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Research Article

Strain Improvement of *Saccharomyces cerevisiae* by Bridge-induced Chromosome Translocation (BIT) and YAC Recombineering Technology

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Abstract

The present research deals with implementation of the bridge-induced chromosome translocation (BIT) technology and yeast artificial chromosome (YAC) recombineering for strain improvement of bioethanol producing transgenic yeast. We aimed to construct a YAC that carries cellulose degradation genes on it and then to apply the BIT technology. BIT technology allowed us to gain two advantages; one of them was to stabilize YAC into the yeast genome and the other one was to have increased a gene expression level consequent to the translocation event. Selection strategies were implemented to obtain novel genetic regulation that would achieved the final phenotype originally desired with the high cellulose degradation and high ethanol producing features.

In conclusion, in our study, we utilized two novel technologies (Yeast Artificial Chromosome (YAC) recombineering and Bridge-Induced Translocation (BIT) technology to introduce new, multi-factorial genetic traits into a yeast strain, a process that would otherwise take several time-consuming and labor-intensive rounds of genetic engineering. This work describes the successful recombinant translocant yeast that is able to efficiently utilize cellulosic material as a carbon source with highly stable recombinant translocant chromosome and has high level of cellulases capacity.

Keywords: Bridge-Induced Translocation (BIT); Yeast Artificial Chromosome (YAC); *Saccharomyces cerevisiae*

Introduction

Bioethanol is a renewable energy source that can be produced from different biological substrates and the production of bioethanol from cheap biomass, like waste of plant material, is fundamental for fuel industry. There are numerous studies about bioethanol production from different biological sources and lignocellulosic biomass is one of them, having several environmental and social advantages for bioethanol production [1-3].

Saccharomyces cerevisiae is an ideal model organism for bioethanol production studies because of its high ethanol productivity, high tolerance to ethanol and simplicity of genetic engineering. Therefore, the development of a recombinant yeast strain that is able to produce ethanol by fermenting cellulosic substrates captured the attention of the science world over recent years [4-6]. Since *S. cerevisiae* can't utilize the cellulose molecules, at least three groups of enzymes have to be expressed, namely endoglucanases (*EGC*), cellobiohydrolases (*CBH*) and β-glucosidases (*BGL*), for cellulose conversion. Cellulose chains can be efficiently degraded to glucose dimers by the synergetic action of *EGC* and *CBH.* In the last stage of the enzymatic cellulose degradation, glucose dimers get hydrolyzed to glucose by *BGL* activity [7]. There are bacterial and fungal strains that have specific set of enzymes for cellulose degradation [8]. In our study we used *Pichia stipitis* (for *BGL* and *EGC*) and *Trichoderma reesei* (for *CBH*) as source organisms for the cellulase genes. We extracted these genes to clone into different plasmids under different promoters with secretion signal to make cellulases' constructs ready for cloning into the YAC. Later on, con-

structs were used for the next step, which is improving the degradation by applying BIT and YAC technologies.

Bioethanol production studies aim to achieve efficient degradation of biomass, to obtain high amount of ethanol, to simplify the process and to decrease the cost of the system [9]. To improve the current bioethanol production processes, we designed our research to utilize two novel technologies (YAC recombineering and BIT) to introduce new, multi-factorial, complex genetic traits into yeast strains. In our laboratory, we have been previously developed a new genomic system, called bridge-induced translocation, BIT [10], which induces the chromosomal translocation between any desired two genomic loci of *S. cerevisiae,* by the help of a selectable DNA cassette that integrates into genome by homologous recombination and results many different effects on cellular morphology, physiology and genome organization. All of these BIT effects state that one single translocation event can result in a mixture of chained molecular events in the cells [10-12]. We wanted use BIT technology for the strain improvement by the help of its effect on gene expression level and genome re-organisation. To do this, we started to engineer the yeast artificial chromosome. YACs are generally used for cloning and manipulation of large DNA inserts in yeast cells. Moreover, they can also be amplified in bacterial cells. They contain centromere, telomeres, origin of replications and restriction sites for cloning experiments. YACs are important in biotechnology field since they are transportable to other organisms and they can carry big inserts. For these reasons, it is generally used for production of heterologous protein, recombination studies and analysis of foreigner genomic sequences [13].

