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Metabolic engineering of *Neurospora crassa* for increasing carotenoids synthesis

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Carotenoids are essential nutrient compounds with numerous biological functions. *Neurospora crassa* is a model filamentous fungus with orange pigmentation which is attributed to the accumulation of carotenoids containing neurosporaxanthin (NX) and neutral carotenoids (NC). To enhance carotenoids synthesis in *N. crassa*, isoprene diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) were increased using the genes, xylulose-5-phosphate phosphoketolase (*XPK*), phosphotransacetylase (*PTA*), and NADH-specific-3-hydroxy-3-methyl glutaryl coenzyme A reductase (*HMGR*), as single, fused and three combined expressions to channel more carbon source into the mevalonate pathway (MVP). The single (*PTA*, *XPK*, *HMGR*), fused (*PTA:HMGR* & *XPK:HMGR*) and three combined gene (*PTA* with fused *XPK:HMGR*) expressions in engineered fungal resulted in carotenoid titers with contents of NX accumulated up to 4.5 mg/g DW and NC up to 1.7 mg/g DW as compared to the wild-type with NX up to 1.54 mg/g DW and NC up to 0.8 mg/g DW. The optimized MVP with metabolic engineering methods is a key method to increase the synthesis of carotenoid and other active terpenoids in *N. crassa*.

Key words: *Neurospora crassa,* mevalonate pathway, carotenoids, neurosporaxanthin, neutral carotenoids.

INTRODUCTION

Carotenoids, which are a general term for xanthophylls and carotenes depending on weather they contain or lack oxygen in their molecules, can be found in fungi, plants and algae (Lee et al., 2012). They have numerous biological functions in the human body. In the food industry, carotenoids are used in many products such as cheese, pastry and some non-alcoholic beverages. It is also used as an active ingredient in cosmetic products as it provides protection against UV radiation exposure (Barreiro and Barredo, 2018). Originally, carotenoid was extracted from plants, over the years, various classifications of carotenoids have been synthesized through microbial sources such as bacteria (Yoon et al., 2007, 2009), yeast (Marova et al., 2012; Simova et al., 2004) and algae (García-González et al., 2005). The rising market demand of the various classifications of carotenoids has stimulated the development of its diverse production methods. Although current titers in many engineered fungi hosts except *Fusarium fujikuroi* (Parra-Rivero et al., 2020) still lack economic feasibility, future

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research leading to better approach in alternative hosts may be established for large scale production with less cost.

Neurospora crassa is a filamentous orange bread mold whose potential as a model organism has been recognised for several reasons (Davis and Perkins, 2002). It is known to have a relatively simple life cycle, rapid growth rate and ease of propagation on cheap growth media. Its haploid nature allows for dominant and recessive traits to be expressed in their offsprings (Turrà et al., 2016). It is non-toxic and is traditionally used for food production in south eastern Asia and is considered safe for other technical applications (Perkins and Davis, 2000) making it a suitable strain for synthetic biology. It also has an added advantage where it can grow on discarded lignocellular biomass for itself secreting cellulose and semi-cellulose degrading enzymes (Collier et al., 2020; Liu et al., 2020), and is more tolerant (Feldman et al., 2019; Tiwari et al., 2018) in active synthesized terpenoidal compounds such as steroidal or triterpenoid saponins which has a potential of reducing future production cost of active terpenoid compounds used in pharmaceutical or functional food industry. Neurosporaxanthin (NX) a carboxylic xanthophyll is the major carotenoid product of *N. crassa* which is usually accumulated with varying amounts of its neutral carotenoids (NC) (Hornero-Méndez et al., 2018). NX provides the typical orange pigmentation to *N. crassa* because of its accumulation in the airborne spores (Avalos et al., 2012).

In the mevalonate pathway (MVP) for terpenoid synthesis, isoprene diphosphate (IPP) and its interconvertible isomer, dimethylallyl diphosphate (DMAPP), are key intermediate metabolites which are accumulated starting with acetyl-CoA in non-photosynthetic hosts, hence, our optimized synthetic strategy in an engineered fungus by accumulation of acetyl-CoA and channelling its carbon flux into MVP (Moser and Pichler, 2019). In this study, 3 genes; Xylulose-5-phosphate phosphoketolase (*XPK,*NCBI: CP000414.1:1934181-1936622), phosphotransacetylase (*PTA*, ABAJ01000001.1:3408314-3409315) and NADH specific-3-hydroxy-3-methyl glutaryl coenzyme A reductase (*HMGR*, CP000032.1:179002-180303) as single genes, fused (*PTA:HMGR* and *XPK:HMGR*) and three combined gene expressions (*PTA* with fused *XPK:HMGR*) were introduced into *N. crassa* with the aim of channelling more carbon source to acetyl-CoA by concert action of XPK and PTA, then acetyl-CoA conversion to IPP and DMAPP in MVP was enhanced by NADH-specific HMGR.

MATERIALS AND METHODS

Strains, plasmids and chemicals

The 301-6 strain, mating type "A" with a mutated his-3 gene (Δhis, cannot grow without histidine) is the *N. crassa* strain used in this study. Other strains and plasmids used in this study are listed in Table 1. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, (Woburn, USA). Common reagents and kits for molecular cloning were purchased from Shangon Biotech (Shanghai, China).

Cultural media and growth conditions

Cloning host, *Escherichia coli* (DH5α), was grown on LB media with Ampicillin antibiotics. The 301-6 strain was cultivated on minimal slants supplemented with histidine (20 ml/L 50x Vogel's (Vogel, 1956), agar 15 g/L, sucrose 30 g/L and histidine 0.15 g/L) for four days at 30°C under the condition of light and the spores were harvested with distilled water. To improve carotenoid synthesis, fermentation was carried out with 3 different methods. Method 1: fermented with media1 (20 ml/L 50x Vogel's, 20 g/L glucose), method 2: fermented with media 2 (50 ml/L 50x Vogel's, 50 g/L glucose), and method 3: after 4 days inoculation in media 2, additional 2% glucose was added at 30°C at 160 rounds per minute (rpm) in the shaker incubator.

