-use modification, which frequently involves conversion of both the natural and managed forests into rubber plantations, thus destroying the local flora and fauna ecology, as well as the ecosystems of numerous species (Warren-Thomas *et al*., 2018). Furthermore, Kaupper *et al*., (2020) stated that the expansion in the plantations through destruction of the native forests distorts the soil microbiota, resulting in the release of greenhouse gases such as carbon dioxide and nitrous oxide from the deposited carbon and nitrogen. Moreover, as natural gas supplies have increased globally over the last decade, ethylene production; that is used for polyethylene synthesis, has shifted away from the naphtha and gas oil cracking towards ethane cracking. This caused a significant reduction in the isoprene production (a co-produced component of naphtha cracking), which is required for the manufacture of the synthetic rubber (Batten *et al*., 2021). The energy-intensive extraction and distillation processes needed for chemical manufacture of pure isoprene (Heracleous *et al*., 2020), and the negative effects of isoprene obtained from petroleum that causes a climate change, are both noteworthy.

Accordingly, Heracleous *et al*., (2020) have examined bioisoprene production in various cell factories through several technologies that have been classified into the non-mevalonate (MEP-DXP) pathway, which has produced microbial strains with marginal productivity. This is in addition to the mevalonate (MVA) pathway, which has overcome the productivity challenges; however, it has been constrained by the minimal bioisoprene yield. The objectives of this study were to focus on the direct fermentation of the cellulosic materials using a *S. cerevisiae* strain, in order to produce the bioisoprene that is required for rubber synthesis, overcome the productivity and yield challenges, and to have more environmental and economic benefits.

2. Materials and methods

2.1. Treatment of lignocellulose residue

Wholesome peels were removed from dried cassava peels that were purchased from Osogbo, Osun State, Nigeria. The residual soil on the wholesome cassava peels was rinsed with sterile dist. water, air-dried at 30°C for 72 h, milled into a powdery form, and sieved using a mesh of 5.0 m size to obtain a uniform smooth powder. The prepared powder was

kept at 30°C in an airtight container for further analysis.

2.2. Metabolic activities of *Aspergillus niger* strains

2.2.1. Amylolytic and cellulolytic potentials of *A. niger* strains

In the polysaccharide media (containing starch agar and cellulose agar), the metabolic activities of the characterized *A. niger* strains that were previously isolated from garden soil samples (Jimoh *et al*., 2009) were determined; using starch agar medium that was composed of 2.8 % (w/v) nutrient agar and 0.2 % (w/v) soluble starch, and cellulose agar medium [2.8 % (w/v) nutrient agar and 0.2 % (w/v) carboxyl methyl cellulose]. The *A. niger* strains were inoculated individually at the center of triplicate plates using the point inoculation technique of Jimoh *et al*., (2022), and then incubated at 30°C for 5 d. After incubation, the inoculated starch agar plates were flooded with an iodine solution, while the cellulose agar plates were flooded with a zinc chloride-iodine solution. Presence of zones of clearance around the fungal growth in a blue-black background of either the starch agar and\ or the cellulose agar indicated the presence of amylolytic and cellulolytic enzymes, respectively. The best amylase and cellulase-producing strains of *A. niger* that showed the widest diameter of zone of clearance (measured using a calibrated ruler) were selected for further analysis, in reference to Jimoh *et al*., (2017).

2.2.2. Synthesis of crude enzymes

The amylase and cellulase enzymes were synthesized using starch broth [nutrient broth; 2.8 % (w/v) and soluble starch; 0.2 % (w/v)], and cellulose broth [nutrient broth; 2.8 % (w/v) and carboxyl methylcellulose 0.2 % (w/v)]. Both broth media were inoculated individually with a 1 % (v/v) standardized sterile dist. water suspension of a 72-h-old culture of *A. niger* JK21 strain (1.2×10^5 cfu\ ml), and incubated at 30°C for 72 h on a rotary shaker at 200 rpm. After incubation, the inoculated broths were centrifuged individually at 4°C and 6000 rpm for 20 min., thus the obtained cell-free supernatants were used as crude sources of the amylase and cellulase enzymes (Dutta *et al*., 2016). The amylase and cellulase activities were determined using a dinitrosalicylic acid reagent, where the absorbance was measured at 540 using a Microfield UV-Spectrophotometer (MF-752N, England) in contrast to a blank (Jimoh and Ajibise, 2017). All treatments were carried out in triplicates. The amylase and cellulase activity is defined as the amount of an enzyme that produced one mol of a reducing sugar every 30 min. (Akintola *et al*., 2019).

2.3. Enzymatic hydrolysis of the lignocellulosic residue

The cassava peel powder was hydrolyzed using the produced crude cellulase and amylase extracts. Briefly, the cassava peel powder (1 % w/v) was immersed in a 50 mM sodium citrate buffer (pH 4.8), which contained 0.01 % (w/v) of sodium azide (Akintola *et al*., 2019). About 30 ml of each crude enzyme extract was added to the cassava mixture individually and then suspended on a rotary shaker for 72 h (200 rpm) at 30°C. The resulting cassava peel hydrolysate was used for the synthesis of isoprene using the slightly modified method of Wang *et al*., (2016). All the enzymatic assays were carried out in triplicates, and the concentration of the resulting reducing sugar was determined using a dinitrosalicylic acid reagent, in reference to Jimoh *et al*., (2018).

2.4. Biosynthesis of isoprene

According to the method conducted by Wang *et al*., (2017), isoprene was produced in triplicates using a shake flask fermentation medium that was composed of sterile substrate (cassava peel hydrolysate, 2g\ l), beef extract (9 g\ l), K_2HPO_4 (9.8 g\ l), ferric ammonium citrate (0.3 g\ l), $MgSO_4$ (0.06 g\ l), citric acid monohydrate (2.1 g\ l), and 1 ml of a trace elements solution {$(NH_4)6Mo_7O_{24}•4H_2O$ (0.37 g\ l), $ZnSO_4•7H_2O$ (0.29 g\ l), $CuSO_4•5H_2O$ (0.25 g\ l), H_3BO_4 (2.47 g\ l), and $MnCl_2•4H_2O$ (1.58 g\ l)}. The fermentation medium was inoculated with a characterized *S. cerevisiae* 19KB strain that was

previously isolated from fermented beverage during the study of Jimoh *et al*., (2012), and then incubated at 37°C for 72 h on a rotary shaker (180 rpm). The biomass yield from the cultured broth was evaluated at 24-h intervals, while the crude extract obtained after centrifugation at 10,000 rpm for 15 min. was examined for concentration of the reducing sugar and isoprene production.

