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Full Length Research Paper

Anaerobic and micro-aerobic 1,3-propanediol production by engineered *Escherichia coli* with *dha* genes from *Klebsiella pneumoniae* GLC29

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1,3-Propanediol (1,3-PDO) is a bifunctional molecule, and used in applications similar to those of ethylene glycol, propylene glycol, 1,3-butanediol and 1,4-butanediol. The use of glycerol as a feedstock is an alternative to reduce production costs for both 1,3-PDO and biodiesel, since biodiesel glycerol can be used for the production of 1,3-PDO by bacteria. Also, using metabolic engineering, it is possible to manipulate the metabolic routes and obtain high value products, reduce or eliminate the formation of undesirable byproducts. The aim of the study was to produce 1,3-propanediol in *E. coli* cloned with *dha* genes from *Klebsiella pneumoniae* GLC29. Six genes responsible for 1,3-PDO production in *Klebsiella pneumoniae* GLC29 were cloned. These genes were assembled in pSB1C3 as an expression vector: Genes *dhaB1*, *dhaB2*, *dhaB3* and *dhaT* (pSB1C3dhaB123T), and another vector with genes *dhaF* and *dhaG* (pSB1C3dhaB123TFG) were derived using Gibson's Assembly technique. *Escherichia coli* TCS099 and SZ63 stains were used as hosts for 1,3-PDO production, and kept at -80°C for long-term storage. Glycerol was used as the sole or main carbon source in all experiments. Fermentations were performed in flasks in aerobic and anaerobic conditions using minimal media. Also, two stage fermentation (aerobic-anaerobic) was performed for 1,3-propanediol production. Only pSB1C3dhaB123TFG was able to produce high amounts of 1,3-PDO in shake flasks experiments, producing 2.5 g/L in micro-aerobic conditions, using *E. coli* TCS099 as host. Besides, *E. coli* SZ63 hosting pSB1C3dhaB123TFG was able to produce high amounts of 1,3-PDO, corresponding to 11.3 g/L of 1,3-PDO using a two-stage fermentation process using low concentration of vitamin B12 (1 mg/L). Plasmid pSB1C3dhaB123TFG shows potential for producing high amounts of 1,3-PDO, specially because of *dhaF* and *dhaG*, reaffirming the importance of this genes on 1,3-PDO production, especially with the addition of low amounts of vitamin B12, which is an expensive compound.

Key words: 1,3-Propanediol, *Escherichia coli*, glycerol, *Klebsiella pneumoniae*, metabolic engineering.

INTRODUCTION

1,3-Propanediol (1,3-PDO) is a starting point for a new generation of polymers with improved properties for the textile industry. It can be obtained by chemical or biochemical route, however in the chemical route the co-

production of its isomer 1,2-propanediol cannot be avoided, being produced in a 1:1 ratio, resulting in a costly separation process. DuPont and Genencor International uses a genetically modified *Escherichia coli* strain to produce 1,3-PDO from glucose (Maervoet et al., 2011). Glucose is a high cost feedstock, and biodiesel-derived glycerol is becoming an abundant alternative feedstock, due to the increasing biodiesel production (Pyne, 2014). The price of corn-derived glucose is approximately US$ 0.28/kg (Gallardo et al., 2014) while the current price of biodiesel-derived glycerol varies from US$ 0.04 to 0.11/kg (Quispe et al., 2013). Some processes of biodiesel production generate crude glycerol a byproduct considered as waste, generally with no commercial value or acceptance, and its disposal costs is attributed to the biodiesel producers (Yazdani and Gonzalez, 2007).

Glycerol may be used as carbon source in many bioprocesses, and one promising exploitation is to produce 1,3-PDO by *Klebsiella pneumoniae* and *Clostridium butyricum* (Papanikolaou and Aggelis, 2009; Rymowicz et al., 2006). Production from crude glycerol from biodiesel can contribute to the reduction of environmental pollution and commercial valorization of this carbon source and to lower the production of 1,3-propanediol. But intrinsic bottlenecks limiting these processes are the potential pathogenicity of *K. pneumoniae,* and the requirement of total anaerobic conditions for *Clostridium* spp., which although it is not vitamin dependent, the enzyme from *Clostridium* spp. is oxygen sensitive, requiring therefore, totally anaerobic cultures (Kaur et al., 2012).

The production of 1,3-PDO is connected to the process of glycerol oxidation. Glycerol enters the cell by *glpF* (glycerol facilitated transport), or by diffusion (Maervoet et al., 2011). After entering the cell, it may follow two routes. In the first one, it suffers oxidative dehydrogenation by a NAD^+ dependent glycerol dehydrogenase, becoming dihydroxyacetone (DHA). DHA is then phosphorylated to dihydroxyacetone phosphate by an ATP-dependent DHA kinase. Through a parallel process, glycerol is dehydrated to form 3-hydroxypropionaldehyde (3-HPA) by glycerol dehydratase (EC 4.2.1.30), which in *K. pneumoniae* is B12-dependent, composed by three peptides encoded by *dhab1, dhaB2,* and *dhaB3*. Then, 3-HPA is reduced to 1,3-PDO by 1,3-propanediol oxidoreductase (EC 1.1.1.202) linked to NADH (Oh et al., 2012; Yazdani and Gonzalez, 2007).

In *K. pneumoniae* the overall reductive reaction rate is limited, firstly because this reaction is mediated by cyanocobalamin (vitamin B12). Furthermore, substrate inhibition may occur, with an irreversible binding of cobalamin with the enzyme to form alkylcobalamines. However, reactivation factors, encoded by genes gdrA and gdrB (or dhaF and dhaG), swap the inactivated cobalamin for a new molecule of vitamin B12, requiring the presence of magnesium ions (Mg^{2+}) and with consumption of 1 ATP. The resultant Apo enzyme rebinds coenzyme B12, and glycerol conversion to 3-HPA resumes. To avoid low activity of the enzyme, the amount of glycerol should be controlled and vitamin B12 to the medium should be added (Yamanishi et al., 2012; Nakamura and Whited, 2003; Shibata et al., 2002; Kajiura et al., 2001, Daniel et al., 1998). As a consequence of the normal catalytic cycle with glycerol, the coenzyme B12 is occasionally rendered inactive (B12-inact). The B12-inact remains tightly bound to the dehydratase and catalysis ceases. An auxiliary enzyme, glycerol dehydratase reactivase, facilitates the dissociation of the B12-inact and glycerol dehydratase (EC 4.2.1.30). The resultant apoenzyme rebinds and glycerol conversion to 3-HPA resumes (Nakamura and Whited, 2003).

E. coli naturally grows on glycerol under aerobic conditions, but several researchers have been trying to genetically modify it to produce 1,3-PDO, thus making glycerol a valuable carbon source (Ma et al., 2009). By metabolic engineering, it is possible to manipulate metabolic routes, obtain high value products, and reduce or eliminate the production of undesirable byproducts (Cheng et al., 2005). *E. coli*, which does not have a *dha* system, is unable to grow anaerobically on glycerol without an exogenous electron acceptor and does not produce 1,3-PDO (Tong et al., 1991). Although several researches have been done on 1,3-PDO production using glycerol, the 1,3-PDO productivities and, in particular, the product concentrations obtainable with engineered organisms harboring the 1,3-PDO pathway have been low (less than 0.1 g/L with recombinant *S. cerevisiae* and 6.5 g/L with recombinant *E. coli* AG1) compared to those of natural 1,3-PD producers (Biebl et al., 1999). While other papers have reported high amounts of 1,3-PDO produced by Engineered *E. coli* harboring genes from *Clostridium sp.*, the limitation of being very sensitive enzymes to oxygen is a drawback in large scale use. Also, very few papers were successful using genes from *K. pneumoniae*, which is a great natural producer of 1,3-PDO and requires little vitamin B12, as we demonstrated in this work.

The objective of this study was to construct two plasmids, expressing genes related to synthesis from *K. pneumoniae* GLC29 in order to produce 1,3-PDO from glycerol: One plasmid harboring the genes *dhaB1*, *dhaB2*, *dhaB3*, encoding glycerol dehydratase, and *dhaT* encoding 1,3-PDO oxidoreductase, under control of the R0010 promoter, while the other plasmid had in addition

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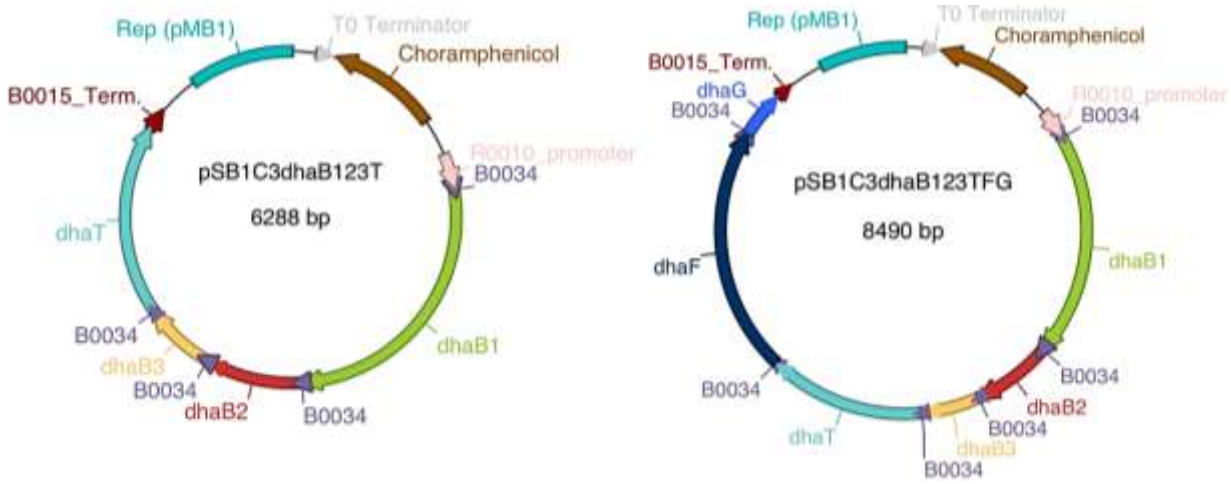

Figure 1. Designed plasmids and contruct containing *dha* genes for 1,3-propanediol - Plasmid pSB1C3_JJ04450 was used as template to derive pSB1C3dhaB123T and pSB1C3dhab123TFG.

the coding DNA for the glycerol dehydratase reactivation factors *dhaF* and *dhaG.* This approach was done using standard parts from iGEM and genes extracted from a wild type bacterium (*K. pneumoniae* GLC29), which have been previously reported as a good 1,3-PDO producer. Furthermore, Gibson's Assembly (Gibson et al., 2009) was used for all cloning, eliminating the need to previously sequence or modify the wild genes for traditional cloning, cloning multiple genes at once, and eliminating scars from restriction enzymes. Both constructs for 1,3-PDO production were compared in micro-aerobic and anaerobic conditions, and then evaluated for 1,3-PDO production in bioreactors using anaerobic conditions and two-stage fermentations.