Construction of expression vectors

The pPcfp_Myc_His and pPcfp_Myc_HyR with constitute expression promoter of cfp (Alvarez et al., 1993) pyruvate decarboxylase (NCBI: NC_026507.1:3384904-3387654), with *his-3* and hygromycin selection markers, respectively were used as starting vectors for construction (Table 1). After, the codon was optimized for expression in *N. crassa*, the coding sequence of *XPK* (2484 bp) from *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 829, *PTA* (1049 bp) from *Clostridium kluyveri* DSM 555 and *HMGR* (1349 bp) from *Ruegeria pomeroyi* DSS-3 with their start codon (ATG) up flanking with a fused sequence of "GGCGGTGCTGCA" coding for peptide linker "Gly_Gly_Ala_Ala" were obtained by chemical synthesis from Shangon (Shanghai, China). To construct the single gene expression vectors, *XPK*, *PTA* and *HMGR* were amplified by PCR with primer pairs of XPK_P1 & XPK_P2, PTA_P1 & PTA_P2 and HMGR_P1 & HMGR_P2, respectively to add *BamH* I site on 5′ end and both *Bgl* II and *EcoR* I sites on 3′ end (Table 2). After being digested with both *BamH* I and *EcoR* I, the fragments were inserted into *Bgl* II and *EcoR* I sites of pPcfp_Myc_His to get intermediate vectors, pPcfp_XPK_His, pPcfp_PTA_His, and pPcfp_HMGR_His (Table 1). Meanwhile, the terminator, glyceraldehyde-3-phosphate dehydrogenase (NCBI: NC_026502.1:3180104-3182245) of *N.* crassa (Shinohara et al., 1998) was amplified with primer pairs of T_gdph_P1 & T_gdph_P2, and the amplified *T_gdph* fragment was further digested with restriction enzymes *Bgl* II and *EcoR* I, followed by insertion into the intermediate vectors (pPcfp_XPK_His, pPcfp_PTA_His, and pPcfp_HMGR_His) to get the final expression vectors pXPK, pPTA and pHMGR, respectively (Table 1, Figure 2A).

For the construction of two fused gene expression vectors, the *HMGR* was re-amplified with primer pairs HMGR_P1 & HMGR_P2 (Table 2), digested with both *BamH* I and *EcoR* I and inserted into *Bgl* II and *EcoR* I sites of pPcfp_XPK_His and pPcfp_PTA_His to get fused expressed intermediate vectors, pPcfp_XPK:HMGR_His and pPcfp_PTA:HMGR_His. The *T_gpdh* of *N. crassa* was inserted into the fused expressed intermediate vectors (pPcfp_XPK: HMGR_His and pPcfp_PTA:HMGR_His) after it was digested with both *Bgl* II and *EcoR* I to get the final fused vector pXPK:HMGR and pPTA:HMGR (Table 1, Figure 2A). The pPTA_H vector with hygromycin resistant marker was constructed for combining aforementioned three gene expressions in *N. crassa.* It was constructed by inserting the CDS of *PTA*, followed by *T_gdph* fragment into pPcfp_Myc_HyR (Table 1 and Figure 2A).

Table 1. Strains and plasmids used in this study.

Name of plasmids/strains	Characteristics	Source
pPcfp_Myc_His	*cfp* promoter, Histidine resistance (His)	He Qun China Agricultural University
pPcfp_Myc_HyR	*cfp* promoter, hygromycin resistance (HyR)	lab's collection
pPcfp_XPK_His	With Linker (GGCGGTGCTGCA encoding Gly_Gly_Ala_Ala)	This study
pPcfp_PTA_His	same as below	This study
pPcfp_HMGR_His	same as below	This study
pPcfp_PTA:HMGR_His	same as below	This study
pPcfp_XPK:HMGR_His	same as below	This study
pPcfp_PTA_HyR	same as below	This study
pXPK	same as below	This study
pPTA	same as below	This study
pHMGR	same as below	This study
pPTA:HMGR	same as below	This study
pXPK:HMGR	same as below	This study
pPTA_H	same as below	This study
*Escherichia coli (*DH5α)	Cloning host (Δ lac)	Lab's collection
Neurospora crassa (301-6, A)	Fugal host (Δ his-3)	Lab's collection
PTA/NC_{301-6}	pPTA transformed N. *crassa* 301-6 Strain	This study
XPK/NC_{301-6}	pXPK transformed N. *crassa* 301-6	This study
HMGR/NC_{301-6}	pHMGR transformed N. *crassa* 301-6	This study
PTA_HMGR/NC_{301-6}	pPTA_HMGR transformed 301-6 N. *crassa* 301-6	This study
XPK_HMGR/NC_{301-6}	pXPK_HMGR transformed 301-6 N. *crassa* 301-6	This study
PTA & XPK_HMGR/NC_{301-6}	pPTA_H and pXPK:HMGR co-transformed N. crassa 301-6	This study

Transformation of *N. crassa*

The single or fused gene expression vectors were transformed into the 301-6 strain using electroporation as described by Vela-Corcía et al. (2015) with some modifications. The electroporated *N. crassa* spores were then inoculated on minimal agar media and incubated at 30°C. The histidine free growing fungal colonies were picked unto minimal slants to grow again without histidine at 30°C for about 4 to 6 days. Their respective genomic DNAs were extracted from new growth colonies as described by Zhang et al. (2010) were used as template, and the transformed strains were finally confirmed by amplifying the inserted genes with detecting primers XPK_P1 & XPK_P2, PTA_P1 & PTA_P2 and HMGR_P1 & HMGR_P2 (Table 2, Figure 2B). For combing three genes in *N. crassa*, the expression vector of pPTA_H was transformed again into the successfully confirmed *XPK:HMGR* strain by selection on minimal agar media with hygromycin, and the strains were further confirmed by PCR amplification.

