World Academy of Science, Engineering and Technology
International Journal of Chemical and Molecular Engineering
Vol:13, No:6, 2019

Application of Thermoplastic Microbioreactor to the Single Cell Study of Budding Yeast to Decipher the Effect of 5-Hydroxymethylfurfural on Growth

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***Abstract*—**Yeast cells are generally used as a model system of eukaryotes due to their complex genetic structure, rapid growth ability in optimum conditions, easy replication and well-defined genetic system properties. Thus, yeast cells increased the knowledge of the principal pathways in humans. During fermentation, carbohydrates (hexoses and pentoses) degrade into some toxic by-products such as 5-hydroxymethylfurfural (5-HMF or HMF) and furfural. HMF influences the ethanol yield, and ethanol productivity; it interferes with microbial growth and is considered as a potent inhibitor of bioethanol production. In this study, yeast single cell behavior under HMF application was monitored by using a continuous flow single phase microfluidic platform. Microfluidic device in operation is fabricated by hot embossing and thermo-compression techniques from cyclo-olefin polymer (COP). COP is biocompatible, transparent and rigid material and it is suitable for observing fluorescence of cells considering its low auto-fluorescence characteristic. The response of yeast cells was recorded through Red Fluorescent Protein (RFP) tagged Nop56 gene product, which is an essential evolutionary-conserved nucleolar protein, and also a member of the box C/D snoRNP complexes. With the application of HMF, yeast cell proliferation continued but HMF slowed down the cell growth, and after HMF treatment the cell proliferation stopped. By the addition of fresh nutrient medium, the yeast cells recovered after 6 hours of HMF exposure. Thus, HMF application suppresses normal functioning of cell cycle but it does not cause cells to die. The monitoring of Nop56 expression phases of the individual cells shed light on the protein and ribosome synthesis cycles along with their link to growth. Further computational study revealed that the mechanisms underlying the inhibitory or inductive effects of HMF on growth are enriched in functional categories of protein degradation, protein processing, DNA repair and multidrug resistance. The present microfluidic device can successfully be used for studying the effects of inhibitory agents on growth by single cell tracking, thus capturing cell to cell variations. By metabolic engineering techniques, engineered strains can be developed, and the metabolic network of the microorganism can thus be manipulated such that chemical overproduction of target metabolite is achieved along with the maximum growth/biomass yield.

***Keywords*—**COP, HMF, ribosome biogenesis, thermoplastic microbioreactor, yeast.

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I. Introduction

THERE is an increasing attention to the production of biofuels from renewable resources due to global warming and the reduction of fossil fuels. Bioethanol is one of the biodiesel fuels and it can be produced from non-food lignocellulosic biomass. Bioethanol is very promising alternative energy source and it offers several opportunities such as reduced CO_2 emissions and cheap raw material [1]. Besides that, lignocellulosic raw materials are often found on earth and they can be used in the sustainable production of fuels, chemicals and materials. Cellulose, hemicellulose and lignin are the building blocks of the lignocellulose. Enzymatic saccharification of pretreated biomass is the method of biochemical conversion of lignocellulose [2]. Pretreatment process is very important step to convert lignocellulose and to reduce the bioethanol fermentation cost. Unfortunately, several undesirable toxic compounds such as phenolic compounds, furan derivatives or weak acids show up during pretreatment process and they could inhibit the microbial fermentation [1].

The furan derivatives HMF and furfural are the degradation compounds of hexoses and pentoses, respectively. HMF and furfural influence the ethanol yield and ethanol productivity by inhibiting enzymes such as alcohol dehydrogenase, aldehyde dehydrogenase, pyruvate dehydrogenase and glycolysis [3]. The most striking effect of these chemicals on the livings is that they interfere with the microbial growth and they are considered as the most potent inhibitors in bioethanol production [4]. HMF and furfural decrease enzymatic biological activities, break down DNA, inhibit protein and RNA synthesis, damage cell structure, generate cellular reactive oxygen species (ROS) and reduce the specific cell growth rate [3], [5]-[8].