MATERIALS AND METHODS

Strains and maintenance

K. pneumoniae CLG29 was isolated from bryophytes grown on the base of leaf stalks of *Terminalia catappa* at UNESP – Universidade Estadual Paulista, Rio Claro, Brazil and characterized as a new 1,3-PDO producer (da Silva et al., 2014). *E. coli* TCS099 - Δ*mgsA*, Δ*ldhA*, Δ*fdrA*, Δ*zwf*, Δ*ndh*, Δ*maeB*, Δ*pta*, Δ*poxB*, Δ*mhp*F, Δ*adh*P and Δ*adhE* (Trinh and Srienc, 2009), was used at University of Tennessee Knoxville, and *E. coli* SZ63, W3110 mutant, Δ*focA-pflB*::FRT, Δ*frdBC* Δ*adhE*::FRT, *ackA*::FRT (Zhou et al., 2003) was kindly sent from the University of Florida to the Department of Biochemistry and Microbiology, Biosciences Institute of Rio Claro, Univ. Estadual Paulista – UNESP**.** Both used as hosts for vectors assembled and experiments. Cultures were kept at -80°C for long-term storage, and reactivated in Luria-Bertani medium (LB) prior to experiments.

Cloning

Plasmid backbone pSB1C3_RFP containing BBa_R0010 promoter, sensitive to LacI and CAP protein, and BBa_B0015 double terminator (Figure 1) was used as template for the plasmid backbone. BBa_B0034 was designed within the primers, which sequence was obtained from iGEM. Primers corresponding to genes were designed using known sequences from *K. pneumoniae* strains 342, MGH78578, and KTCC 2242, in which complete genome is available at ncbi.nlm.nih.gov (three genomes homology regions were identical to the primers designed). All primers were designed using Gibson's Assembly (Gibson et al., 2009), with a 40 base pair overlap with the next sequence (Table 1), dhaB1_F was designed with a 40 base pair overlap with the promoter and dhaT_term_R was designed with a 40 base pair terminator overlap. Genes were isolated from *K. pneumoniae* GLC29 using PCR and the primers listed in Table 1.

All genes (Figure 2) used for cloning were amplified using the primers designed using Phusion® polymerase HF, gel and purified using Zymo Research® Zymoclean™ Gel DNA Recovery Kit and ligated using Gibson Assembly (2009), cleaned and concentrated using Zymo Research® DNA Clean and Concentrator™. Plasmids were extracted from *E. coli* cultures after 6 h growth in LB media with 50 µg/ml chloramphenicol, using Zymo Research® - Plasmid Miniprep™ - Classic. Construction of plasmids were performed using Gibson's Assembly (GA) isothermal protocol (Gibson et al., 2009). Plasmid pSB1C3-dhaB123T carries the genes *dhaB1, dhaB2, dhaB3* (glycerol dehydratase), *dhaT* (1,3-propanediol oxidoreductase), while pSB1C3-dhaB123TFG harbors the above-mentioned genes and also *dhaF* and *dhaG* (glycerol dehydratase reactivase). Plasmids were sequenced by capillary electrophoresis ABI 3730 genetic analyzer.

Production of 1,3-propanediol

Confirmed plasmids were extracted from Top10 using plasmid mini-prep and transformed into competent *E. coli* TCS099 by heat shock. Transformed *E. coli* TCS099 hosting pSB1C3dhaB123T and pSB1C3dhaB123TFG were characterized for 1,3-PDO production in minimal medium (glycerol 20.0 g/L; KH_2PO_4 3.5 g/L; K_2HPO_4 5.0 g/L; $(NH_4)_2HPO_4$ 3.5 g/L; $MgSO_4.7H_2O$ 0.25 g/L; $CaCl_2.2H_2O$ 0.015 g/L; vitamin B12 0.25 mg/l; chloramphenicol 30.0 µg/ml). Experiments were performed in test tubes in aerobic, anaerobic and micro-aerobic conditions. Periodically, samples of 1 ml were collected and centrifuged at 10,000 g for 10 min. The cell-free

Table 1. Primers designed for 1,3-propanediol constructs - Gibson's assembly.

Primer		Sequence
dhaB1_F	For	aattgtgagcggataacaatttcacacaaaagaggagaaaATGAAAAGATCAAAACGATT
dhaB1_R	Rev	GTTGTCTGTTGCATtttctcctcttTTATTCAATGGTGTCAGGCTG
dhaB2_F	For	ACACCATTGAATAAaaagaggagaaaATGCAACAGACAACCCAAATTC
dhaB2_R	Rev	GTTTTCTCGCTCATtttctcctcttTCACTCCCTTACTAAGTCGAC
dhaB3_F	For	TAGTAAGGGAGTGAaaagaggagaaaATGAGCGAGAAAACCATGCGCG
dhaB3_R	Rev	ATACGATAGCTCATtttctcctcttTTAGCTTCCTTTACGCAGCTTATG
dhaT_F	For	GTAAAGGAAGCTAAaaagaggagaaaATGAGCTATCGTATGTTTGATTATC
dhaT_R	Rev	GCTATTAACGGCATtttctcctcttTCAGAATGCCTGGCGGAAAATC
dhaF_F	For	GCCAGGCATTCTGAaaagaggagaaaATGCCGTTAATAGCCGGGATTG
dhaF_R	Rev	GGTGAAAGCGACATtttctcctcttTTAATTCGCCTGACCGGCCAG
dhaG_F	For	GTCAGGCGAATTAAaaagaggagaaaATGTCGCTTTCACCGCCAGGCG
dhaG_R	Rev	cagtctttcgactgagcctttcgttttatttgatgcctggTCAGTTTCTCTCACTTAACG
dhaT term_R	Rev	cagtctttcgactgagcctttcgttttatttgatgcctggTCAGAATGCCTGGCGGAAAA
pSB1C3_F	For	Ccaggcatcaaataaaacgaaaggctcag
psB1C3_R	Rev	Ttctcctcttttgtgtgaaattgttatcc

Dotted underlined, Promoter overlap; underlined, ribosome binding site; Dot, dash underlined, terminator overlap

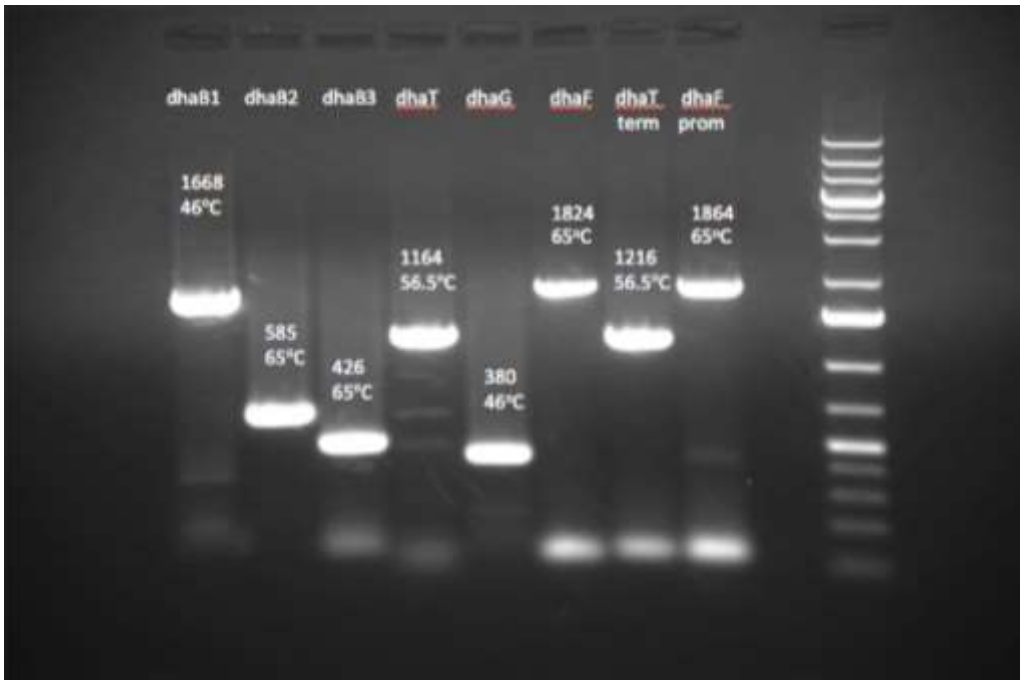

Figure 2. Genes amplified with Phusion® polimerase. GeneRuler 1 kb Plus DNA Ladder 75 to 20,000 base pairs.

supernatant was filtered (0.22 µm) and analyzed by high performance liquid chromatography (HPLC) using ion exchange column Phenomenex Rezex ROA (300 mm × 7.8 mm) at 60°C and 0.005 M H_2SO_4 solution as mobile phase at 0.5 ml/min flow rate, equipped with UV and RI detectors. External standards were ethanol, 1,3-PDO, propionic acid, acetic acid, 2,3-butanediol and glycerol.