2.4.1. Assay of biomass yield

According to Jimoh *et al*., (2009), approximately 3 ml of each culture broth was withdrawn individually at 24-h intervals and examined for cell density at 650 nm using a Microfield UV-Spectrophotometer (MF-752N, England).

2.4.2. Quantitative analysis of reducing sugar

The concentration of the reducing sugar was determined by adding 1 ml of 3,5-dinitrosalicylic acid to 1 ml of the crude extract and heated for 5 min. before adding 10 ml of sterile dist. water. The absorbance of each sample was measured at 540 nm using a Microfield UV-Spectrophotometer (MF-752N, England). In turn, the corresponding reducing sugar concentration was calculated using a glucose standard curve (Jimoh *et al*., 2018).

2.4.3. Qualitative analysis for isoprenoids

In reference to Khanal, (2021), about 3 ml of conc. H_2SO_4 was carefully added to 2 ml of a stirred solution of crude extract and 2 ml of chloroform. Development of an interphase's reddish-brown color indicated the existence of terpenoids that is also known as isoprenoids, which are the skeletons of isoprene.

2.4.4. Preliminary quantitative analysis of isoprenoids

A mixture of 50 ml crude extract and 4.5 ml of ethanol kept for 24 h was filtered, using a membrane filter and the obtained filtrate was extracted with 5 ml of petroleum ether using a separating funnel. The separated ether extract was placed in a pre-weighed crucible and allowed to evaporate, thus the percentage yield of isoprenoid (terpenoid) was calculated according to the methodology of Khanal, (2021), using the following formula;

Total isoprenoids = $\{(Wi - Wf)\backslash Wi\} \times 100$

Where, Wi = initial weight of the crude extract, Wf = final weight of the extracts after evaporation

2.4.5. Preliminary screening of enzymatic activity

The enzymatic assay was based on the detection of the reducing sugars produced during enzymatic hydrolysis of the cassava peel substrate using the dinitrosalicylic acid reagent (DNS) technique and 100 µl of a crude extract as the crude enzyme, as described by Oliyad and Dawit, (2018). Furthermore, 900 µl of the substrate was prepared by mixing 0.5 % (w/v) of a substrate in 0.1 M of phosphate buffer (pH 7.5) and then transferred into 3 clean glass vials marked independently as an enzyme (E), enzyme blank (EB), and a reagent blank (RB). About 100 µl of the crude enzyme was added to the glass vial E, 100 µl of dist. water was added to the glass vial RB, and neither the crude enzyme nor dist. water was added to the glass vials labeled EB. All the solutions were incubated in a water bath for 10 min. at 50°C before the reaction was stopped on the addition of 200 µl of DNS reagent, while 100 µl of the crude enzyme was added to the EB vial following the DNS. Furthermore, the solutions were immersed for 10 min. in a 92°C water bath, cooled, and the absorbance was measured at 540 nm using a Microfield UV-Spectrophotometer (MF-752N, England). The enzymatic activity was evaluated against an enzyme blank and a reagent blank. The enzyme unit was defined as the quantity of enzyme required to release one µmol of galacturonic acid per min. (µmol\min). The relative enzyme activity was estimated as the percentage enzyme activity of the sample compared with the sample with the highest activity using the formulae of Akintola *et al*., (2019);

% Relative enzyme activity = Activity of sample (U)\ Maximum enzyme activity (U) × 100

2.5. Enzymes assay

2.5.1. Isoprene synthase assay

According to Silver and Fall, (1995), synthesis of isoprene synthase was quantified using a Gas chromatography (GC) with a flame ionization detector (GC\ FID). The reaction mixture consisted of 15 µl of the crude enzyme, 12 mM $MgCl_2$, and 10 mM dimethylallyl diphosphate (DMAPP), which was then incubated at 32°C for 10 min. before GC-headspace evaluation. Although the DMAPP concentration was slightly greater than the Michaelis constant (Km), which is the affinity of an enzyme to a substrate; however, isoprene production was proportional to time and to enzyme concentration. To stabilize the enzyme, isoprene synthase was diluted using a IPB buffer, which was composed of 50 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$, 1% (v/v) polyethylene glycol 400 (PEG 400), and 2 mM dithiothreitol (DTT) complemented with 5 mg\ ml of bovine serum albumin.

2.5.2. Mevalonate-5-phosphate decarboxylase assay

The activity of mevalonate-5-phosphate decarboxylase enzyme was evaluated by linking the release of adenosine diphosphate (ADP) with nicotinamide adenine dinucleotide reduced (NADH) oxidation by pyruvate kinase\ lactate dehydrogenase (PK\ LDH) at 340 nm, using a Microfield UV-Spectrophotometer (MF-752N, England) at 10 sec intervals (Rossoni *et al*., 2015). The assays were carried out at 30°C in 1 ml polymethyl methacrylate cuvettes with the addition of 0.2 mM NADH, 0.5 nM phosphoenolpyruvate, 5 mM adenosine triphosphate (ATP), aqueous glycerol solution of PK\ LDH (20 µl), and 10 µg of the pure enzyme. The assays were equilibrated for 2 min. before addition of the substrate. The NADH concentrations were computed using an extinction coefficient of 6220 $M^{-1}cm^{-1}$. Where, one unit of an enzyme activity equals the synthesis of one µmol of NADH\ min.