In order to generate the industrial bioethanol, *Saccharomyces cerevisiae* (*S. cerevisiae*) is the most widely employed microorganism; it is well-known food-grade yeast with a long history on alcohol fermentation from various raw materials. The yeast cells are able to decrease HMF and furfural into less harmful compounds. 2.5-bis-hydroxymethylfuran [furan-2,5-dimethanol (FMD)] and 2-furanmethanol (FM) are the reduced chemicals of HMF and furfural under anaerobic conditions, respectively. Moreover, NAD(P)H and $NAD(P)^+$ usage are necessary for furfural and HMF detoxification, respectively (Fig. 1) [3], [8]. When HMF and furfural concentrations decrease to a lower concentration, a recovery of cell growth can be observed. Thus, it was

proposed that, genomic adaptation might occur during the lag phase [6], [9]. However, cellular stress response caused by HMF is not fully understood in yeast cells.

In this study, the yeast behavior under 4g/L HMF treatment was investigated on single cell basis by using a continuous flow single phase microfluidic platform, which allows capturing cell to cell variations. The microfluidic bioreactor was fabricated via hot embossing and thermo-compression bonding by employing COP. The cell responses with and without HMF effects were recorded under brightfield and fluorescence microscopy. The understanding of the HMF effect on growth will help us design new industrial strains with coupled growth and product formation at a high yield using metabolic engineering techniques.

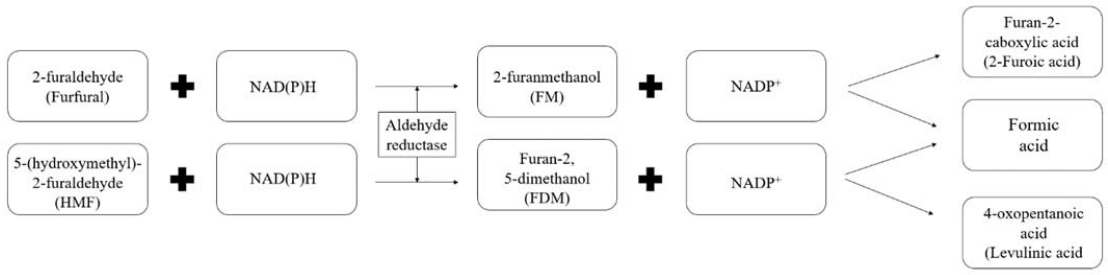


Fig. 1 Conversion pathways of furfural and HMF

II. Materials and Methods

A. Device Design and Fabrication Steps

Designing a microfluidic device for a specific goal is a very important step of microfluidic device fabrication. The design should meet all kinds of requirements to achieve the intended aim. In this work, a microfluidic bioreactor is created in order to study the HMF effect on yeast cells. Among several thermoplastics, COP is found to have lower auto-fluorescence and give clearer images for fluorescently tagged proteins of the yeast cells [10], [11]. Therefore, the designed device is made of COP. It has two distinct microbioreactor regions, that have 8 chambers along with inlet and outlet channels for yeast and nutrient flows (Fig. 2 (a)). Every chamber has its own C-shape to trap the yeast cells.

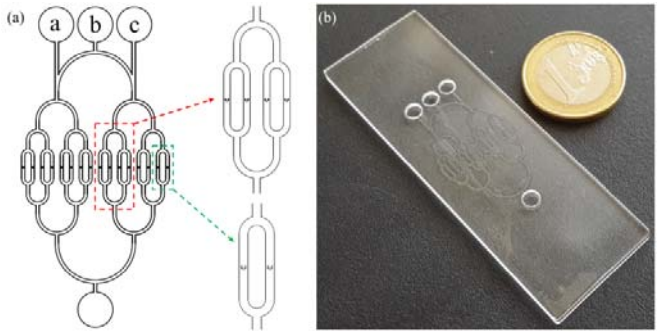


Fig. 2 (a) Design of the microfluidic device (a, b and c can be used as yeast or medium inlets) (b) Fabricated microfluidic device

Device fabrication includes 4 fundamental steps; mold fabrication, planarizing, hot embossing and thermo-compression bonding. Before planarizing, inlet and outlet holes of the device are drilled via CNC machining on a blank COP. Blank COP is then submerged into the ultrasonic bath to remove any burrs caused by CNC drilling. Planarization process is applied to flatten COP uniformly via hydraulic press machine at 130 ^{o}C and 30 bar. In order to create the design on the blank COP with drill holes, stainless steel mold is used. This mold is created by the application of photolithography and electro-chemical wet etching methods [10]. COP is superimposed on the mold, and heat and pressure at 130 ^{o}C and 30 bar is applied via hydraulic press machine. As the last step, thermo-compression bonding at 125 ^{o}C and 25 bar is performed to cover the hot embossed COP with another blank COP. The fabricated device is shown in Fig. 2 (b).