Total RNA of the transformed and wild-type strains were extracted using the Hi pure RNA isolation kit from Magen (Beijing, China) according to the manufacturer's instructions. The qualified isolated RNA was then transcribed into complimentary DNA (cDNA) using Fastking gDNA dispelling RT supermix from Tiangen (Beijing, China) according to the manufacturer's protocol. The primers XPK_D_P1&XPK_D_P2, PTA_D_P1&PTA_D_P2, HMGR_D_P1& HMGR_D_P2, PTA_H_D_P1& PTA_H_D_P2, and XPK_H_D_P1& XPK_H_D_P2 (Table 2) were used to amplify the fragment, respectively to detect gene expressions with the cDNA derived from mRNA as templates respectively (Figure 2C).

Growth rate determination

Fermentation for growth rate determination was carried out in a 500 mL erlenmeyer flask with 100 mL media and 1 mL of spore suspensions (10^6 spores/mL) of transformed and wild type strains obtained from minimum slant media

growth fungi was inoculated into the 3 different fermentation media, respectively (see cultural media and growth conditions) at 30°C with rotation of 160 rpm having 3 replications. During the cultivation, replicate flasks were drawn from the shaker cultivated at 5 different sampling time (48, 96, 144, 192 and 240 h), and their mycelia were filtered and dry weights determined after drying at 80°C to constant weight. The growth curves of the strains were then recorded from their dry weights (g/L) versus incubation time in hours.

Carotenoid quantification using different concentrations of carbon source

Fermentation was further carried out with only media 1, media 2 and starting from media 2 then adding glucose to final concentration of 2% (see culture media and growth conditions) after 4 days inoculation to investigate carotenoids titers among engineered and natural strains. Carotenoids were extracted from the accumulated biomass by bleaching with methanol after they have been weighed and ground with sea sand and the NX and NC concentrations were determined using the subtraction protocol as described by Avalos et al. (2012) with at least three independent duplications. NX concentration was estimated using its widely accepted specific Extinction Coefficient 1715 (Avalos et al., 2012) while NC concentration was estimated using β-carotene purchased from Jinang technology (Kunming, China), as standard with the measured specific Extinction Coefficient of 2590.

Microscopic observations of spores

Spore samples were picked directly from the solid media of different strains with nearly the same concentration by suspension in 80% glycerol, and a tiny drop of the suspended spores of about (10 μL) were viewed under the microscope.

Statistical analysis

Data was analysed using GraphPad prism version 9.0 (California, USA). The data was reported as means of each strain and statistical significance determined using analysis of variance (ANOVA). The level of statistical significance was set at ($p < 0.05$) with Tukey's multiple comparism analysis for strains that showed significance.

RESULTS

Verification of gene expression in *N. crassa*

The engineered strains were constructed to increase either only acetyl-CoA with single gene expression (*XPK* or *PTA*), increase only MVP influx by *HMGR* or both acetyl-CoA and MVP (Figure 1) with 2 fused gene expression (*XPK:HMGR, PTA:HMGR)* or 3 gene co-expression *(PTA with XPK:HMGR)* (Figure 1).

The constructed expression vectors (Figure 2A) were transformed with the method of electroporation and selected on minimal media. The histidine/hygromycin selected fungal colonies were further confirmed as transformed strains by PCR (Figure 2B) using primer pairs from Table 2. Expressions of single gene *PTA*, *XPK & HMGR*, fused *PTA:HMGR & XPK:HMGR* gene and *PTA* with the fused *XPK:HMGR* in *N. crassa* were confirmed (Figure 2C) at the mRNA level with primers using RT_PCR amplification (Table 2).

Changes in growth rate

The engineered *N. crassa* strains, either as single expressions of *XPK, PTA* & *HMGR*, fusion expressions of *PTA:HMGR* & *XPK:HMGR* or co-expression of *PTA* with *XPK:HMGR* were grown with media 1, media 2 and first grown in media 2 than adding final 2% glucose 4 days after inoculation, respectively. The engineered *N. crassa* strains did not reduce the growth rate (Figure 3A, 4A and 4B), as compared with 301-6 strain transformed with an empty vector, pPcfp_Myc_His (Table 1), and nearly all the transformed strains cumulated higher biomass with the strain of co-expression genes of *PTA* with fused *XPK:HMGR* having the highest biomass accumulation (7.86 g/L). When the strains were grown in media1 the exponential growth started after 48 h inoculation and entered into stationary phase after 96 h cultivation. When the strains were grown in media 2 (Figure 3A), the biomass accumulation increased about 2 times as compared with media 1 cultivation, however, the same single "S" pattern of growth was observed as cultivated in media 1. The growth pattern was changed to nearly double "S "type with the highest biomass accumulated recorded 192 h after cultivation first with media 2 and additional final 2% glucose media 4 days after inoculation. This indicated that, the biomass accumulation of the strain is dependent on available carbon source. The increase in the biomass of the engineered strains may be due to the channelling of the intermediate metabolites to terpenoid synthesis partially reducing the primary metabolic feedback inhibition and/or accumulation of terpenoids (such as carotenoid) increasing the stress tolerance of strain in fermentation condition.