In this study, the engineered YAC that carries the cellulases was transformed into *PEP4* mutant yeast strain, which has a decreased degradation of expressed recombinant proteins (Zubenko., *et al*. 1982). BIT technology was applied in the following term. BIT allowed us to gain two advantages. One of them was to make YAC stabilize into yeast genome and the other one was to have increase gene expression level. Selection strategies studied and implemented to obtain novel genetic regulation that achieved the final phenotype originally desired with the high cellulose degradation feature.

In the present work, by the application of the novel technologies, we demonstrated the successful recombinant translocant yeast strain that is able to efficiently utilize cellulosic material as a carbon source with highly stable recombinant translocant chromosome and has high level of cellulases capacity.

Aim of the Study

We aimed to study the evolutionary effect of bridge induced translocation (BIT) technology to improve *S. cerevisiae* capacity to produce ethanol via the acquisition of an advantageous homoeostasis following the de-regulation of cellulose degradation genes carried on yeast artificial chromosome (YAC).

Material Methods

Strains and media

E. coli XL10-Gold and Stbl2 strains were used for preparation of all the plasmids and constructs described in this work. Cellulose degradation genes were amplified from *P. stipitis* NBRC 10063 (CBS 6054) and *T. reseei* QM9414 strains. *S. cerevisiae* BAP4 strain was used as a host for the cellulose degradation experiments. BAP4 strain is a diploid *PEP4* mutant strain, it is produced by mating CBL1-20 strain (Ludwig D.L. and Bruschi C.V., 1991): *a, [cir°], ura3-52, leu2-3,112, trp1-289, pep4-3* and *PEP4* gene deleted YPH4 strain: a, *ura3-52, lys2-801, ade2-101* and *his3-A200.*

E. coli cultures were grown in LB broth supplemented 100 mg/ml ampicillin for the selection of recombinant plasmids as previously described [14]. *S. cerevisiae* BAP4 strain was grown in YPD medium and BAP4 strains with the plasmids were selected and maintained on synthetic complete lacking URA (SC-URA) drop-out medium. YPD with 200 mg/ml G418 (geneticin, G418 sulfate Gibco BRL, Gaithersburg, MD) was used for the selection of the translocant strain [15]. To check cellulose degradation, recombinant yeast cells were grown at 30°C in YPCMC (1% yeast extract, 2% peptone, 1% CMC).

DNA manipulations and vector construction

Standard protocols were followed for DNA manipulations [16]. The enzymes for DNA cleavages and ligation were purchased from New England Biolabs (NEB) and used as recommended by the supplier. Features of all the recombinant *S. cerevisiae* strains are listed in table 1.

Recombinant Yeast Strains	Relevant features
BAP4	Parental strain, Diploid, [cir°], pep4-3, ura3-52, leu2-3,112, trp1-289, lys2-801 ade2-101, his3-A200.
BAP4-y3	BAP4 strain transformed with linear YAC3 that contains all three constructs (promoter-secretion signal-gene-terminator).
BAP4-ciry3	BAP4 strain transformed with circular YAC3 that contains all three constructs (promoter-secretion signal-gene-terminator).
BAP4-bity3	BAP4-y3 strain with the translocation between YAC and chromosome 4 by BIT technology.

Table 1: Genetic properties of all the yeast strains.

To construct *BGL5, EGC1* and *CBH2* expressing yeast strains, we used *Pichia stipitis* NBRC 10063 (CBS 6054) and *Trichoderma reseei* QM9414 strains as a source for these genes. *BGL5* and *EGC1* were amplified in two-step from *P. stipitis* NBRC 10063 (CBS 6054) with Pwo (Roche) DNA polymerase. *CBH2* cDNA (from *T. reseei* QM9414 strain) was provided to us by Dr. Sezerman from Yeditepe University/Istanbul. The sequences of all PCR primers used are summarized in table 2. These three genes were cloned with the secretion signal (alpha factor) into three different plasmids that contain different constitutive promoters and terminators; pVT100u, pJL49 (Designed by Jean-Luc Parrou, CNRS Toulouse/France) and pTEF. The resulting constructs were amplified, cloned into pYAC3 vector (Figure 1). Genetically engineered pYAC3 was checked by DNA sequencing (BMR Sequencing service) and transformed to BAP4 yeast strain through the LiAc method [15].