B. Yeast Strain, Medium Preparation and Microscopy

Wild type EY0987 haploid strain (EY0987 ATCC 201389: *MAT***α** *his3Δ1* leu2Δ0 lys2Δ0 ura3Δ0 (S288C)) with RFP tagged NOP56 protein was kindly provided by Dr. P. Arvidson (Harvard University/HHMI). YNB medium including 2% glucose, 1.7 g/L YNB, 5 g/L ammonium sulfate, 0.1 g/L leucine, 0.02 g/L histidine, 0.03 g/L lysine and 0.02 g/L uracile was prepared for yeast cells (Nop56:RFP). HMF was added dry to final concentration of 4g/L (prepared from powder form and not sterilized). OD_{600} of the preculture fed to the microfluidic device was 0.5. Nikon Ti-E inverted fluorescence microscope with Nikon DS-Ri2 detector and texas red filter were used for image recording. Brightfield and fluorescence microscopy images of the cells were taken at 10 min time intervals during the experiment and ImageJ (NIH, Bethesta, MD) software was used for image processing.

III. Effect of HMF on Yeast Cell Growth

In order to understand the link between growth and HMF inhibition, the response of RFP:Nop56 yeast cells upon HMF treatment was followed in a microfluidic platform. At first, YNB medium was sent through the microfluidic bioreactor for priming step. Then, yeast cells were loaded and trapped in the c-shaped regions of the chambers. After cell loading, fresh YNB medium was sent for 3 h followed by HMF solution for another 3 h. Finally, fresh YNB medium was fed for 10 h till the end of the experiment.

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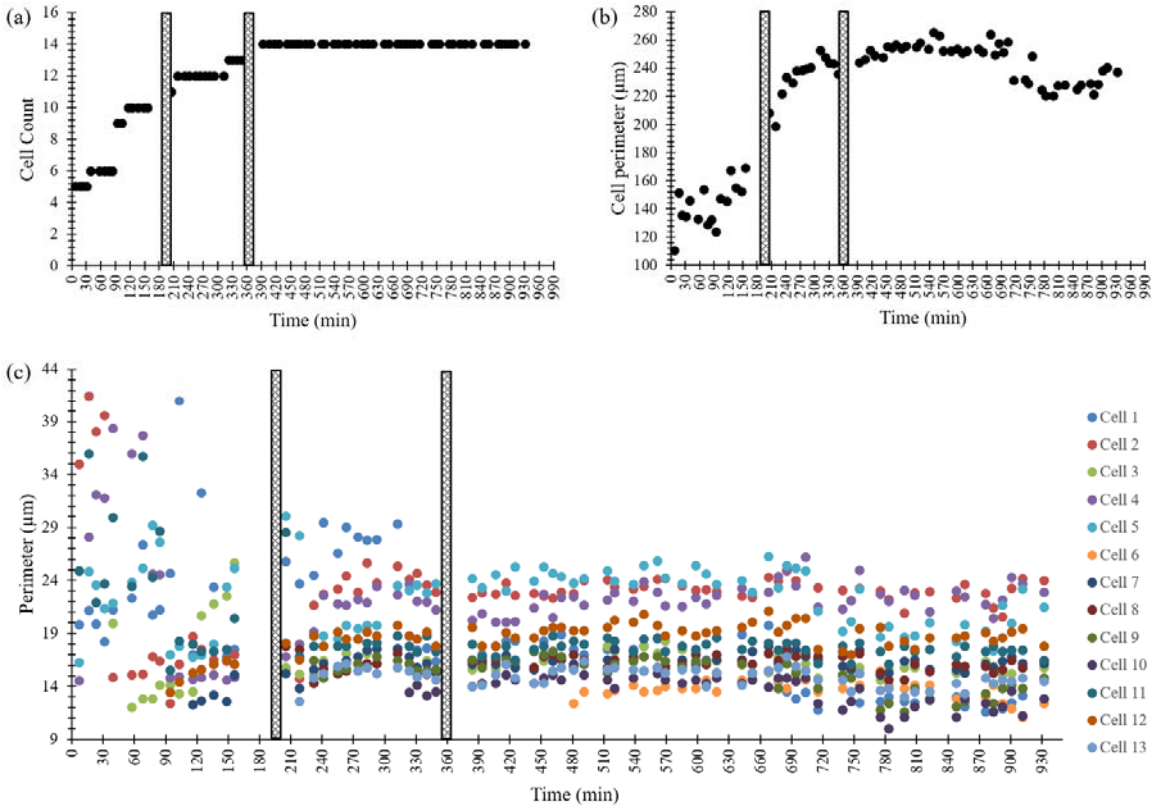