2.5.3. Assay for isopentenyl phosphate kinase

Appearance of ADP coupled with the disappearance of NADH was determined through a pyruvate kinase (PK)-lactate dehydrogenase (LDH)-coupled assay using a Microfield UV-Spectrophotometer (MF-752N), according to the modified protocol of Vannice *et al*., (2014). The effect of an enzyme concentration on phosphomevalonate decarboxylase (PMD) enzyme activity was investigated in 50 mM of 4-(2-hydroxyl) piperazine-1-ethane sulfonic acid)-potassium salt (HEPES-KOH) (pH 7.5) and 10 mM $MgCl_2$. This was coupled with 40 U of both the pyruvate kinase and lactate dehydrogenase enzymes on increasing the concentrations of the crude extract from 0 to 4 M, in the presence of 2 mM (R, S)-Mevalonate 5-phosphate and 2 mM ATP at 30°C.

2.5.4. Cellulase assay

The cellulase enzyme activity was evaluated through detecting its effect on microcrystalline cellulose leading to glucose formation, according to the method of Worthington and Worthington, (2011) with a slight modification. The release of glucose in the hexokinase\ glucose-6-phosphate dehydrogenase system was monitored at 340 nm. One unit of cellulase enzyme activity released 0.01 mg of glucose\ h from the microcrystalline cellulose that was incubated at 37°C (pH 5.0). The enzyme was dissolved in reagent grade water at a concentration of 1mg\ ml. The assay mixture concentration is presented in Table (1).

The mixture was stirred at 37°C for 2 h before being transferred to an ice bath for the formation of sediment and then was further centrifuged. The supernatant was stored in an ice bath for subsequent analysis. To achieve temperature equilibrium, 3 ml of the glucose reagent was placed into a cuvette and 0.1 ml supernatant from each reaction mixture was added, and then allowed to acclimatize to 25°C before analysis using a Microfield UV-spectrophotometer (MF-752N, England). The absorbance of the glucose reagent-supernatant mixture glucose reagent was measured at 340 nm against glucose reagent (blank).

Table 1: Cellulase enzyme assay mixture

	Test	Blank
Microcrystalline cellulose	200 mg	200 mg
0.05 M acetic acid	4.0 ml	4.0 ml
Reagent grade water	------	1.0 ml
Enzyme dilution	1.0 ml	-----

2.5.5. Amylase assay

An aliquot of 0.5 ml of (1 %) starch and 0.5 ml of the crude enzyme were incubated at 37°C for 30 min. After incubation, the reaction was terminated by adding 1 ml of 3, 5 dinitrosalicylic acid and placing the mixture in a water bath at 100 °C for 10 min., for the development of a brown color. Furthermore, the final volume of the mixture was made up to 5 ml with dist. water, and the absorbance was measured at 540 nm using a Microfield UV-Spectrophotometer (MF-752N, England), in reference to Dharani Aiyer, (2012). Using the standard maltose curve of Mehrabadi and Bandani, (2009), the concentration of the liberated maltose was estimated. Where, one unit of an amylase enzyme activity is defined as the quantity of an enzyme that liberates 1 mol of maltose per min.

2.6. Quantification of isoprenoids

According to the modified method of Jimoh *et al*., (2017), the supernatant obtained from the fermentation medium with a silicon seal was transferred to the headspace vial and further placed in the headspace jacket that was connected to the GC system (HP 6890 Powered with HP ChemStation Rev. A 09.01 [1206]) software. The operational conditions of the GC system that was equipped with a pulse flame photometric detector (PFPD) and a CP-Sil 5 CB column (25 m × 0.32 µm × 0.12 µm) at a flow rate of 1 ml\ min., included an initial oven temperature that was maintained at 35 °C for 3 min.; injection temperature (150°C), detector temperature (300°C), and a final temperature of 100°C. Commercial isoprene (Sigma-Aldrich) was used as a standard for the analysis.

2.7. Statistical analysis

The obtained results were analyzed with GraphPad Prism 8.0.1 using one-way and two-way analysis of variance (ANOVA).

3. Results and Discussion

3.1. Cellulolytic and amylolytic activities of *A. niger* strains

Among the 3 fungal strains screened *in vitro* for their enzymatic activity, *A. niger* 11JK strain expressed the widest zone of clearance on cellulose and starch agar media, recording 37 mm and 60 mm, respectively. Accordingly, as this strain showed the highest cellulolytic and amylolytic activities, it was selected for further research and isoprene production (Fig. 1). These activities expressed by *A. niger* 11JK indicated its ability to break down lignincellulolytic material crystalline structure, which hinders its solubility in water. The statistical level of significance analysed by a one-way ANOVA was followed by Tukey post hoc pairwise multiple comparisons test: where $^{a}p < 0.05$ compared to the cellulolytic activity of *A. niger* 11JK, $^{b}p < 0.05$ compared to the cellulolytic activity of *A. niger* 12PC, $^{c}p < 0.05$ compared to the amylolytic activity of *A. niger* 11JK, and $^{d}p < 0.05$ compared to the amylolytic activity of *A. niger* 12PC.

3.2. Hydrolytic activity of *A. niger* IIJK

Starch is a substantial part of the agricultural wastes that is being processed into starch hydrolysate,

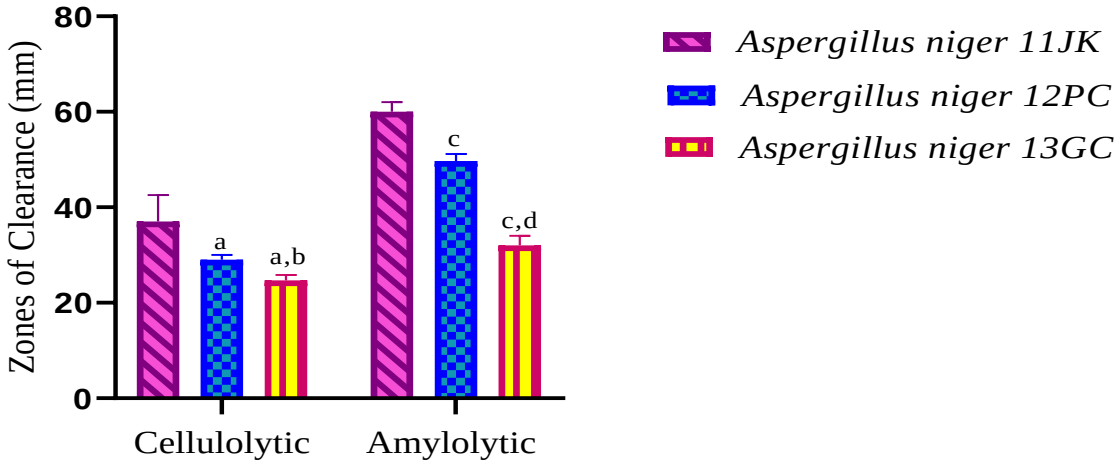

Fig. 1: *In vitro* cellulolytic and amylolytic activities of the 3 *A. niger* strains. The error bars represent the mean ± SD (Standard deviation, n= 2). The values followed by the same superscript letters are significantly different at $p < 0.05$