Two-stage fed-batch fermentations were performed with four different aeration conditions on initial 24 h (first stage). Medium composition was glycerol 20 to 45 g/L, tryptone 10 g/L, KH_2PO_4 0.136 g/L, $(NH_4)_2HPO_4$ 3.5 g/L, $MgSO_4.7H_2O$ 0.48 g/L, $CaCl_2$ 0.15 g/L, vitamin B12 1 mg/L, sodium selenite 1 µM, and chloramphenicol 30 µg/ml. After 24 h, remaining glycerol was quantified, and one pulse of glycerol was fed to reach 50 g/L in the bioreactor. The second stage was set into anaerobic conditions, and pure nitrogen gas was pumped at 0.05 L/min. Samples were

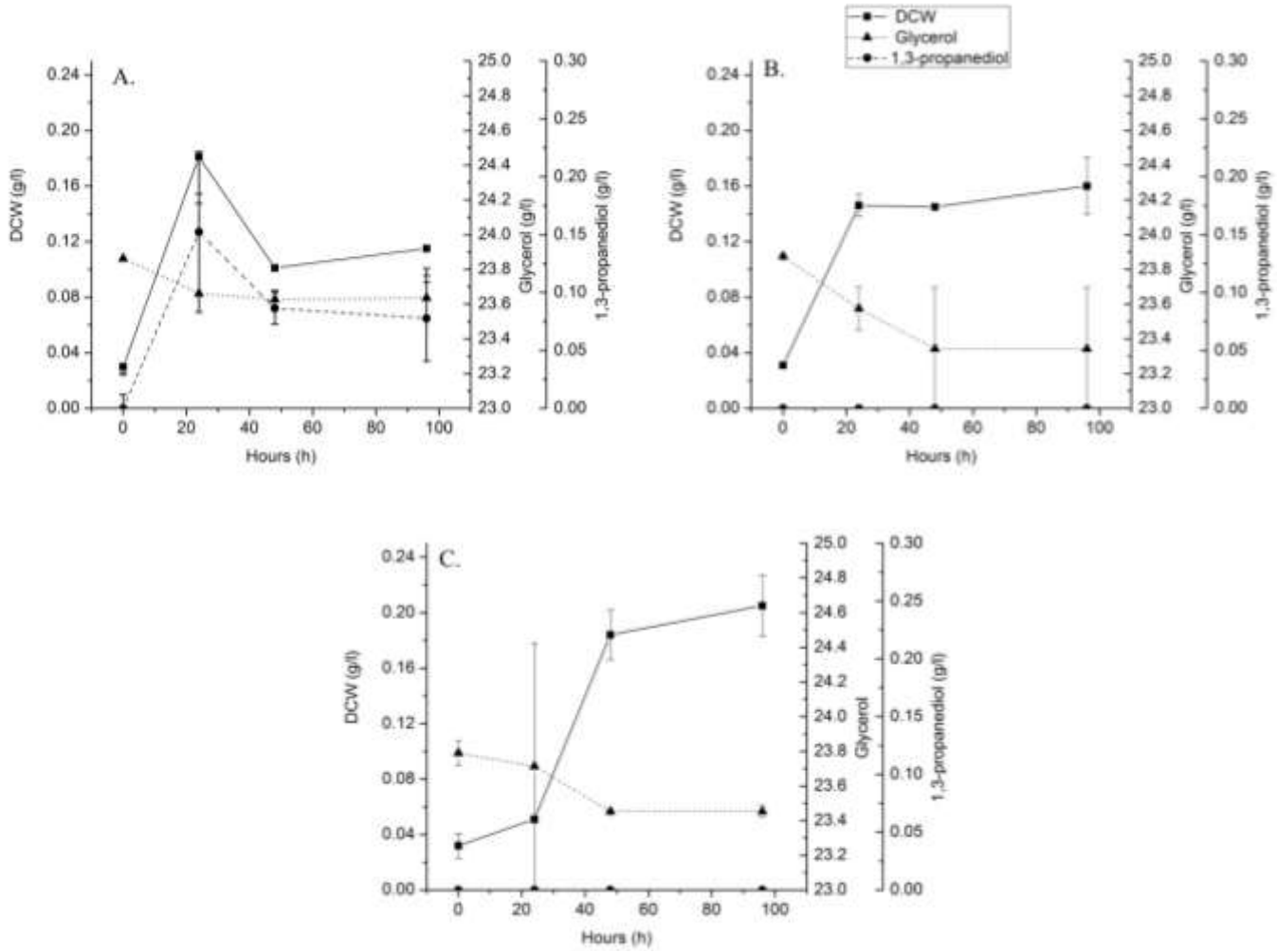

Figure 3. Production in anaerobic conditions (induction at time zero with IPTG 500 µM). pSB1C3dhaB123TFG; B. pSB1C3dhaB123T; C. pSB1C3_RFP.

collected and analyzed as described previously.

RESULTS AND DISCUSSION

Production of 1,3-propanediol

Production of 1,3-PDO and influence of glycerol dehydratase reactivase (*dhaF* and *dhaG*) were evaluated in micro-aerobic and anaerobic conditions. Induction was performed with 500 µM of IPTG at the first minute of the process and TCS099 with pSB1C3_RFP was used as negative control. Anaerobic culture showed little growth and low 1,3-PDO production. Host with pSB1C3dhaB1234TFG presented the fastest growth and the best production, followed by pSB1C3dhaB123T, both presented faster growth than the negative control (Figure 3).

Using micro-aerobic condition (Figure 4), growth and 1,3-PDO production were higher than in anaerobic cultures, in which the strain containing pSB1C3dhaB123TFG reached up to 2.5 g/L of 1,3-PDO in 72 h (0.41 g/g yield). Glycerol consumption was more consistent and it was possible to verify that pSB1C3dhaB123TFG consumed more glycerol than the other experiments.

It is important to note that in this experiment, 250 µg/l of vitamin B12 was added to a minimal medium; besides, fermentation strategies and media were not optimized yet. In a similar result, Skraly et al. (1998) demonstrated production of 1,3-PDO up to 6.3 g/L out of 9.33 g/L of glycerol in a 4-liter fed-batch *E. coli* AG1/pTC53 fermentation using 14 µg/l of vitamin B12. Using statistical design, Zhang et al. (2006) constructed a novel

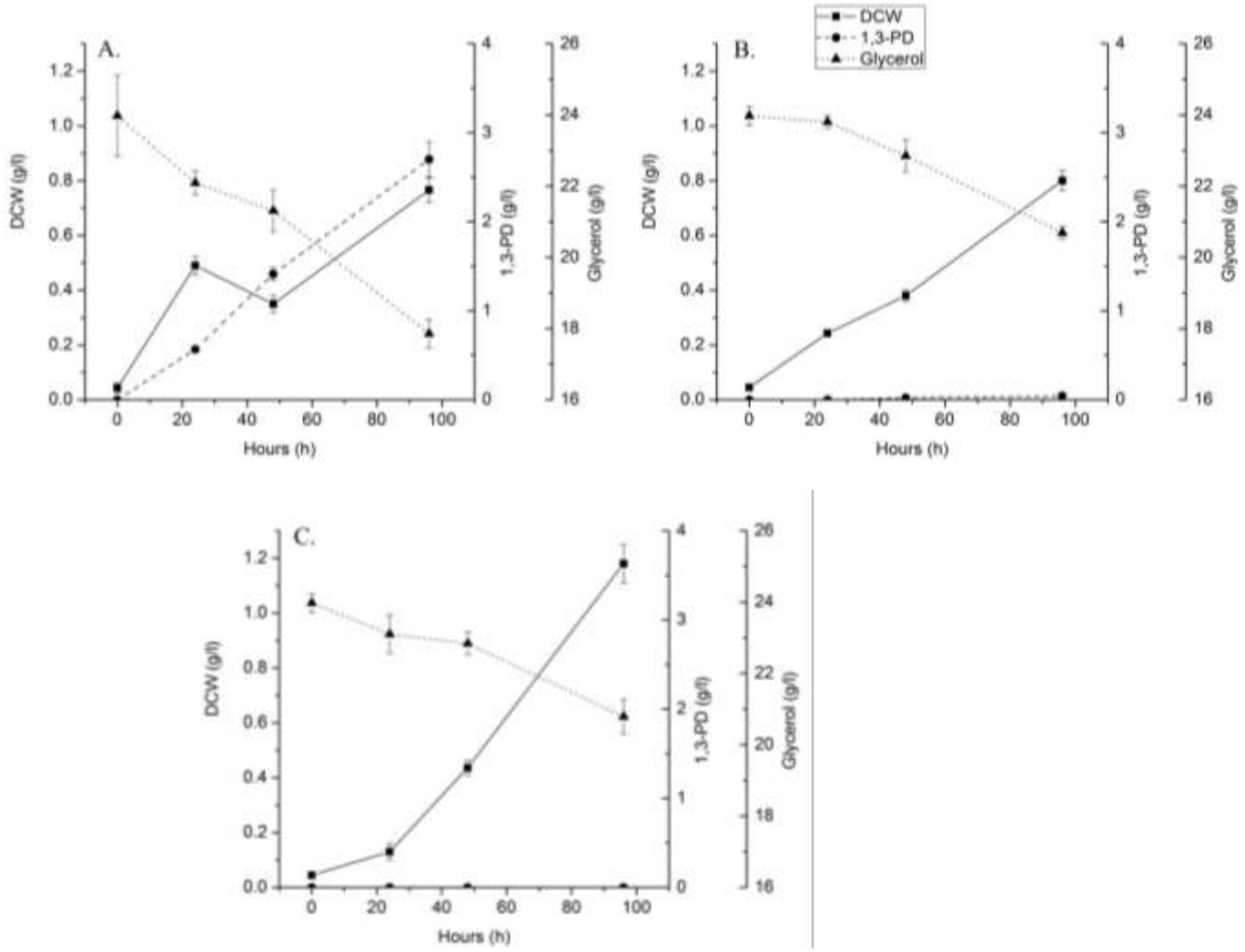

Figure 4. Growth in micro-aerobic conditions (induction at time zero with IPTG 500 µM). A. pSB1C3dhaB123TFG; B. pSB1C3dhaB123T; C. pSB1C3_RFP.