Bridge induced translocation

Translocants were obtained by using the BIT technique that is developed previously at Yeast Molecular Genetics Laboratory in ICGEB/Italy [10] by exploiting the EUROFAN protocol [15] for PCR-based gene replacements with the lithium acetate transformation method [17]. The list of primers used for BIT is shown in table 3. The correct chromosomal integrations were confirmed by PCR and then by DNA sequencing (BMR Sequencing service).

Southern blot analysis

Chromosomal separation was performed in a 1% pulse- field certified agarose (Bio-Rad) gel electrophoresis with the CHEF DR-

FwBGL5-Ext.	ACATCCCGTTTTGACGCTAC
RevBGL5-Ext.	GGGGGTTCGATTCCTATTGT
FwBgl5ecoR1	cccgaattccccATGGGT-GTTCAAGAATTAGA
RevBgl5xho1	ccgctcgagcggCTATAATCCCAAC-CAGTAGA
FwEGC1-Ext.	GATGAAACAACACGGTTACG
RevEGC1-Ext.	GATCTTTGATAAGGAGTAGGG
FwEGC1xho1	ccgctcgagcggATGTCTACAG-GATTCTTAACC
RevEGC1bamH1	gccgggatcccggTTAATTCTTGTA-ATCCTTCAAGA
FwCBH2ecoR1	cccgaattcgggCAAGCTTGCT-CAAGCGTCT
RevCBH2cla1	cccatcgatgggCAGGAAC-GATGGGTTTGCG
Fwalphafactor (fused to each gene with the appropriate restriction sites)	ATGAGATTTCCTTCAATTTTTAC
Revalphafactor (fused to each gene with appropriate restriction sites)	CTTTTATCCAAAGATACCCCT

Table 2: PCR primers used for recombinant genes' amplifications.

Figure 1: Schematic representation of the vectors with the cellulase genes (EGC1 gene in pVT100-U plasmid, CBH2 gene in pTEF plasmid and BGL5 gene in pJL49 plasmid) and of the YAC with all three constructs.

FwYackan	tggcccgctttatcagaagccagacattaacgcttctggagaaact-caacgagctggacgcggatgaacaggcagacatcgtcgacg-gatccccgggttaa
RevPAD-1kan	tttcagacaggaatgcaaagtctgtaaaatacatccaatttcttgtctg-caatttatgtcataacatgttaagcgttttccgcgcgttggccgattcat
Control a1	TCAGTAACCCGTATCGTGAG
Control b1	TGCGGTAAAGCTCATCAGCG
K1	acaatcgatagattgtcgcac
Control a2	GCGGTCTTGCCATGATATTC
Control b2	AACGGTAAACAGGAAAGGCG
K2	tcagtcgtcactcatggtgat

Table 3: Bridge-induced translocation primers.

FwBgl5	GGTTCCATCAACGGTCAGGT
RevBgl5	ATCGGCTGCAACAGACAAGT
FwEGC1	ACATGGGTCGCTGGTTTCAA
RevEGC1	CCATGGAGTAGGTGTTGGCA
FwCBH2	CGGGAACCGCTACGTATTCA
RevCBH2	AAGAGGGAACCTTTGCGACA
FwACT1	GAAATGCAAACCGCTGCTCA
RevACT1	TACCGGCAGATTCCAAACCC
FwPAD1	GAAATGCAAACCGCTGCTCA
RevPAD1	TACCGGCAGATTCCAAACCC

Table 4: RT-PCR primers.

II apparatus (Bio-Rad) [18]. Hybridizations were optimized from the standard protocols [16]. Probes were labeled by using the PCR digoxygenin probe synthesis kit (PCR DIG, Roche).