which serves as a microbial substrate during amylase synthesis (Abalaka and Adetunji, 2017). The enzymatic assays used to monitor the hydrolytic activity of the crude enzymes (cellulase, amylase, and both enzymes) that were produced by *A. niger* 11JK during hydrolysis of the cassava peel to simple sugars required for isoprene synthesis, revealed maximum metabolic activity (Fig. 2), which was further confirmed through the higher enzymatic activity and the corresponding molecular weight recorded using GC-FID (Table 2). The high synergistic effect (0.237) of both amylase and cellulase enzymes obtained in this study was attributed to the high enzymatic activity of amylase (0.126) compared with 0.06 that was expressed by cellulase individually. These results indicated that amylase is a strong hydrolyzing enzyme that mediates the hydrolysis of polysaccharides to monosaccharides, by hydrolyzing the bonds between the adjacent glucose units (Padma and Pallavi, 2016). The enzymatic activity serves as the biological pretreatment of lignocellulose using *A. niger* 11JK, which possess hydrolytic characteristics that will loosen the crystalline structure and facilitates the lignocellulose degradability, in order to release the fermentable sugars. Statistical level of significance analysed by a two-way ANOVA followed by Tukey post hoc pairwise multiple comparisons test revealed a significant difference among the means of the amylase and cellulase enzymatic activities.

3.3. Reducing sugars concentration and biomass yield

The enzymatic ability of *A. niger* 11JK to metabolize cassava peel medium through the liberation of cellulase and amylase enzymes led to the increased reducing sugar concentration within 24 h, which later decreased gradually on the 4th day as the fermentation period increased. Later, the recorded decrease was attributed to the activity of *S. cerevisiae* 19KB strain inoculated on the 3rd day, which converted the reducing sugar to isoprenoid intermediates. *S. cerevisiae* 19KB potential to metabolize the reducing sugars also led to an increase in its biomass yield as the fermentation period increased (Fig. 3 and 4). This implies that *S. cerevisiae* 19KB metabolised the simple sugars such as lactose and maltose with an anomeric carbon that was capable of reducing the

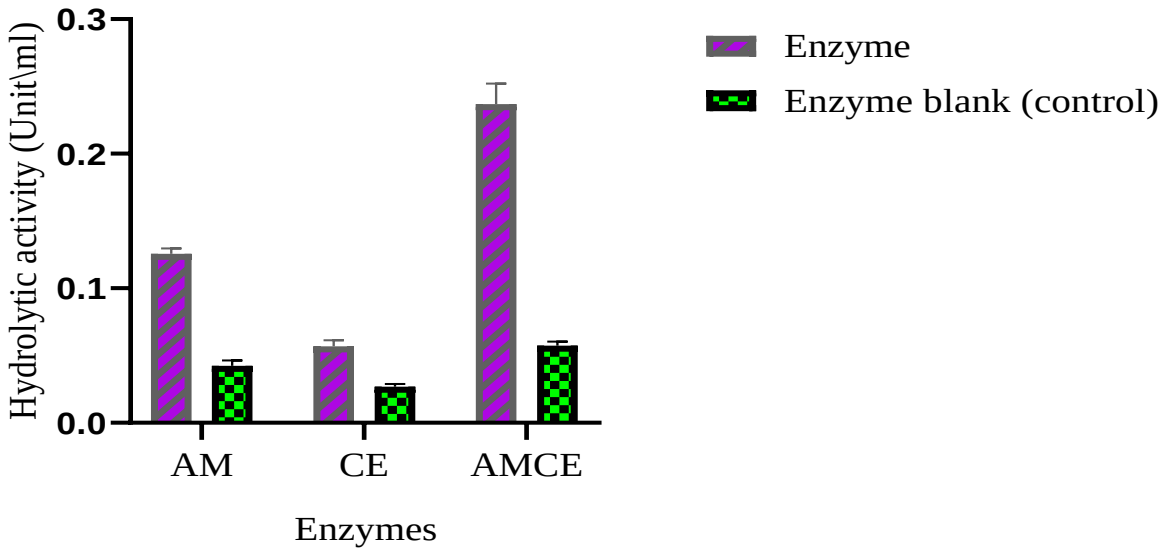

Fig. 2: Hydrolytic activity of *A. niger* 11JK strain

Where; AM =Amylase; CE= Cellulase; AMCE= Amylase and Cellulase. The values are significantly different at p <0.05. The error bars represent the mean ± SD (Standard deviation, n= 2). The statistical level of significance analysed by a two-way ANOVA is followed by the Tukey post hoc pairwise multiple comparisons test

Table 2: Enzymatic activity and molecular weights of the hydrolytic enzymes

Enzyme	**Activity (U\ ml)**	**Molecular weight**
Amylase	344.2 ± 2.623	51.8
Cellulase	145.5 ± 2.255	41.9[a]

Where; the values followed by the same superscript letters are significantly different at p <0.05. Data are presented as mean ± SD (standard deviation, n=3). The statistical level of significance analysed by a two-way ANOVA is followed by Tukey post hoc pairwise multiple comparisons tests: [a]p< 0.05, compared to amylase and cellulose