E. coli recombinant using the complete *dhaB* gene set comprised of three different subunits dhaBCE, (2.8 kb) from *Citrobacter freundii* assembled into pHsh-yqhD. By the experimental design using 61.8 g/L of glycerol, 6.2 g/L of yeast extract, and 49 mg/l of vitamin B12, Zhang et al. (2006) produced 43.8 g/L of 1,3-PDO in a 5-liter bioreactor in aerobic conditions (0.8 vvm). However, due to the current high price of vitamin B12, the use of 49 mg/l is not economically feasible.

Cameron et al. (1998) cloned from *K. pneumoniae* the genes *dhaB* and *dhaT* to *E. coli*, including several ORFs (dhaB3, dhaB3a, dhaB4, and dhaB4a) from *dhaB* complex. A series of synthetic plasmids with *dhaB* and *dhaT* genes disposed in the same transcription direction and under the same promoter were built, and 1,3-PDO concentration reached over 70 g/L in 5 L fermenter, using fed batch fermentations, reaching a yield of 0.39 $g_{1,3\text{-}PDO}/g_{glycerol}$ (0.48 mol/mol), but using aerobic conditions. The main byproduct was 2,3-butanediol, reaching nearly 20 g/L. Importantly, in our case; no 2,3-butanediol was detected on fermentations using the different plasmids and strains. Different from this work, the aim was to produce 1,3-PDO anaerobically using glycerol.

Reactor fermentation with *E. coli* SZ63

In an effort to maximize 1,3-PDO yield and concomitantly minimize production time and byproduct, Tang et al. (2009) established a two-stage two-substrate fermentation for producing 1,3-PDO by an engineered *E. coli* K-12 ER2925 strain, and dhaB1 and dhaB2 from *C. butyricum* SYU 20108 were cloned and expressed in the host strain. On the first stage from 0 to 10 h, dissolved oxygen was maintained above 40% air saturation, glucose was added continuously to maintain up to 25 g/L until a final biomass

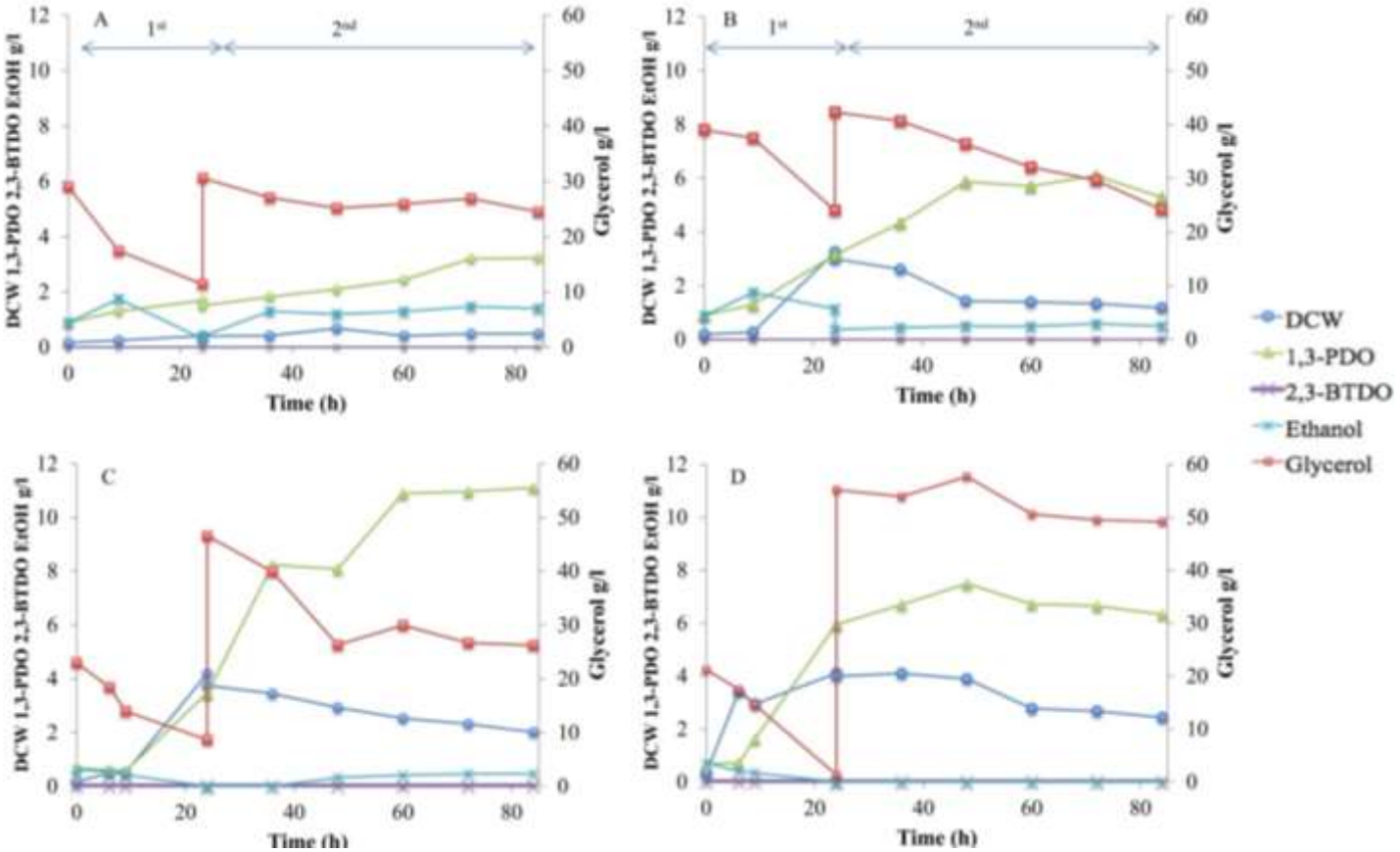

Figure 5. Fed-batch culture of *E. coli* SZ63+pSB1C3dhaB123TFG - 1,3-propanediol g/L production in two stage fermentation. First Stage (0-24 h): a. Anaerobic; b. 5% pO_2 ; c. 15% pO_2; d. 20% pO_2. All second stages (24-84 h) is anaerobic; feed pulse is at 24 h.

of 26 g/L DCW was reached. Then, the second stage involved replacement of glucose medium and byproducts from the first stage with new fresh glycerol fermentation medium every 2 h, shifting the temperature to 42°C, 1,3-PDO the authors claim reaching a final concentration of 104.4 g/L. Therefore a similar approach was figured out enriching the culture with pO_2 and comparing with a anaerobic control.

E. coli SZ63 harboring pSB1C3dhaB123TFG was used for these experiments in bioreactors at UNESP - Universidade Estadual Paulista, Brazil, since *E. coli* TCS099 was not available for importation from the host university. Fermentations in bioreactors are shown in Figure 5. The first fermentation (Figure 5a) started anaerobically while nitrogen gas was used to purge oxygen out of the fermenter prior to the start. Despite the 18 g/L of glycerol consumed in the initial 24 h, little 1,3-PDO was produced (1.69 g/L) and 0.41 g/L of DCW was reached. *E. coli* SZ63 harboring our plasmid improved DCW by 3 fold, glycerol consumption improved 90 fold, and 1,3-PDO production improved by 11 fold on the first 24 h of process when compared to essays in tubes using *E. coli* TCS099 and minimal media. Glycerol was fed (18 g/L) at 24 h, but even though recreating Gonzalez (2012) optimal conditions for glycerol fermentation had tried, only 3.23 g/L of 1,3-PDO was produced, and 6.12 g/L of glycerol consumed after 84 h. However, this represents a 9-fold improvement in 1,3-PDO production. Still, conversion rates were limited by the amount of cells, due to slow growth and consequent low cell density. No 2,3-butanediol was observed, but 1.4 g/L of ethanol was detected in the end of the fermentation, that could be residual from the antibiotic mixture dissolved in ethanol.

Setting up to 5% pO_2 for the initial 24 h fermentation (Figure 5b) resulted in 15 g/L of glycerol consumed, 3.27 g/L of DCW and 3.15 g/L of 1,3-PDO produced in the initial 24 h. This means that more cells were able to produce more 1,3-PDO. In the second stage, fermentation was shifted to anaerobic conditions with nitrogen purge, 18 g/L of glycerol was fed into the bioreactor, and after 60 h, 6.1 g/L of 1,3-PDO was reached. This result suggests that *E. coli* SZ63 is not able to grow in anaerobic condition using glycerol as sole carbon source, even when 1,3-PDO pathway was inserted as a way to recycle NADH, so 1,3-PDO could be the last electron acceptor. It is suggested that micro-aerobic conditions are therefore necessary for growth using glycerol as sole carbon source.

The best conditions were reached using 15% pO_2 (Figure 5c), which resulted in 3.5 g/L of 1,3-PDO, 4.2 g/L of DCW, and 14.4 g/L of glycerol consumed on the initial 24 h. Compared to initial essays in tubes previously described, DCW was improved 8.5-fold, 1,3-PDO was improved 6.5-fold, and glycerol consumption was improved 16.8 fold. Production continued on the second stage in anaerobioc conditions, reaching 11.1 g/L after 60 h, which improved 4.1-fold. No 2,3-butanediol was observed, and residual 0.5 g/l of ethanol was detected.

When 20% pO_2 was employed (Figure 5d), glycerol was depleted after 24 h. During the first 24 h, 6.5 g/L of 1,3-PDO was produced. On the second stage, in anaerobic conditions, however, there was no consumption of glycerol nor substantial 1,3-PDO production. This could mean that cells were stressed from glycerol depletion on the initial 24 h. Similar to the other experiments, no 2,3-butanediol or ethanol was observed during the 84 h of experiment.

Transferring a biosynthetic pathway to a non-native producer faces several difficulties, such as the non-native pathways overexpression can disrupt the intrinsic metabolism in the host, using most of the essential precursors for growth or maintenance. Furthermore, pathways re-engineering frequently leads to imbalanced gene expression, which creates bottlenecks in the biosynthetic pathway that could that reduce production of the wanted compound (Atsumi et al., 2008). New investigations on protein expression or mRNA could elucidate better comprehension on limiting factors on the production of 1,3-PDO in this work.