Fig. 3 (a) Time profile of cell count (b) Time profile of total cell perimeter (c) Time profile of single cell perimeters (Vertical lines on the graphs shows the HMF feeding period

Cell count increases gradually throughout the experiment till the end of the HMF treatment period (Fig. 3 (a)). When the HMF feeding is ceased, no proliferation is observed until the end of the experiment. Yeast cells continue growing and increasing in dimension including the HMF treatment period. Around the 720th minute, there is a decreasing trend in the total cell perimeter (Fig. 3 (b)). Each cell is also treated separately and the response is monitored, capturing cell to cell variations (Fig. 3 (c)). The initial increase and subsequent decrease in cell size during the HMF treatment period can be seen more clearly on single cell basis. Towards the end of the experiment, the cells are able to recover themselves without losing their viability.

RFP tagged Nop56 expression is analyzed to observe the changes in protein expression within the cells. In the first 150 min, the fluorescence intensities of the cells are very high, as shown in the inset graph of Fig. 4. During the HMF treatment, the fluorescence intensity has a decreasing trend, i.e. Nop56 expression is suppressed by the HMF. After the HMF treatment, the fluorescence intensity continues to decrease for a while, however through the end of the experiment, under the fresh nutrient medium, the cells fluoresce at an increasing trend. Since Nop56 has a role in ribosome biogenesis and ribosome biogenesis is closely linked to cellular activities like growth and division [12], we can speculate that the cells are trying to regain their cellular functions and recover themselves.

Fig. 5 shows the time profiles of the perimeter and fluorescence intensity of mother and its daughter cells. M defines the mother cell and the numbers next to M show the daughter and granddaughter cells. For example, M1 is mother cell 1, M1-2 is second daughter of the M1 and M1-2-1 is the daughter cell of second daughter of M1. Before the HMF treatment, all mother cells continue their normal life cycle and proliferated. The doubling time of the cell was 70-80 min before the HMF treatment. During the exposure to HMF, all of the mother cells still continued proliferation; however, the doubling times of these cells extended, ranging between 124 and 207 minutes depending on the exposure time of the individual cells to HMF, confirming cell to cell variation in the population (Figs. 5 (a)-(d)). For example, M4 entered G_1 phase 22 min before the M1, that is more exposed to HMF than M4. Moreover, if the daughter cell was born before the HMF treatment, it can make a new bud despite the presence of HMF. But, if the daughter cell was born after the HMF treatment, it could not bud.

In order to see clearly the rises and falls of fluorescence intensity of the cells, some noisy data at the beginning are discarded. Before HMF treatment (in fresh medium) there is sharp peak of the expression of Nop56 in G1 phase as well as in budding phase. If a daughter cell appears towards the end of the HMF treatment period, Nop56 expression is not seen, probably due to the HMF suppression (Fig. 5). However, if the cell division occurred within 20-30 min of HMF application

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period, Nop56 expression was seen in both G_1 and budding phases of cell cycle, however at the end of the M phase Nop56 fluorescence (expression) disappears. Finally, if the cell is divided after HMF treatment, it behaves as if it were in fresh medium at the beginning of the experiment, and Nop56 expression was seen (Fig. 5 (c)).