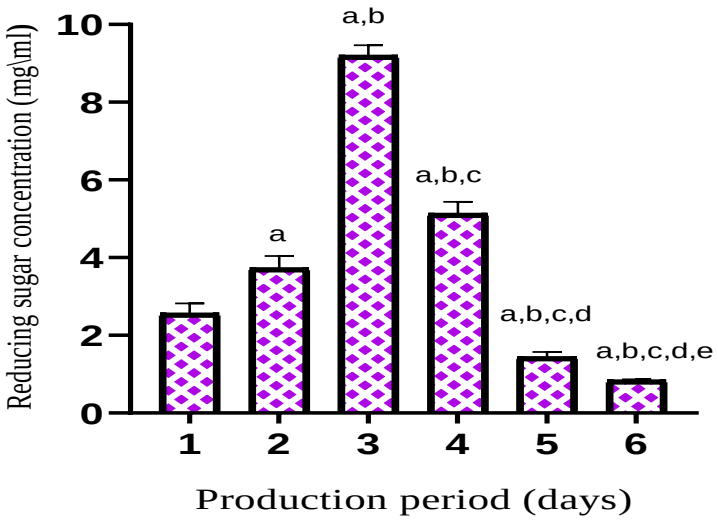

Fig. 3: Cassava peel hydrolysate yield

Where; the values followed by the same superscript letters are significantly different at p <0.05. Data are presented as mean ± SD (standard deviation; n=3). Statistical level of significance analysed by a two-way ANOVA is followed by Tukey post hoc pairwise multiple comparisons test; $^{a}p < 0.05$ compared with day 1, $^{b}p < 0.05$ compared with day 2, $^{c}p < 0.05$ compared with day 3, $^{d}p < 0.05$ compared with day 4, $^{e}p < 0.05$ compared with days 5 and 6

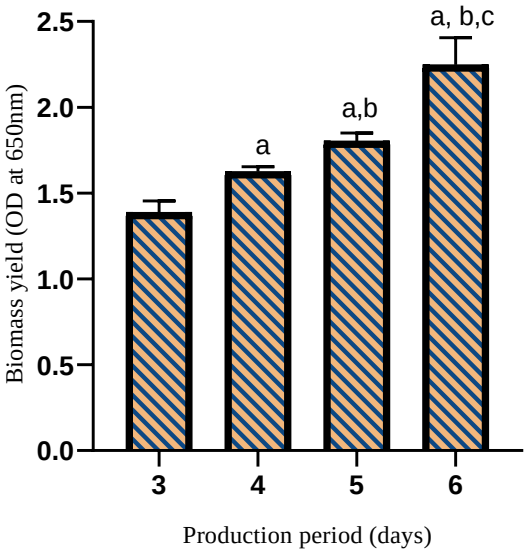

Fig. 4: Yield of *S. cerevisiae* during isoprene production

Where; the values followed by the same superscript letters are significantly different at p <0.05. Data are presented as mean ± SD (standard deviation; n=3). Statistical level of significance analysed by a two-way ANOVA is followed by Tukey post hoc pairwise multiple comparisons tests; ^{a}p <0.05 compared to day 3, ^{b}p <0.05 compared to day 4, ^{c}p <0.05 compared to days 5and 6

ferric ions in the DNS reagents, which were present in the cassava peel medium (Jimoh *et al*., 2018).

Furthermore, hydrolysis of the cellulosic waste (cassava peel) by *A. niger* 11JK into simple sugars such as glucose provided high MVA\ MEP flux for *S. cerevisiae* 19KB strain, through the generation of Glyceraldehyde-3-phosphate\ pyruvate or acetyl-CoA (Caureuters and Lee, 2021). The generated simple sugars served as carbon sources that were required for isoprene synthesis through the MVA\ MEP pathway, thus solving the problem of high production costs. This was the greatest challenges of isoprene biosynthesis starting from carbon feed that accounted for over 90 % of the production costs and yield (Wu and Maravelias, 2018). The results obtained in this study confirmed that cassava peel is a promising solution to the exorbitant substrate costs, thus switching of the carbon sources to the carbon wastes such as cellulosic biomass will reduce the high isoprene production costs, which are not expensive to compete with the petroleum-derived analogs (Carrutters and Lee, 2021). The two-way ANOVA revealed a significant difference (p <0.05) between the reducing sugars concentrations obtained between day 1 and day 6, and also *S. cerevisiae* 19KB (biomass) yield between day 3 and day 6.

3.4. Metabolic activities of *S. cerevisiae* 19KB strain

Using GCFID, the enzyme assay employed during isoprene synthesis by *S. cerevisiae* 19KB strain revealed the presence of several metabolic enzymes in the crude extract that were recovered after the fermentation period. These metabolic enzymes included isoprene synthase (IspS), mevalonate-5-diphosphate decarboxylase (MvaD), and isopentyl phosphate kinase (IPK), based on their recorded molecular weights (62.6, 90.5, and 30.2 kDa) and enzymatic activities (Fig. 5). Similarly, the molecular weight of isoprene synthase correlated with the 62-kDa band that was previously reported by Silver and Fall, (1995). Furthermore, the obtained mevalonate-5-diphosphate decarboxylase activity (122.1 U\ mg) of *S. cerevisiae* 19KB was extremely high, and the expression of these significant enzymes confirmed the metabolic activity of this strain during biosynthesis of isoprene, which was synthesized via the classical mevalonate pathway rather than the alternative 2C-methyl-D-erythritol 4-phosphate (MEP) pathway (Reichert *et al*., 2018). The inability of *S. cerevisiae* 19KB to utilize the MEP pathway may be attributed to the higher demand of redox partners for the MEP pathway along with hydroxyl-methylbutenyl 4-diphosphate (HMBPP) synthase, HMBPP reductase, and DXP reductase enzymes; because each enzyme in MEP pathway requires 1 NADPH for the accomplishment of a particular step rather than 2 NADPH that are demanded by the MVA pathway for the production of mevalonate through the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (Hussain *et al*., 2021). This result confirmed that the three steps of the MEP pathway rely heavily on NADPH's availability for proper functioning, which is mainly supplied by the pentose phosphate pathway, while the MVA pathway is regulated by only a single step, as reported by Hussain *et al*., (2021). *S. cerevisiae* 19KB utilized the latter pathway to ensure the supply of enough NADH molecules through the oxidation of pyruvate to acetyl-CoA, while consuming glucose as a major carbon source (Ward *et al*., 2018). According to Reichert *et al*., (2018), the ability of *S. cerevisiae* 19KB to utilize the mevalonate pathway specified the capacity of this strain to convert acetyl-CoA and acetoacetyl-CoA into 3-hydroxy3-methylglutaryl-CoA (HMG-CoA). This compound was further reduced to mevalonate, phosphorylated to phosphomevalonate, and subsequent phosphorylation led to the production of diphosphomevalonate. This product was further decarboxylated to the C5 building blocks isopentenyl pyrophosphate (IPP), which had undergone isomerization to generate DMAPP.