Previous studies on D-lactate production have shown that an initial period of aeration in complex media can be used to boost the growth of D-lactate-producing *E. coli* strains containing mutations in phosphoenolpyruvate carboxylase and phosphotransacetylase genes resulting in shorter time for fermentation. Initial aeration of an SZ58 culture eliminated the lag phase resulting in 10-fold increase in cell yield within the initial 24 h, which accelerated glucose conversion to lactate and reduced the time required to complete the process (Zhou et al., 2003).

Further fermentations should be performed to optimize these conditions. Among the evaluated strains, glycerol was not efficiently fermented to support cell growth and 1,3-PDO production. Glycerol is a highly-reduced substrate and maintenance of redox balance is challenging specially in anaerobic conditions. In the absence of oxygen or other electron acceptor, *E. coli* is not able to use glycerol as sole carbon source efficiently. The incorporation of glycerol as a carbon source to cell mass results in production of reducing equivalents, in which H_2 plays an important role, participating as electron donor for several reactions. If H_2 is decreased, this does not happen and fermentation proceeds. Increasing headspace dilutes H_2, and also flushing it out with an inert gas, such as argon, nitrogen, or CO_2, improves fermentation (Gonzalez, 2012).

Glycerol can be oxidized to dihydroxyacetone (DHA) by the GldA enzyme, a type II glycerol dehydrogenase, which is encoded by *gldA* in *E. coli,* however it is usually not expressed in wild type strains. Activation of this gene requires inactivation of *glpK*, *glpR* and *glpD* followed by mutagenesis and selection procedures, which resulted in a strain that recovered the ability to metabolize glycerol, but not the ability to ferment glycerol (Gonzalez, 2012). In *E. coli*, there are two glycerol-3-phosphate dehydrogenases, but only one can use NAD^+ as an electron acceptor (Lin, 1976). Only one of these enzymes is able to donate electrons to the fumarate reductase complex, producing succinate from fumarate. It was demonstrated that the quantity of succinate produced by their *E. coli* strain corresponded to only 4% of the glycerol metabolized, therefore, it is not able to grow in anaerobic environment (Skraly et al., 1998). Production and growth of 1,3-PDO was reported by Tong et al. (1991) using a cosmid harboring *dha* genes from *K. pneumoniae* ATCC 25955, in which *E. coli* AG1/pTC1 produced up to 0.46 mol/mol of 1,3-PDO after 120 h, but little 1-3-PDO was produced. *DHA* and glycerol were added to a defined medium, and also the cosmid had a *dha* kinase and glycerol dehydrogenase from *K. pneumoniae*, which might explain the cell growth reported in anaerobic environment. New experiments cloning *dha* kinase from *K. pneumoniae* should be performed to evaluate cell growth in anaerobic conditons.

Optimum glycerol fermentation by *E. coli* occurs at slightly acid pH of 6.3, 10-20% CO_2 or higher, high concentrations of glycerol, up to 100 g/l, 200 rpm, 37°C, 0.01 L/min argon or nitrogen, low potassium (less than 10 mM) and phosphate concentrations (from 50 to less than 1.3 mM) are preferred, because high concentration of these ions inhibits glycerol dehydrogenase and *DHA* kinase, and furthermore increases methylglyoxal toxicity. Tryptone supplementation is additionally required when *DHA* is not added (Gonzalez, 2012). These conditions were replicated in these experiments on bioreactors, but fermentation of glycerol did not result in good cell growth and high productivity.

High concentration of glycerol is required to GldA, due to its low K_m, and acid condition favors its reductive activity, while neutral or alkaline conditions increases its oxidative activity. Also, alkaline conditions increase methylglyoxal toxicity. To prevent cytoplasmic acidification, *E. coli* produces CO_2 and H_2 from formic acid, but H_2 can negatively influence glycerol fermentation (Gonzalez, 2012). Besides, high concentrations of glycerol, over 49 g/L, is known to decrease 1,3-PDO production, since it favors the inactivation of glycerol dehydratase (GDHt) (da Silva et al., 2014).

The overexpression of GDHt leads to serious growth deficiency of *K. pneumoniae*. Instability of the plasmids bearing the genes encoding GDHt and/or 1,3-PDO oxidoreductase were responsible for the observed

phenomena due to an imbalanced conversion of glycerol to 3-HPA and its toxicity. Similar research using resting cell systems, in which growth was stopped while metabolic activity was maintained, eliminates disturbances associated with cell growth. Overexpression of 1,3-PDO oxidoreductase led to faster glycerol conversion and 1,3-PDO production. After 12-h conversion, it improves 1,3-PDO yield by 20.4%, and boosts product/substrate yield from 50.8 to 59.8% (mol/mol) (Song et al., 2010). Further investigations should be done to address if 3-HPA is occurring in *E. coli* SZ63 during 1,3-propanediol production and see the need to overexpress 1,3-PDO oxidoreductase.

Zhang et al. (2006) constructed a similar plasmid in *E. coli* with genes from *Citrobacter freundii*, and using experimental design from fixed concentrations: 61.8 g/L glycerol, 6.2 g/L of yeast extract and 49 mg/L of vitamin B12, achieved 1,3-PDO production of 41.3 g/L. Ma et al. (2009) constructed a plasmid containing *dhaB* and *dhaT* genes in *E. coli* and expressed both genes in the same direction, successfully producing 11.3 g/l of 1,3-PDO from 40 g/l glycerol.

Conclusion

E. coli hosting pSB1C3dhaB123TFG was able to produce higher amounts of 1,3-propanediol than the plasmid pSB1C3dhaB123T. Genes *dhaF* and *dhaG* are very important for the production of 1,3-propanediol, specially with lower concentrations of vitamin B12 (from 0.25 to 1 mg/L). Moreover, aeration on the initial 24 h up to 15% pO2 is able to increase 1,3-PDO production and productivity with *E. coli* SZ63, reached 11.3 g/L of 1,3-PDO produced in 60 h. Bottlenecks on glycerol fermentation by *E. coli* still need to be addressed, adding 1,3-PDO as a NADH recycle metabolic pathway did not improve *E. coli* growth on glycerol on the absence of oxygen, that could mean regulation issues such as 3-HPA toxicity or an anaerobic glycerol-3-phosphate dehydrogenase deficiency from *E. coli*. New fermentation strategies could be performed to improve productivity and production, such as using glucose and glycerol together, and further engineer *E. coli* to efficiently ferment glycerol. Also, protein expression and mRNA analysis could elucidate bottlenecks on glycerol metabolism by *E. coli* hosting pSB1C3dhaB123TFG and improve 1,3-PDO and cell growth. Improving glycerol uptake via cloning *K. pneumoniae dhaD*, *dhaK*, *dhaL* and *dhaM* to convert glycerol to DHA and then to glyceronephosphate and pyruvate could increase cell growth in anaerobioc conditions, furthermore generating more NADH for 1,3-PDO production.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Obtaining mutant fungal strains of *Aspergillus niger* with high production of fructooligosaccharides (FOS) using ultraviolet light irradiation

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A strain of *Aspergillus niger* PR-142 native to northern coast of Peru was subjected to successive processes of mutagenesis by ultraviolet light (UV) irradiation at 253.7 nm to increase the production of fructooligosaccharides (FOS). An initial selection was made by considering the mutants with increased invertase activity followed by the measurement of β-fructosyltransferase (FTase) activity both in mycelium and extracellular environment. Five selected mutants, which showed increased values of mycelium invertase activity (ranging from 101 to 128% as compared to the parent strain) at 40°C and sodium dodecylsulfate 0.15 (w/v), were grown in a fermentative medium in 50 mL conical tubes on a rotary shaker, and their FTase activity was determined. The 6-M69 mutant showed the most active mycelium activity of 1.5 fold as compared to the parent strain. When the same reaction was performed between 1 to 4 h, at the 3rd h, the mycelium FTase activity significantly increased up to 7 and 3 times in the mutant and parental strain, respectively. Finally, 4 mutants and the parental PR-142 were genetically characterized using inter simple sequence repeat polymerase chain reaction (ISSR-PCR) molecular markers. This analysis showed a significant 33% polymorphic bands between the parent and mutant markers, and 20 bands were unique to the mutants.

Key words: *Aspergillus*, mutagenesis, β-fructosyltransferase, fructooligosaccharides, inter simple sequence repeat polymerase chain reaction (ISSR-PCR).

INTRODUCTION

Fructooligosaccharides (FOS) are prebiotics with known beneficial properties, including growth promotion of *Bifidobacterium* species, prevention of colon cancer, and reduction of cholesterol levels and triglycerides in the

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blood. It can also be considered as a calorie-free and non-cariogenic sweetener (Maiorano et al., 2008). FOS can be obtained from different vegetables such as garlic onions, artichokes, asparagus, bananas, rye wheat and tomatoes (Sangeetha et al., 2005). However, commercial FOS are enzymatically produced either by hydrolysis of inulin (to produce oligofructoses) catalyzed by inulinases (EC 3.2.1.7) or by transfructosylation of sucrose employing sucrose fructosyltransferases (FTase, EC 2.4.1.99) and β-D-fructofuranosidases (also termed invertases) (FFase, EC 3.2.1.26) (Maiorano et al., 2008). These classes of enzymes are widely distributed in plants, fungi and bacteria kingdoms and most of them belong to the Glycoside Hydrolase families 32 (GH32) and 68 (GH68) (Olarte et al., 2016). Although, the physical and chemical characteristics of the molecular structure vary in many microorganisms, they all have both hydrolytic and transfer activities (Dominguez et al., 2013).