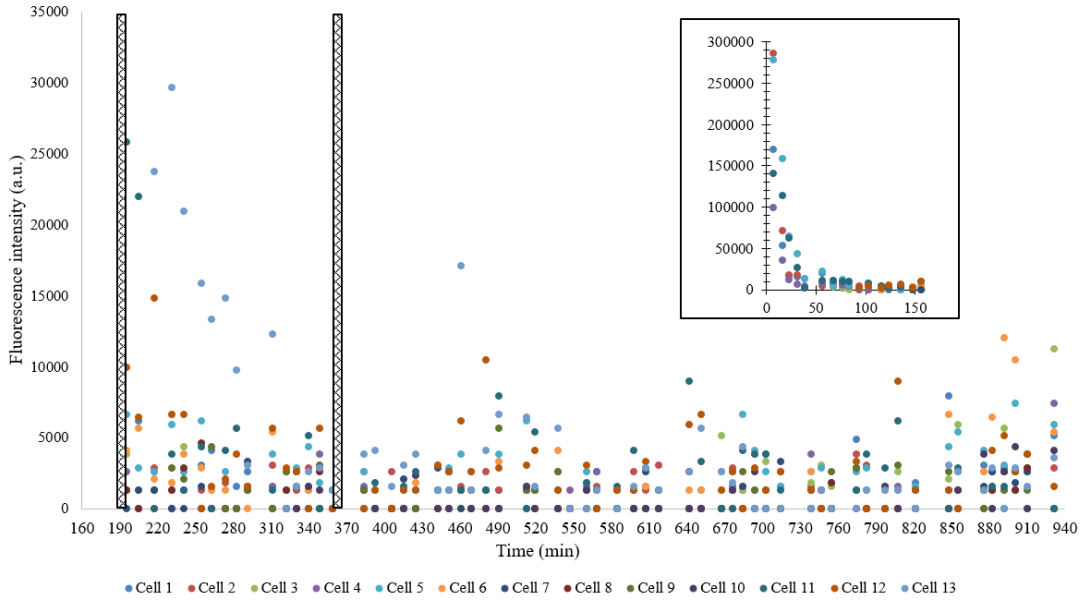


Fig. 4 Time profile of fluorescence intensity at single cell level (inset shows the fluorescence intensity in the first 150 min)

IV. Discussion

The relationship between growth and HMF, which has negative effects on cells like break down of DNA, inhibition of protein and RNA synthesis, is studied in continuous flow single phase microfluidic device for the first time. In cells, the snoRNAs have two main functional groups; one of them is the box C/D snoRNAs that manages the methylation of ribosyl-2'-hydroxyl groups and the other one is the H/ACA snoRNAs that direct the conversion of uridine to pseudouridine [13]. Nop56 is an essential evolutionary-conserved nucleolar protein, and it is found as the member of the box C/D snoRNP complexes. Nop56 is involved in the early to middle stages of 60S ribosomal subunit biogenesis, required for the assembly of 60S ribosomal subunit and joined in pre-RNA processing. Yeast cells with RFP tagged Nop56 gene product were used to observe the changes in protein and ribosome syntheses along with their link to growth within the microfluidic device.

During the HMF treatment, the cell proliferation continued but HMF slowed down the cell cycle, and the budding of cells stopped after the HMF treatment period (Fig. 3 (a)). However, the yeast cells did not lose their viability, and recovered in 6 hours by the addition of fresh nutrient medium after the HMF treatment (Fig. 3 (b)). Thus, 4 g/L HMF treatment suppressed normal functioning of cell cycle but it did not cause cells to die. In order to observe the single cell behavior, each cell in the experiment was individually analyzed. As previously stated for the population, the individual cell proliferation continued during the HMF treatment and new cells (daughter cells) joined the culture. Since HMF inhibits protein and RNA synthesis, there is a sharp decrease in the intensity of Nop56 fluorescence during the HMF treatment, and that decreasing trend continued till the 600th minute. However, the fluorescence intensity increased towards the end in fresh nutrient medium, and the cell recovery can be seen clearly.