3.5. Isoprene biosynthesis

Isoprene is a class of ubiquitous organic molecules that are synthesized from the 5-carbon starter unit

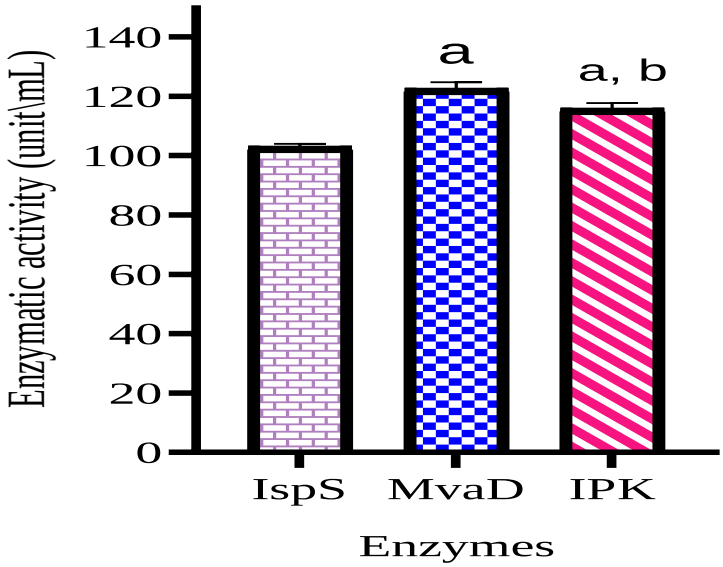

Fig. 5: Enzymes activity expressed through the MVA pathway

Where; IspS: isoprene synthase; MvaD: mevalonate-5-diphosphate decarboxylase; IPK: isopentyl phosphate kinase. The values followed by the same superscript letters are significantly different at p <0.05. Data are presented as mean ± SD (standard deviation, n=3). Statistical level of significance analysed by a two-way ANOVA is followed by Tukey post hoc pairwise multiple comparisons tests; [a]p <0.05 compared with isoprene synthase, and [b]p <0.05 compared with mevalonate-5-diphosphate decarboxylase

known as IPP. The appearance of a reddish-brown interface during the qualitative analysis indicated the presence of terpenoids otherwise known as isoprenoids, which depicts the presence of isoprene, as the isoprenoids are the building blocks of the isoprene polymers. Higher terpenoids (isoprenoids) percentage yield of 27.1 % that was obtained in this study through preliminary quantitative analysis is higher than 12.77 % and 13.13 % isoprene yields, which were recorded in two analysed samples in the previous work conducted by Khanal, (2021).

Currently, results of biosynthesis of several intermediates through the mevalonic acid\ mevalonate (MVA) pathway, including isoprene (0.12 g\ l), isopentyl diphosphate (IPP) (0.35 g\ l), and its isomer dimethylallyl diphosphate (DMAPP) (0.36 g\ l) that are presented in Fig. (5) have revealed the potential of *S. cerevisiae* 19KB strain to synthesize various metabolites, which constitute the mevalonate pathway. These results agree with results of the previous study reported by Liao *et al*., (2016). The higher yield of isoprene (99.18 g\ l) obtained in this study supersedes the 297.5 mg\ l and 430 mg\ l yields reported by Wang *et al*., (2016); Cheng *et al*., (2017). Similarly, *S. cerevisiae* 19KB isoprene yield was higher than 437.2 µg\ l and 1434.3 µg\ l produced by the recombinants *Bacillus licheniformis* and *B. subtilis* (Gomaa *et al*., 2017), and synthesized by the engineered *Escherichia coli* cells (23.3 mg\ l) (Yeom *et al*., 2018). This metabolic product establishes the ability of the *S. cerevisiae* 19KB strain to utilize the mevalonate pathway to synthesize isoprene rather than the alternative 2C-methyl-D-erythritol 4-phosphate (MEP)

pathway, which requires 8 enzymatic steps that proceed via the condensation of pyruvate and glyceraldehyde 3-phosphate to produce 1-deoxy-D-xylulose 5-phosphate (DOXP) (Reichert *et al*., 2018).

Furthermore, during isoprene synthesis, the lack of DOXP that was required for the synthesis of the intermediate MEP in the crude extract also confirmed that the metabolic processes of *S. cerevisiae* 19KB strain proceeded via the mevalonate pathway. This accounts for the conversion of acetyl-CoA to isopentenyl 5-diphosphate, which is the versatile precursor of the polyisoprenoid metabolites and the natural products. The impacts of the carbon supply, energy demands (ATP\ CTP), and availability of the reducing cofactors (NADH, NADPH), intensely affected the yield and efficiency of the MVA or MEP pathway (Ward *et al*., 2018). However, the current results showed that *S. cerevisiae* 19KB utilized only one precursor involved in the MVA pathway but could not utilize the MEP pathway, which may be attributed to the metabolic problem of the imbalanced supply of several precursors such as pyruvate and glyceraldehyde 3-phosphate (G3P) that had been encountered by the yeast strain (Li *et al*., 2015).

Conclusion

Consecutive condensation of the 5-carbon monomer isopentenyl diphosphate (IPP) to its isomer dimethylallyl diphosphate (DMAPP) validated that the metabolic activities of *S. cerevisiae* 19KB during the synthesis of isoprene from acetyl-CoA took place through the mevalonate pathway. This indicated that the budding yeast *S. cerevisiae* 19KB possessed several genes encoding for isoprene synthase, mevalonate-5-diphosphate decarboxylase, and isopentyl phosphate kinase, which were required for the MVA pathway. The current results ensured that the availability of a suitable precursor supply that is obtained through the use of agricultural residues such as cassava peel and the budding yeast such as *S. cerevisiae* 19KB is essential. Finally, it is concluded that synthesis of rubber via direct fermentation of cheap and abundant carbon sources such as cassava peel and the presence of *S. cerevisiae* 19KB strain, will overcome the productivity and yield challenges, thus generating better environmental and economic benefits.