Although, FOS has been produced by commercial microorganisms for more than twenty years, it is still necessary to continue to identify new FFase(s) of high yield as well as the genes involved in its synthesis. This is the reason why the search for microorganisms with these characteristics such as *Penicillum*, *Aerobasidium*, *Fusarium* and mainly *Aspergillus* species is continuously reported (Muñiz et al., 2016). It should be noted that *Aspergillus niger* is a filamentous fungus of the group of black *Aspergillus* that are widely disseminated in the environment and is widely used in biotechnology industries due to its great secretory capacity of a large amount of enzymes and organic acids, among other things. In addition, many of these products have the generally recognized as safe (GRAS) status by the US Food and Drug Administration (FDA). Different techniques have been used in order to increase the production of FOS and among them are recombinant DNA technologies that have allowed the expression of the FTase gene in plants (Heyer and Wendenburg, 2001), bacteria and yeasts (Zhang et al., 2015). In the specific case of increasing FOS productivity, although there are very few reports on the use of ultraviolet (UV) irradiation mutagenesis, Guilarte et al. (2009) obtained three mutants of *Aspergillus oryzae,* known as IPT-745, IPT-746 and IPT-748, which showed values ranging from 1.5 to 1.8-fold of activity, that was bound to the mycelium of the parent strain. The ability of UV light to produce genetic variation is known and has been used on numerous occasions to produce metabolic changes that increase the efficiency of some particular enzymes of interest. The inter simple sequence repeat polymerase chain reaction (ISSR-PCR) molecular markers have been successfully used to study the genetic variability of *Aspergillus* species, but there are no reports on the use of this marker in UV mutants in order to better compare them with the parent strain.

The present study aimed to 1) increase FOS production, using a native *A. niger* strain, via UV irradiation, and 2) characterize the mutants in reference to the parent strain using an ISSR-PCR as molecular marker.

MATERIALS AND METHODS

Microorganism and fermentative medium

The native strain of *A. niger* PR-142 isolated from sugarcane soil during 2015 at the north coast of Peru were used in this research. The isolate was identified in the Biotechnology Laboratory of the Continental University S.A.C, Huancayo, Peru (Gutarra et al., 2017). All strains including the mutants were grown on potato dextrose agar (PDA) medium at 30°C for 5 to 7 days. The produced spores were filtered and separated from the mycelium with the help of cotton swabs in sterile 0.1% (w/v) Tween 80 medium, and they were quantified using a Neubauer chamber. The fermentative tests were carried out in 250 ml flasks containing 50 ml of fermentative medium (g/L): sucrose 3.0, $NaNO_3$ 0.3, KH_2PO_4 0.2, $MgSO_4.7H_2O$ 0.05, $MnCl_2.4H_2O$ 0.02 and $FeSO_4.7H_2O$ 0.001 with a pH of 5.5.

Growth studies

Adequate doses of sodium dodecylsulfate (SDS) were evaluated in order to restrict the growth of mycelia and to have isolated strains. The evaluation comprised a range of 0.001 to 0.3% (w/v), resulting in the growth of hyphae being measured for a period of between 3, 4 and 5 days. Different doses of ultraviolet (UV) radiation were evaluated by using two 253.7 nm germicidal UV lamp (15W G15T8, Philips Ltd.) within a time range of 1 to 10 min, fixed as an optimal time that allowed the survival of only 5% of the spores used.

Production of mutants

A concentration of 10^5 spores was spread on glass plates coated with PDA medium that was supplemented with SDS and placed at a distance of 15 cm from two germicidal UV lamps (15W G15T8, Philips Ltd.) for 4 min. The spores were maintained for 30 min in absolute darkness and incubated at 40°C overnight and, then, at 30°C for 5 to 6 days. The chosen colonies were those that at first glance, presented typical morphology while those of strange appearance were discarded.

Selection of mutants based on FFase activity

As an initial step, the initial selection of promising mutants was based on the activity of FFase (invertase) secreted by them according to the methodology reported by Guilarte et al. (2009) that consists of taking circular fragments of mycelium of 7 mm in diameter as a source of enzyme. In the second stage, 10^7 spores were grown in 250 ml flasks with 50 ml of the fermentation medium for 72 h at 30°C and 200 rpm in an orbital shaker. The obtained mycelia were vacuum filtered using a polysulfone (PSF) filtration kit (Nalgene Ltd.) and Whatman® grade 1 qualitative filter paper. The enzymatic reaction was carried out in a shaking water bath GFL 1083 (Biovendis Ltd.) at 100 rpm for 60 min at 50°C with 50 ml conical tubes containing 1.2 ml of 0.2 M tris-acetate buffer at pH 5.5 and using approximately 0.02 g of mycelium as the enzyme source. As the substrate, 3.7 ml of a sucrose concentration of 64% (w/v) was used. The amount of reducing sugars obtained was quantified by the 3,5-dinitrosalicylic acid (DNS) assay method as described by Miller (1959). In the first stage, given the considerable number of

Table 1. Effect of sodium dodecylsulfate (SDS) on the colony size of *Aspergillus niger* PR-142.

SDS (%w/v)	Colony diameter (mm)*			Percentage of reduction		
	Day 3	Day 4	Day 5	Day 3	Day 4	Day 5
0	23.7 ± 2.3	30.9 ± 0.8	39.2 ± 0.4	100	100	100
0.01	18.0 ±1.8	28.5 ± 0.6	25.7 ± 0.3	92.2	65.6	65.6
0.02	19.1 ± 1.9	26.3 ± 0.3	24.5 ± 0.4	85.1	62.5	62.5
0.03	20.2 ± 2.0	23.3 ± 0.4	21.8 ± 0.4	75.4	55.6	55.6
0.04	20.1 ± 2.0	23.3 ± 0.5	23.4 ± 0.5	75.4	59.7	59.7
0.05	24.2 ± 2.4	29.0 ± 0.4	26.8 ± 0.6	93.9	68.4	68.4
0.06	22.4 ± 2.2	26.2 ± 0.5	27.0 ± 0.5	84.8	68.9	68.9
0.07	22.1 ± 2.2	27.8 ± 0.3	26.4 ± 0.3	90	67.3	67.3
0.08	18.0 ± 1.8	19.9 ± 0.2	20.3 ± 0.4	64.4	51.8	51.8
0.09	17.9 ± 1.7	19.9 ± 0.4	21.4 ± 0.9	64.4	54.6	54.6
0.10	18.3 ± 1.8	20.0 ± 0.2	23.6 ± 0.1	64.7	60.2	60.2
0.11	14.8 ± 1.4	17.8 ± 0.2	19.0 ± 0.0	57.6	48.5	48.5
0.12	12.2 ± 1.2	13.4 ± 0.3	15.4 ± 0.6	43.4	39.3	39.3
0.15	8.3 ± 0.8	10.3 ± 0.1	12.4 ± 0.2	33.3	31.6	31.6

*Diameter of the colony is the average of 20 measurements.

strains to be evaluated, only the absolute values of absorbance (optical density, OD 540 nm) were considered as the selection factor. In the second stage, FFase activity linked to mycelium was defined as that catalyzing the formation of 1 µmol reducing sugar per minute under the above conditions.

Selection of mutants with high FTase activity

The mycelial and extracellular FTase activities of the mutants that had the highest values of invertase activity were determined. For this purpose, the same methodology as described above was used, but with 0.3 ml of the fermentation broth as an enzyme source for the extracellular activity. The reaction products were analyzed by liquid chromatography with refractive index detector (LC-RID) on an Agilent Technologies 1220 Infinity LC system-1260 RID equipment (Boeblingen, Germany). Separation was performed on a HPLC column Kromasil® (100-NH_2) (Akzo Nobel, Brewster, NY, USA) (250 x 4.6 mm, I.d. 5µm particle size) using 70:30 (v/v) acetonitrile/water as the mobile phase and isocratic elution with a flow rate of 1.0 ml min^{-1} for 120 min. One unit of fructosyltransferase activity was defined as the amount of enzyme producing 1 µmol of FOS (1-kestose and nystose) per min, under the assay conditions.

Temporal profile of the reaction

In a manner that is similar to that described above, the FTase reaction of the mycelium of the best mutant was evaluated for a period of 1 to 4 h using the portions of mycelia of about 0.02 g as the enzyme. The reaction was undertaken on a ThermoMixer® Comfort Shaker (Eppendorf, Cambridge, UK) at 800 rpm and 50°C using 2 mL tubes containing 0.42 ml of 0.2M tris-acetate buffer with pH at 5.5, and 1.3 ml of 64% (w/v) sucrose was used as substrate.

Statistical analysis

Data analysis was carried out using one-way analysis of variance (one-way ANOVA) and Turkey's test by the Statistical Package for the Minitan16. Statistical significance was set at $P < 0.05$ and the results were expressed as means ± standard error of mean.

Scrutiny of genetic variability

The inter simple sequence repetitive (ISSR) molecular markers were used to test the ability of UV irradiation to produce genetic differences between mutants and parental PR-142. Genomic DNA extraction was performed using the methodology developed by Liu et al. (2000). The amplification via polymerase chain reaction (PCR) was performed with a volume of 25 µL having the same number and concentration of components as described by Neal et al. (2011) in a Mastercycler® Nexus Gradient Thermal Cycler (Eppendorf, Germany) using 6 ISSR primers (Integrated DNA Technologies) (Table 5). The thermal cycle of the PCR was also similar to that described by Neal et al. (2011). The PCR products were resolved on 1.5% agarose gels after staining with 1% ethidium bromide. The ISSR band patterns were assigned values of 1 (band presence) and 0 (band absence). The analyses were performed using the NTSYSpc program version 2.11.

RESULTS

Evaluation of the effect of SDS concentration on hyphal growth

A reduction of 68.4% in the diameter of the colonies was achieved at a SDS concentration of 0.15% (w/v) on the fifth day of counting (Table 1). This size was sufficient and facilitated the separation of individual colonies averaging 12 to 15 colonies per plaque.

Resistance to ultraviolet light

The parental strain was characterized by presenting

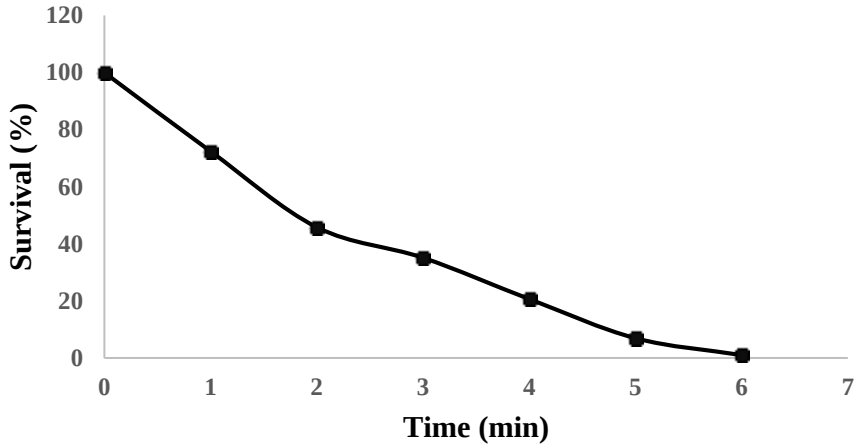


Figure 1. Survival of *Aspergillus niger* PR-142 spores after exposure to UV radiation. Spores were inoculated in PDA medium.