Fig. 6 shows the genes that are significantly affected (induced or repressed) by the HMF in the lag phase of yeast cells and interact with Nop56 [6]. Cbf5 is a pseudouridine synthase catalytic subunit of box H/ACA snoRNPs. It has a role on large and small rRNAs, snRNA U2 and some of the mRNAs. Pseudouridine is known as a rotation isomer of uridine and generally localizes in highly conserved regions of functional RNAs (i.e. tRNA, rRNA and snRNA). RNA pseudouridylation results in an increase of thermal stability of RNA helicases and RNA bases that can collaborate to create new RNA structure and protein binding sites. Cbf5 gene has human ortholog and mutation of this gene causes the dyskeratosis congenital disorder [14]-[18]. In human, Cbf5, Nop10, Nhp2 and Gar1 constitute the core H/ACA RNP proteins. Thus, the changes in Cbf5 influence the ribosomal RNA biogenesis and telomerase operation [19]-[21]. Therefore, having important role in life cycle, Cbf5 is negatively affected by HMF, which is known to inhibit RNA synthesis. Since Nop56 has an interaction with Cbf5, it is expected that Nop56 is also repressed by HMF and shows low fluorescence intensity under HMF treatment. Rrp5 is a RNA binding protein and involves in synthesis of 18S and 5.8S rRNAs. The early pre-rRNA cleavage occurs at sites A_0, A_1 and A_2 and this is necessary for the synthesis of 18S rRNA, and the cleavage at site A_3 is needed for the generation of 5.8S rRNA. Rrp5 is the first rRNA-processing component required for snoRNP-dependent cleavages at $A_0/A_1/A_2$ and RNase MRP cleavage at A_3 [22]-[24]. Rrp5 is the part of the

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ribosomal small subunit (SSU) processome and 90S preribosome. According to proteomic studies Rrp5 participates in both 40S and 60S subunit synthesis thus, it plays an influential role in directing the operations at several sites during early ribosome biogenesis [22]-[24]. Since ribosome biogenesis is a very important step for the growth of yeast cells, a disruption in cell cycle is expected to occur if the ribosome synthesis is affected by an inhibitor. As Rrp5 is one of repressed gene products by HMF [6], thus, Nop56 is also repressed by the HMF treatment due to the genetic interaction between them.

Otu1 is a deubiquitylation enzyme that binds to the chaperone-ATPase Cdc48 which functions in endoplasmic reticulum (ER)-associated and mitochondrion-associated protein degradation events. Controlling ribosomal quality and extraction of chromatin-bound proteins are also duties of the Cdc48 [25]. Otu1 may contribute to the regulation of protein degradation by deubiquitylating substrates that have been ubiquitylated by Ufd2. The abundance of Otu1 causes an increase in the response to DNA replication stress [26]-[28]. Otu1 is an important protein in the proper development of the cell. HMF is known to induce Otu1 expression by 2-3 folds within 2 hrs of treatment [6]. This high expression of Otu1 is expected to be involved in DNA replication stress and consequently might have affected the operations of its interactor Nop56 too. Shp1 is the gene product that has interaction with Nop56 via Otu1, and can therefore be affected by HMF treatment. Shp1 is employed to support the growth and mitotic events with Cdc48 that regulates important cellular processes [29]-[32]. Therefore, Shp1 works for proper Cdc48 functioning. Like Otu1, Shp1 expression is induced by 2-3 fold within 2 hrs of treatment [6]. Shp1 takes part in peptide/protein degradation and is necessary for the yeast cells to survive and adapt the HMF stress. Thus, Shp1 also affects growth and protein Nop56 expression. Like rRNA processing protein Lrp1 (Rrp47) is a nuclear exosome related nucleic acid binding protein and its homolog in mammalian cells is a nuclear matrix protein C1d that involves in regulation of DNA repair and recombination [13], [33]-[37]. Lrp1 and Rrp6 have common responsibilities in RNA processing and degradation. They have roles in nuclear rRNA, snoRNA and snRNA operations [34]. Like Rrp6, Lrp1 is also necessary for the stable RNA processing pathways and both of them have identical relationship in nuclear mRNA surveillance pathways [33], [36], [37]. Nop56 has an interaction with Lrp1 protein, and any agent that affects Lrp1 may also affect Nop56. Since Lrp1 is a slightly upregulated (fold change 0.74) gene product by HMF [38], Nop56 should also be induced, i.e. as Lrp1 is involved in regulation of DNA repair and recombination, the slightly increasing profile of fluorescence intensity of Nop56 after HMF treatment is reasonable. Rpn12 is connected to Nop56 via Lrp1 protein. It is the subunit of the 19S regulatory particle of the 26S proteasome lid. A protein abundance is seen due to DNA replication stress [39]-[41]. Lrp1 fulfils significant tasks such as regulation of DNA repair and recombination in the cell, whereas Rpn12 has GO function of peptidase activity and protein processing (proteolytic). It is induced by 2.5-2.8 fold by HMF and leads to protein degradation in response to HMF [6]. Pdr5 is a plasma membrane ATP-binding cassette (ABC) multidrug transporter. Overexpression of Pdr5 causes the generation of multidrug resistance (MDR) to several structural and functional unrelated cytotoxic compounds such as mycotoxins, cycloheximide, 4-nitroquinoline N-oxide and sulfomethuron methyl [42]-[44]. Four of the 22 putative ABC transporters, which are encoded by the yeast genome (including Pdr5), protect yeast from several drugs and inhibitors [44], [45]. If yeast cells stop growth and quit the exponential phase, a reduction in Pdr5 level occurs rapidly under environmental stress. Pdr5 works like a cellular detoxification determinant in cell's normal life cycle [46]. Pdr5 expression is induced by 20-30 folds by HMF. Since Pdr5 has an interaction with Nop56 via Otu1, its overexpression might affect the Otu1 expression and this influences the Nop56 functioning in cell. Another gene product, which has an interaction with Nop56 via Otu1, is Npl4/Hrd4. Npl4 includes a Zn^{+} binding domain that mediates protein-DNA or protein-protein interactions [47]. Moreover, Npl4 has physical interaction with Cdc48 via Ufd1 to create Cdc48-Ufd1-Npl4 complex. As mentioned before, Cdc48 is an AAA ATPase and necessary for several cellular processes such as cell division, protein degradation and ER membrane fusion [48]. This gene product is also upregulated by 1.5-2.5 fold under HMF exposure.