Acknowledgement

The authors are grateful to the Department of Biological Sciences (Microbiology Programme) of the Fountain University Osogbo, Nigeria, for utilizing its facilities in this study.

Conflict of interest

The authors declare no conflict of interests.

Funding source

This research did not receive specific grants from any funding agencies in the public, commercial or institutional sectors.

Ethical approval

Not applicable.

4. References

Abalaka, M.E. and Adetunji, C.O. (2017). Production and optimization of Amylase and Glucoamylase from *Aspergillus niger* under solid-state fermentation for effective production of Glucose syrup. UMYU Journal of Microbiology Research. 2(1):135-146.

Akintola, A.I.; Oyedeji, O.; Adewale, I.O. and Bakare, M.K. (2019). Production and Physicochemical Properties of Thermostable, Crude Cellulase from *Enterobacter cloacae* Ip8 isolated from Plant Leaf Litters of *Lagerstroemia indica* Linn. Journal of Microbiology, Biotechnology and Food Sciences. 8(4): 989-994. https://doi.org/10.15414/jmbfs.2019.8.4.989-994

Batten, R.; Karanjikar, M. and Spatari, S. (2021). Bio-Based Polyisoprene Can Mitigate Climate Change and Deforestation in Expanding Rubber

Production. Journal of Fermentation. 7(4): 204-217. https://doi.org/10.3390/fermentation7040204

Carruthers, D.N. and Lee, T.S. (2021). Diversifying Isoprenoid Platforms via Atypical Carbon Substrates and Non-model Microorganisms. Frontier in Microbiology. 12: 1-20 https://doi.org/10.3389/fmicb.2021.791089

Cheng, T.; Liu, H.; Zou, H.; Chen, N.; Shi, M.; Xie, C. et al. (2017). Enzymatic process optimization for the *in vitro* production of isoprene from mevalonate. Microbial Cell Factories. 16: 8-15. https://doi.org/10.1186/s12934-016-0622-4

Dharani Aiyer, P.V. (2012). Effect of C:N ratio on alpha amylase production by *Bacillus licheniformis* SPT 27. Frontiers of Agriculture and Food Technology. 2(6): 1-4. http://www.internationalscholarsjournals.org/

Dutta, P.; Deb, A. and Majumdar, S. (2016). Optimization of the Medium for the Production of Extracellular Amylase by the *Pseudomonas stutzeri* ISL B5 Isolated from Municipal Solid Waste. International Journal of Microbiology. 2016:1-7. https://doi.org/10.1155/2016/4950743

Gomaa, L.; Loscar, M.E.; Zein, H.S.; Abdel-Ghafar, N.; Abdelhadi, A.A.; Abdelaal, A.S. et al. (2017). Boosting isoprene production via heterologous expression of the Kudzu isoprene synthase gene (*kIspS*) into *Bacillus* spp. cell factory. AMB Express Journal. 7(1):161.171. https://doi.org/10.1186/s13568-017-0461-7

Heracleous, E.; Pachatouridou, E.; Louie, L.; Dugar, D. and Lappas, A.A. (2020). Efficient Route for the Production of Isoprene via Decarboxylation of Bioderived Mevalonolactone. American Chemical Society (ACS) Catalysis. 10: 9649–9661. https://doi.org/10.1021/acscatal.0c01438

Hussain, M.H.; Hong, Q.; Zaman, W.Q.; Mohsin, A.; Wei, Y.; Zhang, N. et al. (2021). Rationally optimized generation of integrated *Escherichia coli* with stable and high-yield lycopene biosynthesis from heterologous mevalonate (MVA) and lycopene expression pathways. Synthetic and Systems Biotechnology. 6(2): 85–94. https://doi.org/10.1016/j.synbio.2021.04.001

Isikgor, F.H. and Becer, C.R. (2015). Lignocellulosic biomass: A sustainable platform for the production of bio-based chemicals and polymers. Polymer Chemistry. 6(25): 4497-4559. https://doi.org/10.1039/C5PY00263J

Jimoh, S.O.; Adefaye, K.O.; Arowolo, L.A. and Badmos-Oladapo, R.B. (2022). Evaluation of chitinolytic enzymes activities during microbial biotransformation of *Archachatina marginata* exoskeleton to chitooligosaccharide. Novel Research in Microbiology Journal. 6(2): 1530-1542.http://dx.doi.org/10.21608/nrmj.2022.231601

Jimoh, S.O.; Lawal, A.O;, Ashorobi, A.A.; Oyekanmi, E.A.; Bakare, R.I.; Adefioye, Nafisat A. et al. (2018). Microbial Synthesis of Polyhydric alcohol by *Saccharomyces cerevisiae*. Microbiology Research Journal International. 23 (1): 1-6. https://doi.org/10.9734/MRJI/2018/34852

Jimoh, S.O. and Ajibise, F.O. (2017). Evaluation of C19 Steroid Intermediates during Microbial Transformation of Phytosterol. Microbiology Research Journal International, 21(2): 1-8. https://doi.org/10.9734/MRJI/2017/34869

Jimoh, S.O.; Labo-Popoola, H.O. and Alabi, K.A. (2017). Radical Scavenging Capacity and Efficacy of *Myristica fragrans* (Nutmeg) Metabolites on *Cladosporum herbarum* of Food Origin. Microbiology Research Journal International. 20(1): 1-8. https://doi.org/10.9734/MRJI/2017/31962

Jimoh, S.O.; Ado, S. A.; Ameh, J.B. and Whong, C.M.Z. (2012). Characteristics and Diversity of Yeast in Locally Fermented Beverages sold in Nigeria. World Journal of Engineering and Pure and Applied Science. 2(2): 40-44.