NX and NC quantification in engineered strains

Using media 1 as fermentation broth, a preliminary test was carried out to quantify NX and NC; 96 h after inoculation just as the end of exponential growth of the mycelial dry weight was recorded and 144 h when the highest biomass accumulation was recorded (Figures 3A, 4A, and 4B). The 144 h after cultivation was used as the harvesting time from the preliminary testing as it had a significant increase in carotenoid concentrations as compared to that after 96 h (Figure 3B). NX concentrations had titers up to 0.81, 0.76, 1.0, 1.08, 1.11 and 1.39 mg/g with the wild-type having 0.67 mg/g for 96 h while 144 h had NX titers up to 1.09, 1.09, 1.3, 1.55, 1.66 and 1.83 mg/g with the wild-type having 0.78 mg/g. NC concentrations had titers up to 0.28, 0.25, 0.38, 0.47, 0.54 and 0.75 mg/g with the wild-type having 0.14 mg/g

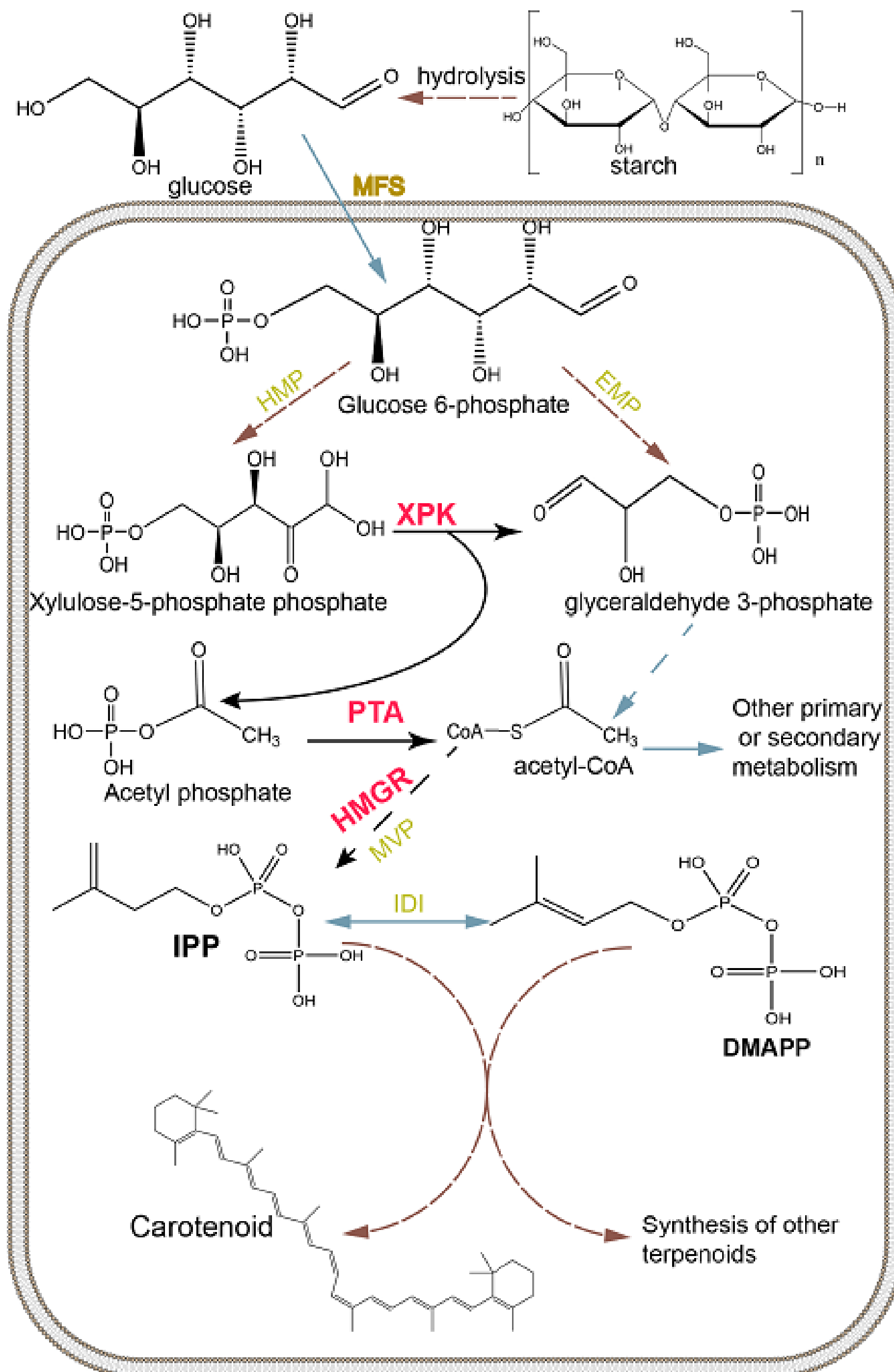

Figure 1. The metabolic engineered *N. crassa* pathway for acetyl-CoA accumulation with expression of *XPK* and *PTA* and increasing IPP and DMAPP by *HMGR* expression for enhancing carotenoid biosynthesis. MFS: Major facilitator superfamily for out cellular sugar transporting, HMP: hexose monophosphate pathway, EMP: Embden-Meyerhof-Parnas pathway, MVP: Mevalonate pathway, IDI; isopentenyl-diphosphate Delta-isomerase, *XPK, PTA* and *HMGR* expression highlighted by red.

for 96 h while 144 h had NC titers up to 0.56, 0.56, 0.65, 0.75, 0.81 and 0.93 mg/g with the wild-type having 0.33 mg/g cultivated with media 1, respectively (Figure 3B and 3C). Fermentation carried out with only media 2 had NX titers up to 1.25, 1.27, 1.72, 2.1, 2.3 and 2.66 mg/g with the wild-type having 1.0 mg/g while NC concentrations