Table 2. Intracellular invertase activity of mutants and parental *Aspergillus niger* PR-142.

Strain	OD (540 nm)[1]	Abs. relation (%)	Mycelial activity A_T (Ug^{-1})[2]	A_T Relation (%)
PR-142	0.718 ± 0.04	100	4.25 ± 0.40[c]	100.0
6-M69	0.920 ± 0.01	128.13	7.43 ± 0.39 [a]	174.8
6-M61	0.890 ± 0.09	123.96	5.49 ± 0.34[b]	129.2
4-M37	0.782 ± 0.09	108.91	6.59 ± 0.80[a]	155.1
5-M40	0.773 ± 0.02	107.77	4.41 ± 0.48[c]	103.8
6-M65	0.731 ± 0.01	101.81	5.33 ± 0. 31[b]	125.4

[1]Sample mean (n=3) ± SD; [2]Mean of six samples (n=6) ± SD. Means followed by the same letter are not significantly different according to the Tukey's test (P<0.05). OD: optical density.

different doses of resistance to UV irradiation which reached a lethality of 50% at 1.5 min (Figure 1). Optimal time occurs at 5 min (5% survival). Consequently, this time was used in all mutagenesis assays.

Selection based on FFase activity

From 666 surviving lineages, 5 of them had higher absorbance values than the parent strain and the 6-M69, 6-M61 and 4-M37 mutants had higher mycelial activity (Table 2). The FFase activity showed that the mutant 6-M69 was the most active but it did not present statistically significant differences with the 4-M37 strain. The other 5 strains had lower activity values without observing significant differences neither between the 6-M65 and 6-M61 strains nor between the parental and the 5-M40 strain (Table 2). All the assays were carried out in sextduplicate.

Selection of mutants with high FTase activity

The 6-M69 and 4-M37 mutants showed the highest values of mycelial activity of between 1.5 and 1.4 times the parental activity, respectively (Table 3). No significant difference in extracellular activity was observed between the parent strain and the 6-M69 mutant except with respect to strains 6-M61 and 4-M37 (Table 3). The cell mass had high values above 15 g/L^{-1} with a slight advantage for the 6-M69 mutant over the parent. The highest FOS production was presented by the 6-M69 mutant strain, being 1.4 times higher than the parental production (Table 4) and the FOS composition was GF2 (1-kestose) and GF3 (1-nistose), which in the case of 6-M69 was 86.2% GF2 and 13.8% GF3.

Temporal profile for 6-M69

Figure 2 shows the course of consumption of sucrose and the synthesis of FOS of the 6-M69 and PR-142 strains. The process reached the maximum FOS production at 2 h (147.7 g/L) in the case of PR-142 and at 3 h (263.78 g/L) in the case of 6-M69 and after that, a gradual FOS decrease concomitant with an increase in sucrose was observed. In both cases, the sucrose underwent a maximum depletion between 2 and 3 h and

Table. 3 FTase activity of mycelial and extracellular parental *Aspergillus niger* PR-142 and its mutants.

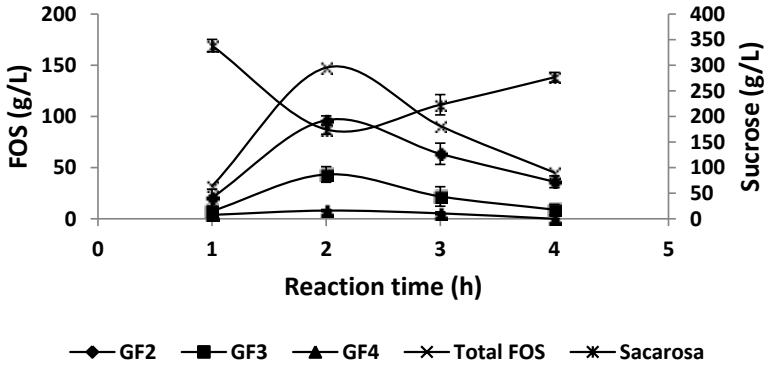
Strain	Mycelial FTase activity			Extracellular FTase activity	
	A_T(U mg^{-1})[1]	A_T (U ml^{-1})[1]	Relative A_T (%)	A_T (U ml^{-1})[1]	Relative A_T (%)
PR-142	0.75 ± 0.14[b]	11.44 ± 0.38[c]	100.0	7.42 ± 0.42[a]	100.0
6-M69	1.15 ± 0.25[a]	17.78 ± 0.48[a]	155.4	6.74 ± 0.21[a]	90.8
6-M61	0.89 ± 0.23[ab]	11.37 ± 0.28[c]	99.3	3.52 ± 0.19[b]	47.4
4-M37	1.140 ± 0.20[a]	16.45 ± 0.40[b]	143.7	3.40 ± 0.02[b]	45.8

[1]Mean of six samples (n=6) ± SD; A_T: fructosyltransferase activity. Means followed by the same letter are not significantly different according to the Tukey's test (P<0.05).

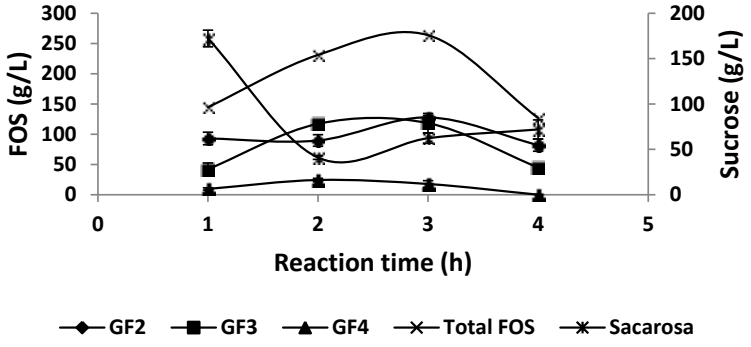
Table 4. Cell growth and FOS concentration of parental *Aspergillus niger* PR-142 and mutants.

Strain	Cellular mass (g/L)	FOS (g/L)	Sucrose (g/L)	FOS (%)*
PR-142	15.75 ± 0.65	18.38 ± 0.80	338.72 ± 20.41	4.70 ± 0.20
6-M69	16.08 ± 1.19	25.72 ± 3.65	305.10 ± 36.03	7.18 ± 1.47
6-M61	13.59 ± 2.55	20.90 ± 6.16	325.56 ± 23.90	5.57 ± 1.80
4-M37	14.77 ± 1.31	25.51 ± 3.58	315.71 ± 27.80	6.92 ± 1.33

*Percentage of FOS as the range between total FOS produced and total carbohydrate concentration.



A



B

Figure 2. Kinetic reaction profile, FOS production and sucrose consumption of *Aspergillus niger* PR-142 (A) and 6-M69 (B) strains in 2 ml tubes in the range of 1 to 4 h. Nomenclature: GF2 (1-kestose), GF3 (1-nistose), GF4 (1-fructosyl-nystose).

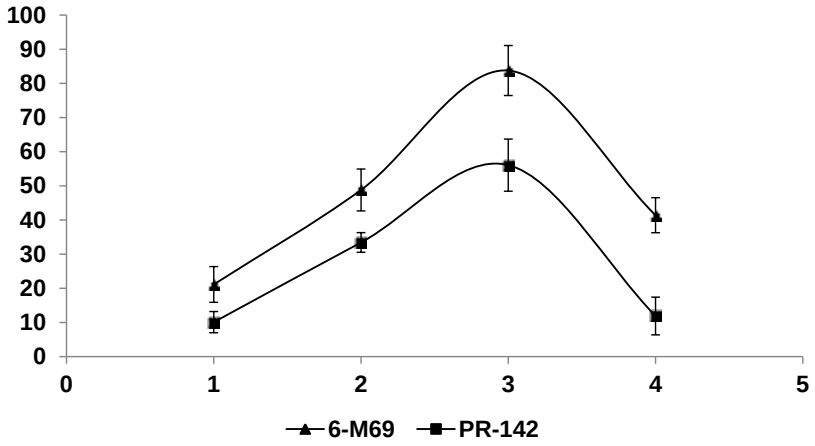


Figure 3. FTase activity of *Aspergillus niger* PR-142 strain and the 6-M69 mutant obtained in 2 ml tubes in the range of 1 to 4 h.

Table 5. Characteristics and level of polymorphism of the primers used in the study.

Code	Sequence (5´- 3´)	SPI*	Polymorphic bands
UBC809	AGAGAGAGAGAGAGAGG	1.46	6.00
UBC817	CACACACACACACACAA	1.42	5.00
UBC834	AGAGAGAGAGAGAGAGCT	1.28	7.00
UBC895	AGAGTTGGTAGCTCTTGATC	2.74	10.00
ISSR1	GTGGTGGTGGTGGTG	1.96	6.00
ISSR2	GACAGACAGACAGACA	2.46	9.00

*****ISSR Initiator Index (Raina et al., 2001).

at the end of this period its concentration increased. During the maximum production of FOS in the PR-142 strain, the final product contained: 1-kestose (GF2-65.2%), 1-nystose (GF3-29.3%) and 1-fructosyl-nystose (GF4-5.4%), while the 6-M69 strain contained: 1-kestose (GF2-48.4%), 1-nystose (GF3-44.8%) and 1-β-fructofuranosyl nystose (GF4- 6.7%) and after that, a gradual decrease of all these products took place. The dramatic increase of FOS under these conditions is far superior to the tests in conical tubes of 50 ml on a rotating shaker, which became evident from the first h of reaction. The FTase activity increased and reached the maximum value at 3 h, of 83.8 and 56.1 U/mL for 6-M69 and PR-142, respectively (Figure 3). This increase kept the activity ratio of 1.5-fold between the mutant and the parental strains which was, in turn, rather similar to that obtained in the test using conical tubes.