All the above mentioned proteins are taking part in important cellular processes like RNA and DNA related events, so the mechanisms underlying the inhibitory or inductive effect of HMF on growth is enriched in functional categories of protein degradation, protein processing, DNA repair and, MDR. By using the metabolic engineering approaches for strain improvement, the HMF formation which is harmful throughout the biofuel production process is needed to be hindered by either manipulating these gene products or the reactions in which they take part without affecting growth/ biomass formation.

V. Conclusion

In the present study the microfluidic platform was successfully established to evaluate the effect of HMF on the growth of *Saccharomyces cerevisiae*. The microfluidic device was fabricated via hot embossing and thermo-compression bonding methods. Dominating the biological activity of the cells, HMF caused a decrease in cell dimensions; however, yeast cells did not lose their viability. Despite the deceleration in the rate of yeast cell proliferation, an increase in the number of cells was later observed in fresh nutrient medium following the HMF treatment. In addition to examining the collective (total) behavior of the yeast cells, each cell was separately examined in the fabricated microfluidic bioreactor and Nop56 expression phases of the cells were determined, which helped us understand the protein and ribosome synthesis cycles along with their link to growth. Our computational study indicated that the protein degradation, protein processing, DNA repair and MDR are the dominating processes governing the up- and down-regulatory effects of HMF on growth.