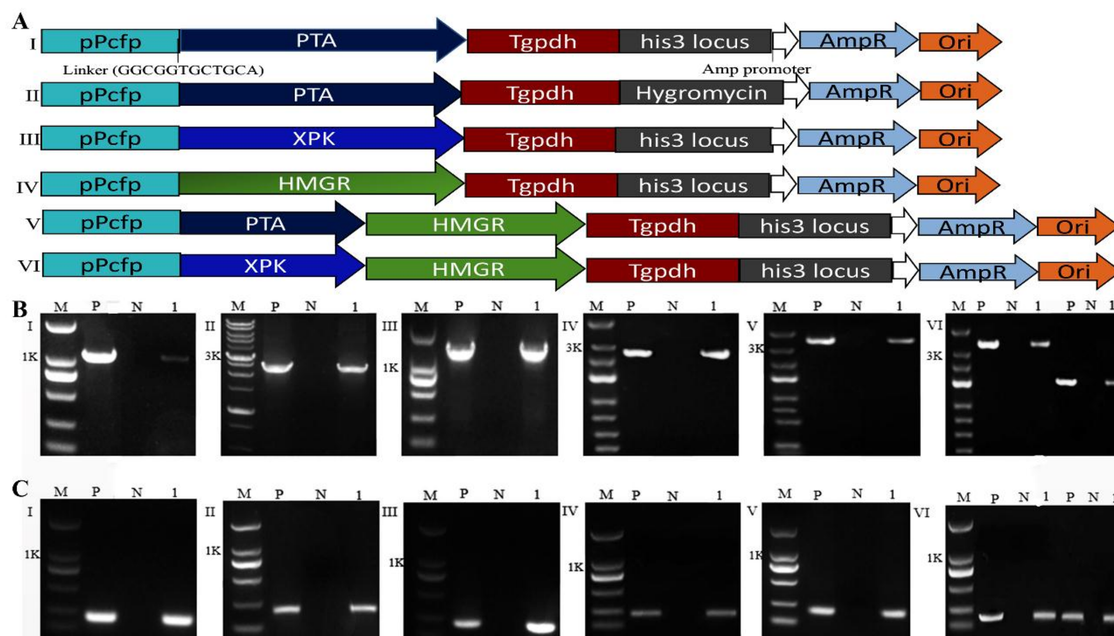

Figure 2. Construction of engineered strains of *Neurospora crassa*. (A) Linear representation of chimeric genes as constructed expressions driven by cfp promoter, a designed sequence linker of "*GGCGGTGCTGCA*" coding peptide of "Gly_Gly_Ala_Ala" for fusion protein link as shown on the chimeric genes; (A I) *PTA*, (A II) *PTA* (with hygromycin resistance), (A III) *XPK*, (A IV) *HMGR*, (A V) fused *PTA:HMGR* and (A VI) fused *XPK:HMGR* with a gpdh terminator of *N. crassa.* (B) Agarose gel electrophoresis of amplified genes from transformed strains. Lane M: 2k, 5k &10k bp DNA ladder, lane P: positive control, lane N: negative control and lane 1: amplified genes from the genomic DNA of the transformed strains where (B I) *PTA* (1049 bp), (B II) *XPK* (2484 bp), (B III) *HMGR* (1349 bp), (B IV) fused *PTA: HMGR* (2393 bp), (B V) fused *XPK: HMGR* (3533 bp) and (B VI) PTA with fused XPK: HMGR (1049 and 3533 bp). (C) Agarose gel electrophoresis of RT_PCR amplified genes from engineered strains. Lane M: 2k bp DNA ladder, lane P: positive control, lane N: negative control and lane 1: amplified genes from the cDNA derived from mRNA of the transformed strains where (C I) *PTA* (196 bp), (C II) *XPK* (198 bp), (C III) *HMGR* (188 bp), (C IV) fused PTA: HMGR (*194*bp), (C V) fused *XPK: HMGR* (168bp) and (C VI) *PTA* with fused *XPK:HMGR* (196 and168 bp), respectively.

had titers up to 0.66, 0.66, 0.75, 0.85, 0.91 and 1.07 mg/g with the wild-type having 0.44 mg/g. (Figure 4C). NX and NC concentrations increased significantly starting from media 2 then adding additional 2% glucose 4 days after inoculation as compared to using only media 2 or media 1, this strategy resulted in NX titers up to 2.54, 2.58, 2.75, 2.97, 3.1 and 4.5 mg/g of dry weight, respectively as compared to the wild-type with 1.70 mg/g after 192 h and NC titers up to 1.06, 1.05, 1.19, 1.27, 1.30 and 1.54 mg/g of dry weight, respectively as compared to the wild-type with 0.80 mg/g after 192 h (the highest biomass accumulation) (Figure 4D).

Phenotypical changes of engineered strains of *N. crassa*

Although there was no changes in mycelial growth among the natural and transformed strains, other phenotypical changes were observed such as deep colouration among transgenes, which is consistent with the increase in carotenoid concentrations determined by its colour enrichment, morphological changes in spores observed under the microscope showed that, the non-transformed strain had jointed spores with regular cell shapes, while that of the transformed strains showed dis-jointed spores with irregular cell shapes. This may be attributed to the insertion of the gene expressions to channel more carbon metabolic flux for terpene synthetic pathway, resulting in reduced polysaccharides biosynthesis of the cell wall leading to weak connections between cells. The irregular shapes of the cells of the transgenic strains may be attributed by its individualistic nature of weak cell wall as compared to the attached with branched appearance of the non-transgenic strain (Figure 5), and the weak wall property may have promoted germination of spores speeding biomass accumulation.

DISCUSSION

With an established easy genetic transforming system, the filamentous fungus *N. crassa*, which has a high

Table 2. Primers used in this study.