Scrutiny of genetic variability

The 6 primers (Table 5) gave a total of 42 loci with an average of 26.5 bands per individual of which 33% were polymorphic. A total of 20 bands were present only in the mutants (Figure 4). Initiators UBC-895 and UBC-834 had the highest number of polymorphic bands and the highest ISSR primer index (SPI) (Raina et al., 2001). The dendrogram obtained (Figure 5) at a similarity coefficient of 0.65 shows two groups, one of which consists of PR-142 parental and the 5-M40 mutant and the other group consists of two subgroups where the first one consists of the 4-M37 and 6-M61 mutants and the second one consists of only the 6-M69 mutant. The highest genetic similarity (0.86) occurred between PR-142 and the 4-M37 mutant and the lowest similarity (0.23) occurred between the PR-142 strain and 5-M40.

DISCUSSION

The 6-M69 and 4-M37 mutants have been shown to have higher FTase activity than the parental strains and this demonstrates the potential of these strains for use in cell immobilization techniques for the production of FOS (Chien et al., 2001). This increase of about 1.5 fold in FTase activity can be compared to the results obtained by Guilarte et al. (2009) for *A. oryzae* strain 1303 and by Skowronek and Fiedurek (2003) for *A. niger* strain 13/36.

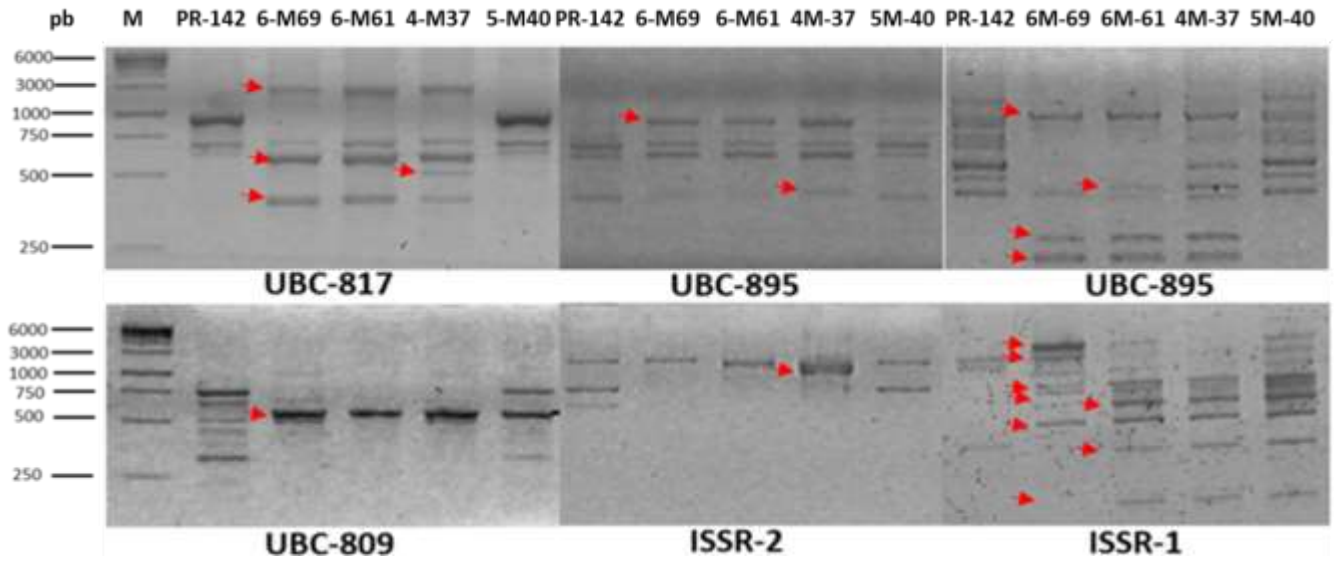


Figure 4. ISSR analysis with 6 primers of *Aspergillus niger* PR-142 parental strain and the 5 mutants with high invertase activity. On the right side is the dendrogram obtained with this analysis.

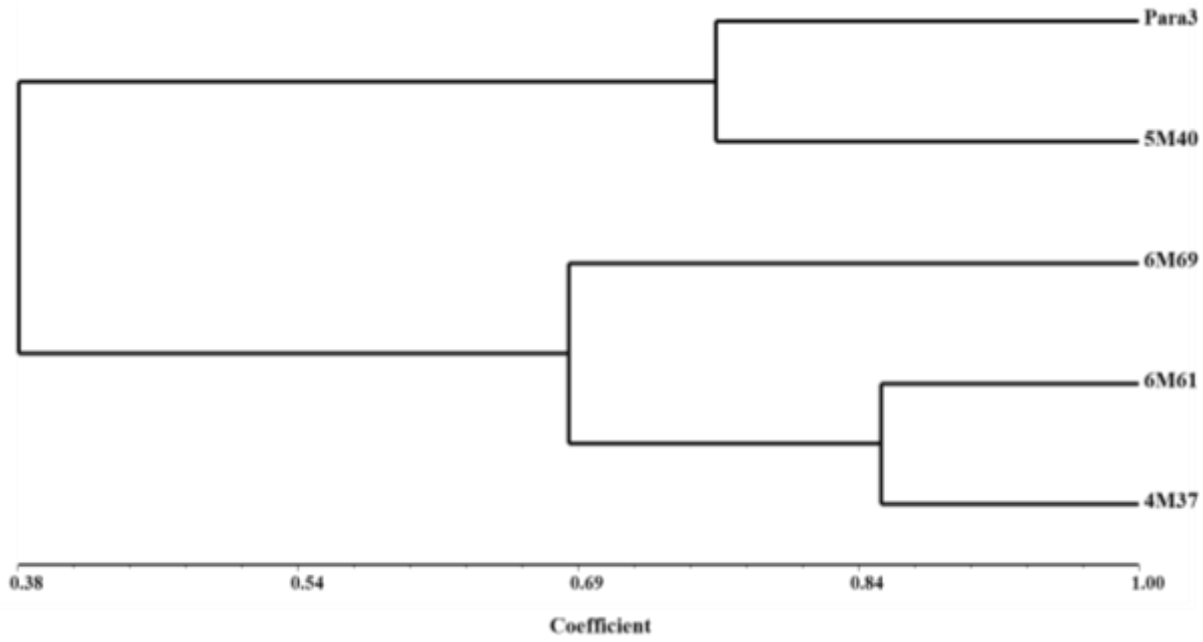


Figure 5. Dendrogram of genetic distance between the evaluated mutants and their parental *Aspergillus niger* PR-142-based ISSR analysis.

The FTase activity and, consequently, the production of FOS increased dramatically by performing the reaction in 2 mL tubes in a ThermoMixer®. This demonstrates that the reaction conditions in 50 ml conical tubes in a rotary shaker were not fully optimal. It must be taken into account that hydrolytic processes are influenced by many factors such as particle size, concentration of the reaction medium, and the geometric and operational form of the medium (Abd Rahim et al., 2015). It is probable that in this case, several factors such as the environment, the type of equipment used, and the agitation elevated to 800 rpm could increase the collision-producing cellular autolysis and morphological changes in the mycelium (Purwanto et al., 2009). These phenomena could allow a

proportion of the enzymes to be free to react with the substrate by drastically changing the results since the cells *per se* would be unable to prolong the reaction for an extended period of time. This would explain the higher FTase activity in the PR-142 strain and M-69M being approximately 3 h in duration, greater than that reported for the *Aspergillus* strain sp.N74 by Sanchez et al. (2008), and which presented a greater performance for up to 1.25 h. A similar finding was reported by Virgen-Ortiz. (2016) for a pure fructosyltransferase from *Aspergillus aculeatus* which reached a maximum yield at 2 h. The decrease of the concentration of FOS after 2 to 3 h of reaction and the gradual increase of sucrose concentration after 2 h was probably due to a loss of hydrolysis capacity of the enzyme produced by the accumulation of glucose which acts as a competitive inhibitor (Sangeetha et al., 2004). However, the typical pattern of FOS formation of double-deletion (Ruiz et al., 2013) comprising the reduction of GF2 and a simultaneous increase of GF3 and GF4 does not seem to occur here after 2 and 3 h in both mutants. This fact could be due to the negative effect of the enzymes shear caused by very high agitation that would be partially inhibiting this behavior. The concentration of 0.15% (w/v) SDS is 10 times higher than that used for the same purposes in *A. oryzae* (Guilarte et al., 2009) which demonstrates the higher resistance of the PR-142 strain. The lethal dose of UV reached 50% at 1.5 min, which is less than the 3 min obtained by other *A. niger* strains as reported by De Nicolás-Santiago et al. (2006) for the production of mutants with high production capacity of xylans and mannans. This reduction could be expected by considering the higher dose of UV rays used in the current work.

It is known that UV rays are inductors of mutations since the pyrimidine bases (thymine and cytosine) of DNA are very sensitive to their effects. These mutations distort the double helix of DNA affecting future replication (Sambrook et al., 2000) and creating genetic variance. In the present study, each strain was differentially affected in its ability to produce FOS after exposure to UV light. The high genetic variability in similar mutants has been previously reported in amylolytic strains of *A. niger* (Shafique et al., 2010) and *Alternaria tennuissima* for hyper-active alpha amylase (Shafique et al., 2009). The presence of unique bands can be considered positive for the mutants, since they could be used in selection programs assisted by molecular markers as previously suggested by Afifi et al. (2013) in mutants of *Penicillium chrysogenum* with a high production of protease.

Conclusions

In this study, the potential of UV radiation mutagenesis to increase FOS production in an *A. niger* strain was reported. The increase of the enzymatic activity and the production of FOS have increased up to 1.5 times and this improvement is dramatically noticeable under conditions of high agitation in 2 ml microtubes. The process of mutagenesis by UV irradiation has been shown to have high capacity to generate great variability with respect to the native strain and the formation of unique molecular patterns.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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