Jimoh, S.O.; Ado, S.A. and Ameh, J.B. (2009). Simultaneous Saccharification and Fermentation of Yam Peel to Ethanol by co-culture of *Aspergillus niger a*nd *Saccharomyces cerevisiae.* Biological and Environmental Sciences Journal for the Tropics. 6(1): 96-100.

Kaupper, T.; Hetz, S.; Kolb, S.; Yoon, S.; Horn, M.A. and Ho, A. (2020). Deforestation for oil palm: Impact on microbially mediated methane and nitrous oxide emissions, and soil bacterial communities. Biology and Fertility of Soils. 56: 287-298. https://doi.org/10.1007/s00374-019-01421-3

Khanal, S. (2021). Qualitative and Quantitative Phytochemical Screening of *Azadirachta indica Juss.* Plant Parts. International Journal of Applied Sciences and Biotechnology. 9(2): 122-127. https://doi.org/10.3126/ijasbt.v9i2.38050

Li, C.; Ying, L.Q.; Zhang, S.S.; Chen, N.; Liu, W.F. and Tao, Y. (2015). Modification of targets related to the Entner–Doudoroff/pentose phosphate pathway route for methyl-D-erythritol 4-phosphate-dependent carotenoid biosynthesis in *Escherichia coli*. Microbial Cell Factories. 14: 117-128. https://doi.org/10.1186/s12934-015-0301-x

Liao, P.; Hemmerlin, A; Bach, T.J. and Chye, M.L. (2016). The potential of the mevalonate pathway for enhanced isoprenoid production. Biotechnology Advance. 34(5): 697-713. https://doi.org/10.1016/j.biotechadv.2016.03.005

Mehrabadi, M. and Bandani, A.R. (2009). Assessing of α-Amylase Activity of Midgut in Wheat Bug *Eurygaster Maura*. American Journal of Applied Sciences. 6(3): 478-483. https://doi.org/10.3844/ajas.2009.478.483

Morais, A.; Dworakowska, S.; Reis, A.; Gouveia, L.; Matos, C.T.; Bogdal, D. et al. (2015). Chemical and biological-based isoprene production: Green metrics. Catalysis Today. 239: 38-43. http://dx.doi.org/10.1016%2Fj.cattod.2014.05.033

Oliyad, J.O. and Dawit, A. (2018). Screening and Molecular Identification of Pectinase Producing Microbes from Coffee Pulp. BioMed Research International. 2018: 1-11. https://doi.org/10.1155/2018/2961767

Padma, S. and Pallavi, K. (2016). Isolation and characterization of amylase producing *Bacillus* spp. from selected soil sample. International Journal of Research in Biosciences. 5(2): 24-29.

Reichert, S.; Ebner, P.; Bonetti, E.J; Luqman, A.; Nega, M.; Schrenzel, J. et al. (2018). Genetic Adaptation of a Mevalonate Pathway Deficient Mutant in *Staphylococcus aureus*. Frontier Microbiology. 9: 1539. https://doi.org/10.3389/fmicb.2018.01539

Rossoni, L.; Hall, S.J.; Eastham, G.; Licence, P. and Stephens, G. (2015). The putative mevalonate diphosphate decarboxylase from *Picrophilus torridus* is in reality a mevalonate-3-kinase with high potential for bioproduction of isobutene. Applied Environmental Microbiology. 81: 2625-2634. https://doi.org/10.1128/AEM.04033-14.

Silver, G.M., and Fall, R. (1995). Characterization of Aspen Isoprene Synthase, an Enzyme Responsible for Leaf Isoprene Emission to the Atmosphere. Journal of Biological Chemistry. 270(22): 13010-13016. https://doi.org/10.1074/jbc.270.22.13010

Vannice, J.C.; Skaff, D.A.; Keightley, A.; Addo, J.K.; Wyckoff, G.J. and Miziorko, H.M. (2014). Identification in *Haloferax volcanii* of phosphomevalonate decarboxylase and isopentenyl phosphate kinase as catalysts of the terminal enzyme reactions in an archaeal alternate mevalonate pathway. Journal of Bacteriology. 196(5): 1055-1063. https://doi.org/10.1128/JB.01230-13

Wang, S.; Wang, Z.; Wang, Y.; Nie, Q.; Yi, X.; Ge, W. et al. (2017). Production of isoprene, one of the high-density fuel precursors, from peanut hull using the high-efficient lignin-removal pretreatment method. Biotechnology for Biofuels , 10: 1-12 . https://doi.org/10.1186/s13068-017-0988-5

Wang, S.; Li, R.; Yi, X.; Fang, T.; Yang, J. and Bae, H.J. (2016). Isoprene production on enzymatic hydrolysate of peanut hull using different pretreatment methods. Biomedical Research International. 1155: 1-8. https://doi.org/10.1155/2016/4342892

Ward, V.C.A.; Chatzivasileiou, A.O. and Stephanopoulos, G. (2018). Metabolic engineering of *Escherichia coli* for the production of isoprenoids. FEMS Microbiology Letter. 365:10-22. https://doi.org/10.1093/femsle/fny079

Warren-Thomas, E.M.; Edwards, D.P.; Bebber, D.P.; Chhang, P.; Diment, A.N.; Evans, T.D. et al. (2018). Protecting tropical forests from the rapid expansion of rubber using carbon payments. Nature Communication. 9: 911-920. https://doi.org/10.1038/s41467-018-03287-9

Worthington, K. and Worthington, V. (2011). Cellulase Assay. Worthington Enzyme Manual. Worthington Biochemical Corporation. Edited by Worthington, K.; Worthington, V. and Santangelo, C. pp: 20. https://www.worthington-biochem.com/products/cellulase/assay

Wu, W. and Maravelias, C.T. (2018). Synthesis and techno-economic assessment of microbial-based processes for terpenes production. Biotechnology Biofuels. 11: 294-303. https://doi.org/10.1186/s13068-018-1285-7

Yeom, S.J.; Kim, M.; Kim, S.K.; Lee, D.H.; Kwon, K.K.; Lee, H. et al. (2018). Molecular and biochemical characterization of a novel isoprene synthase from *Metrosideros polymorpha*. BMC Plant Biology. 18(118): 1-10. https://doi.org/10.1186/s12870-018-1315-4