Primer name	Primer sequence (5' -3')	ize (bp)
PTA_P1	CGC***GGATCC***ATGGATCC**GGCGGTGCTGCA**ATGG (bold and italic shows *BamH* I site and bold underlined shows linker for encoding Gly_Gly_Ala_Ala, same as below)	1049
PTA_P2	CGC**GAATTC**TCATTA**AGATCT**GCCCTGAGCC (bold and italic show shows *EcoR* I &*Bgl* II sites respectively, same as below)	
XPK_P1	GCA***GGATCC***ATGTCC**GGCGGTGCTGCA**ATGG	2484
XPK_P2	CCG***GAATTC***TCATTA***AGATCT***CTTGAGGGAC	
HMGR_P1	CGC***GGATCC***ATGGATCC**GGCGGTGCTGCA**ATGG	1349
HMGR_P2	GCA***GAATTC***TCATTA***AGATCT***GGTGTTCTC	
T_GPDH_P1	CGC***AGATCT***ATGTCGGTTGCGTACCCGCG	719
T_GPDH_P2	GC***GAATTC***AGCGGGCGGCAAGCGGAT	
PTA_D_P1	GCTCCGAGTCCGTCATCAAG	196
PTA_D_P2	ATCATGGTGGCGAAGTAGATGG	
XPK_D_P1	GCACTCCATCAAGTTCCTCAAC	198
XPK_D_P2	TGGTGGTAGGTGGCGTAGT	
HMGR_D_P1	GATATCGAGGTCCACGTCTTCC	188
HMGR_D_P2	AGATCGGCGAGGTTGGAGAG	
PTA_H_D_P1	ACTCCCGCATCGAGAAGAT	194
PTA_H_D_P2	GAACTTGCCGATGACGTTC	
XPK_H_D_P1	TCAAGATGACCGGCAAGAC	168
XPK_H_D_P2	GATGACGTTCTCGATCATGC	

proliferation rate, relatively simple life cycle and is able to grow with cheap media has played significant roles in numerous biotechnological applications including production of important secondary metabolites (Moser and Pichler, 2019). Since it can produce carotenoids naturally, the aim of this study is to optimized IPP and its interconvertible isomer DMAPP accumulation for efficient interested terpenoids biosynthesis from relative simple carbon source such as glucose with easy detection of carotenoid colour enrichment. The *XPK*, catalyses the formation of acetyl phosphate and glyceraldehyde-3-phosphate from xylulose-5-phosphate (Meadows et al., 2016), the PTA, catalyses the reversible reaction between acetyl phosphate and Acetyl-CoA (Castaño-Cerezo et al., 2009), while HMGR, enhancing the conversion of Acetyl-CoA to IPP and DMAPP by MVP (Istvan, 2002), were expressed as single, fused or triple genes together in *N. crassa* to convert more media carbon source to Acetyl-CoA followed by forming IPP and DMAPP (Figure 1). The engineered *XPK* and/or *PTA* overexpression strains not only