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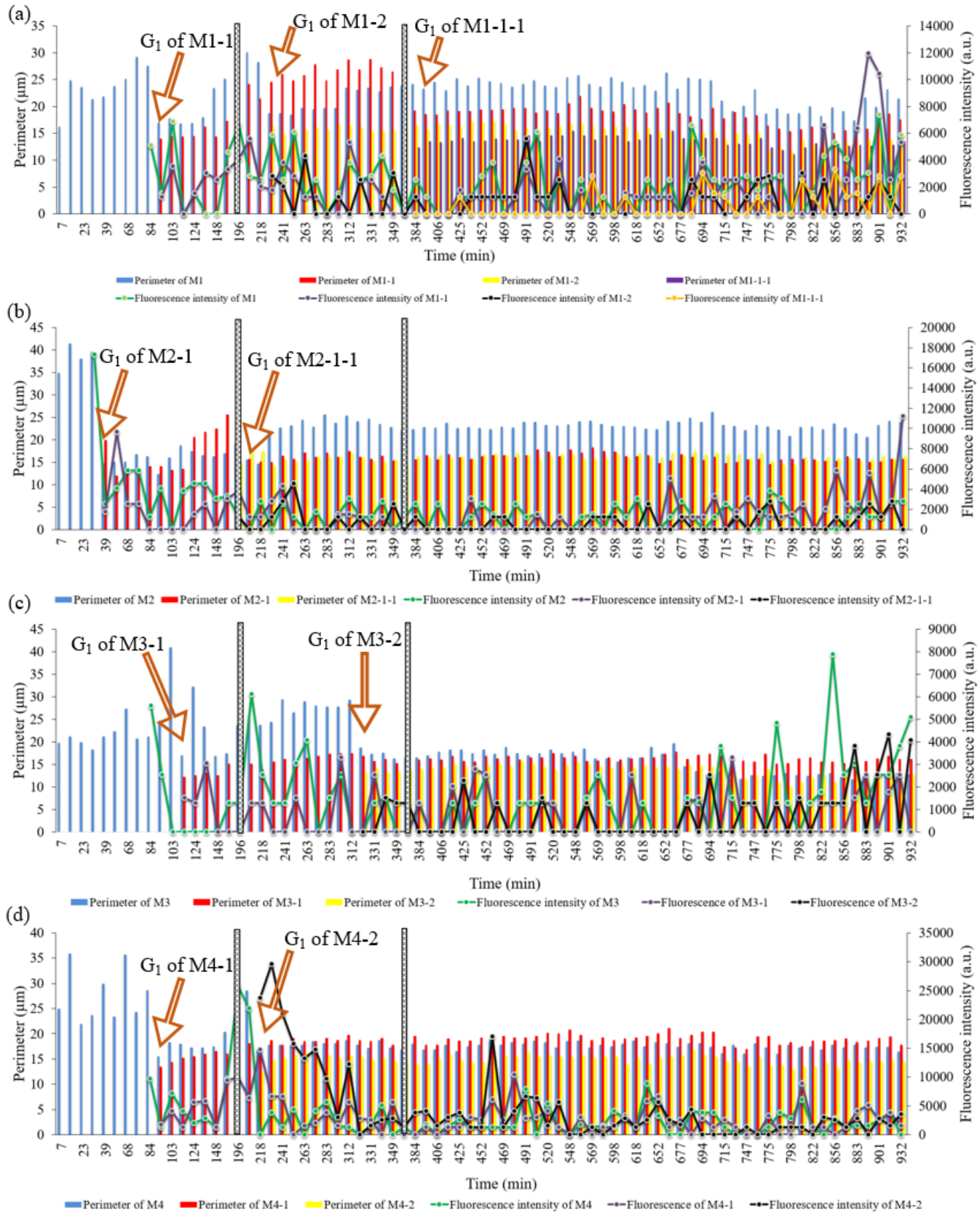


Fig. 5 Time profile of perimeter and fluorescence intensity of individual mother and daughter cells

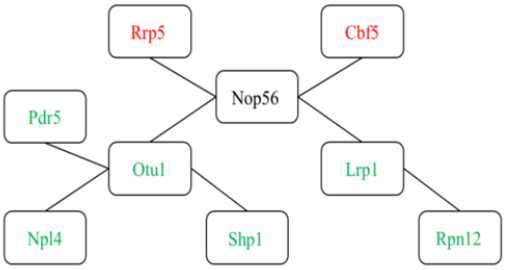


Fig. 6 The interactions of Nop56 with other gene products induced by HMF (red: repressed genes and green: induced genes)

The information gained from this microfluidic study may help us develop new microbial strains, which are engineered towards optimizing growth coupled with the production of biochemicals of industrial value. By metabolic engineering tools, one can use strain engineering strategies, like deletion of specific gene(s) or removal of metabolic reactions that are capable of uncoupling cellular growth from chemical production, and hence the metabolic network regulated by internal cellular objectives can lead to the elimination of toxic by-products and/or overproduction of chemical compounds of interest.

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