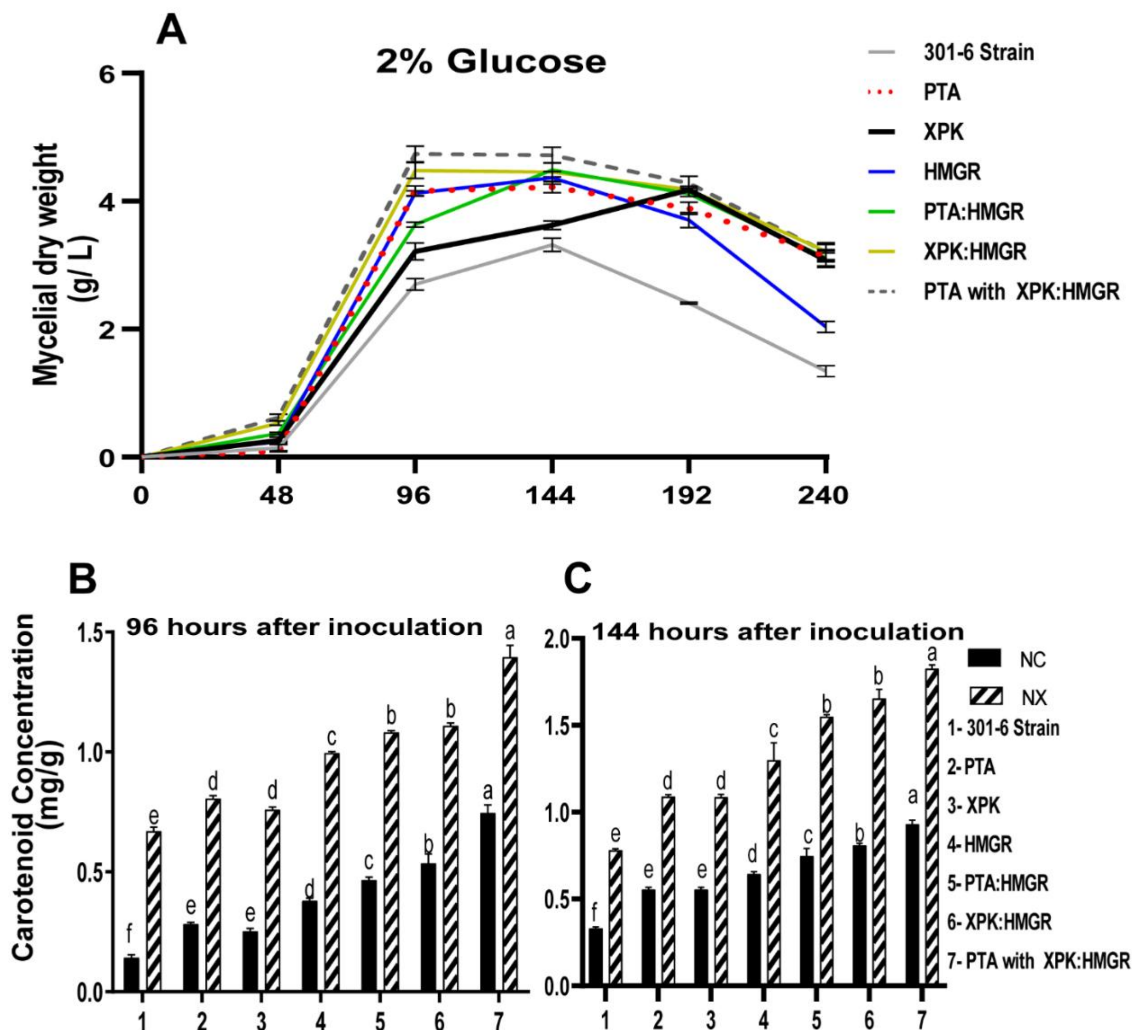

Figure 3. Changes in growth rate among metabolic engineered and wild type strains of *N. crassa*. (A) Growth rate of the transgenes and wild-type using media 1 (2% glucose) from spores at 5 different sampling times (48, 96, 144,192 and 240 h) with at least three independent replications. Carotenoid concentration (NX and NC) titers determined among the engineered and wildtype at (B) 96 h after inoculation just as the first exponential growth was recorded, (C) 144 h after inoculation when the highest biomass accumulation was recorded using media 1 respectively. Bars which do not share the same letters are significantly different with p-values <0.05.

produces more Acetyl-CoA, but also reduce acetic acid accumulation and relieve feedback inhibition of glycolysis, while the HMGR normally use the co-enzyme NADPH and not NADH as its co-enzyme (Beach and Rodwell, 1989; Istvan and Deisenhofer, 2001), however, the HMGR used in this study has the NADH-specific co-enzyme thus (Meadows, 2016), its may channel more carbon flux and energy into MVP responsible for carotenoid biosynthesis for NADH is more abundant than NADPH, but also speeding the recycling between NADH and NAD^+ to remove the glycolysis feedback inhibition by NADH in hyperopic condition. Additionally, the synthesis of carotenoids may have increased the stress tolerance during the growth; these changes in the engineered strains are consistent with the results that all engineered *N. crassa* strains did not reduce the growth rate by transgenes burden, as engineered strains increased their biomass as compared to the non-transgenic wild type (Figures 3 and 4).

Aside the no change in mycelial growth among the strains, dis-jointed spores with irregular cell shapes were observed and may be attributed to the inserted transgenes channelling the more carbon metabolic flux of the β-carotenoids synthesis pathway, resulting in reduced polysaccharides biosynthesis of their cell walls leading to weak connections between cells (Figure 5). As we know, this is the first time, to significantly increase carotenoids by optimizing MVP by channelling the carbon source to synthesize IPP and DMAPP in *N. crassa*. Our engineered N. crassa increased 2~3 folds of carotenoids compared

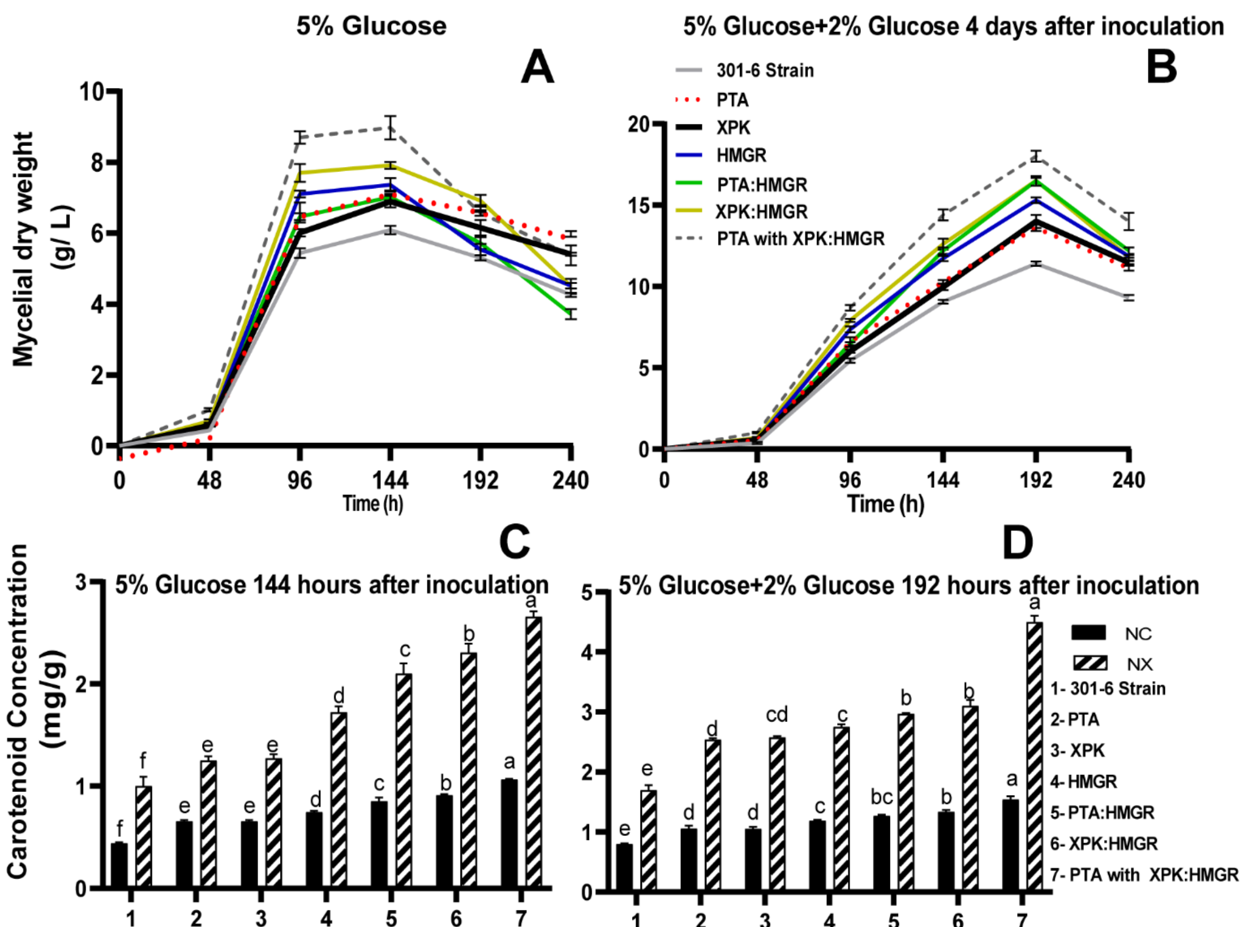

Figure 4. Changes of carotenoid concentration among engineered and wild type strains of *Neurospora crassa*. (A) Growth rate of the transgenes and wild-type using media 2 (5% glucose) and (B) starting from media 2 then add additional 2% glucose 4 days after inoculation from spores at 5 different sampling times (48, 96, 144,192 and 240 h) with at least three independent replications. Carotenoid concentration (NX and NC) titers determined among the engineered and wild type strains at (C) 144 h after inoculation using only media 2 and (D) 192 hours after inoculation starting from media 2 then add additional 2% glucose 4 days after inoculation from transgenes and wild-type strains. Bars which do not share the same letters are significantly different with p-values <0.05.

with wild type, with titers up to 4.5 mg/g for NX and 1.54 mg/g for NC. The currently achieved content of carotenoids in these strains do not reach the popular production in engineered *Fusarium fujikuroi* which produced the highest NX titers up to 8.3 mg/g dry weight (Parra-Rivero et al., 2020), however, the engineered *N. crassa* stains are not fully adapted, and their fermentation broth are not optimized for increasing biomass accumulation for carotenoids production, thus, continuing with a rational design coupled with adaptation and batch fermentation at the early stages, adding more nitrogen source for cell mass accumulation, adding more carbon source at stationary phase and improving the aerobic condition during fermentation may finally catch up to the carotenoids yield achieved in higher yield filamentous fungi or even catch the yields of yeast and the *E. coli*. It is very important as our final goal of the engineered *N. crassa* is to make this fungus a working horse to synthesize other active terpenoids, and this can be achieved by targeting terpenoids synthetic pathway and switching off carotenoid synthesis after the MVP pathway is optimized by carotenoid colour indication.

Conclusion

Optimized mevalonate pathway (MVP) by metabolic engineering is a key step to synthesize carotenoid and other active terpenoids in *N. crassa* as our engineered *N. crassa* strains increased 2~3 folds of carotenoids compared with wild type.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

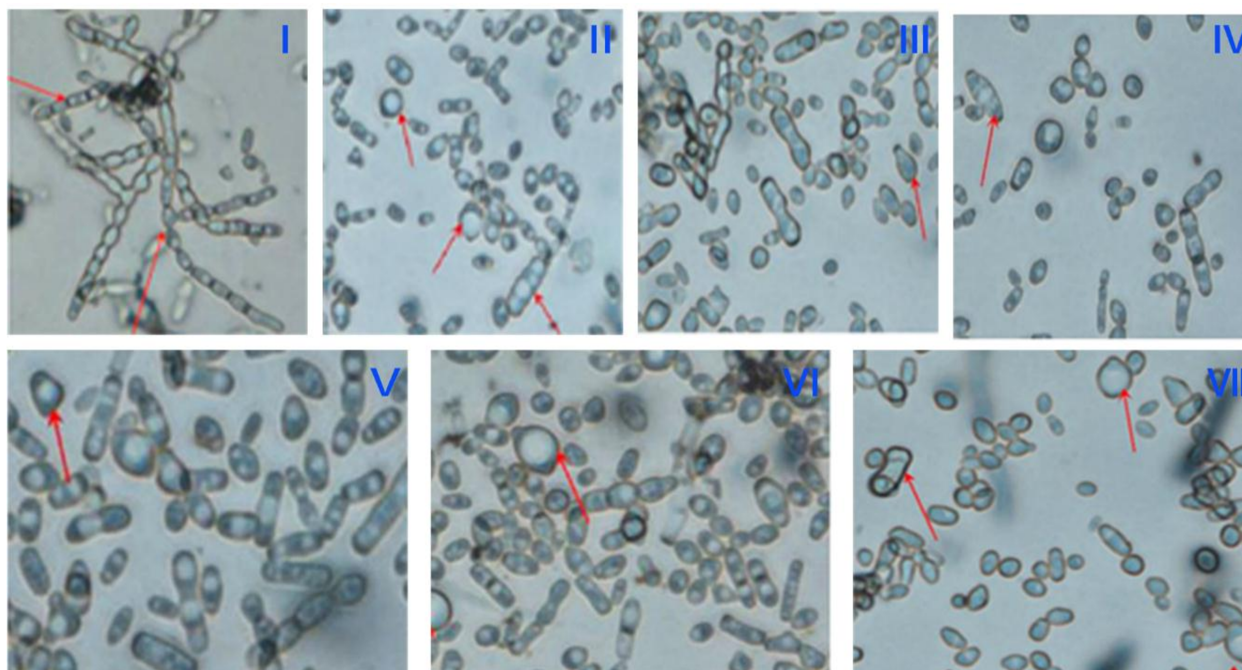

Figure 5. Changes spore phenotype among engineered and wild type strain. (I) 301-6 strain spores viewed under the microscope showing jointed cells shapes and red arrows showing regular cell shapes and sizes. (II~VII) transformed strains (*PTA, XPK, HMGR*, fused *PTA:HMGR*, fused *XPK:HMGR* and *PTA* with fused *XPK:HMGR* strains, respectively) spores viewed under the microscope showing different dis-jointed cells with the red arrows showing irregular cell shapes and sizes.

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