Quantitative PCR

Expression of the genes of interest was analyzed by quantitative PCR. Translocant strains were grown in YPD supplemented with 200µg/ml Geneticin G418 overnight. The following day the RNA was isolated by using Promega Total RNA Isolation System (Z3101, Promega, USA) and the AMV Reverse Transcriptase (M501, Promega, USA) was used to produce cDNA on the RNA template according to the protocol provided by the manufacturer. Resulting cDNA was used as a template for amplification of *BGL5, EGC1, CBH2*, *PAD1* and *ACT1* genes (primers are in the table 4). PCR was carried out by using Rotor-Gene SYBR green kit (Qiagen) with the already provided PCR program designed for the Rotor-Gene Cycler from Qiagen. *ACT1* mRNA level was chosen as a control. The final data was reported as normalized expression level of every gene analyzed with respect to its parental copy BAP4-ciry3 strain considered as the unit. The experiments were repeated at least three times and standard deviations were calculated.

Cellulases enzyme assay

For cellulases activity assay, we used EnzChek Cellulase Substrate (E33953) from Invitrogen and followed the protocol suggested by the supplier. EnzChek Cellulase Substrate is fluorescence-based cellulase substrate that was developed for simple and rapid quantitation of cellulases. Different sample dilutions and standards were run in parallel and read the absorbance at 360nm.

Chromosome stability test

Stability of YAC that has cellulases was checked in BAP4-y3 (contains linear YAC) and BAP4-bity3 (contains translocation between YAC and chromosome 4) strains under non- selective conditions.

At least three individual YAC-containing colonies were picked and used to inoculate separate vials containing 10 ml of YPD to a density of approximately 2 x 10^5 cells/ml. Cultures were incubated at 30°C for at least 10 generations to 2 - 3 x 10^8 cells/ml, where stationary phase was reached. At this time, serial dilutions were made, and cells plated onto several YPD plates to yield 100 - 300 cfu/plate. After three days growth, colonies were replica-plated onto selective medium (SC-URA) and scored for growth as an indication of YAC-containing cells.

Stability was measured as a percentage of prototrophic cfu relative to total cfu plated. In total 2500 colonies were examined per each condition; BAP4-y3 and BAP4-bity3.

Growth curve

To prove the performances of the recombinant *S. cerevisiae* strains and demonstrate its functionality, CMC (Sigma) was used as the sole carbon source. The growth curve of BAP4, BAP4-y3, BAP4-ciry3 and BAP4-bity3 strains were checked in YPCMC (1% yeast extract, 2% peptone, 1% CMC) media where they were inoculated 1 x 10^5 cells of each strains and followed their growth for a week by counting the cells under the microscope.

Results

The bridge-induced translocation system

We transformed exponentially growing BAP4-y3 strain with a PCR-amplified linear DNA cassette harboring the KANR selectable marker (Figure 2). The cassette carried at its ends two nucleotide sequences homologous to two different genomic loci each located on a different chromosome (YAC and chromosome 4). The length of the homologous sequences was 80nt (Table 3). The overall percentage of the induction of chromosomal translocation was equal to or greater than 2%.

Figure 2: Schematic representation of the BIT between YAC and chromosome IV.

To confirm the translocation, the DNA bridge between two chromosomes was amplified by PCR and then sequenced. The list of primers used for BIT control is shown in table 3. Afterwards, we performed a Southern hybridization analysis of the translocant chromosome separated by pulse-field CHEF electrophoresis (Supplementary figure 1) to do the hybridization with kanR DNA probe for the presence of the translocant chromosome. The results of the hybridization of the YAC–IV translocated recombinants with the kanR DNA probes are shown in supplementary figure 2.

According to Southern blotting and PCR analysis, a new translocant chromosome, consisting of the left portion of the YAC and the right portion of the chromosome IV, was generated. PCR, southern blot and sequencing results demonstrate that chromosomal translocation was successfully induced by linear DNA integration

Supplementary Figure 1: Ethidium bromide staining of separated by CHEF gel electrophoresis. BAP4-bity and BAP4 strains were run in the gel. A yeast strain that contains kanamycin was used as a positive control for the KAN probe.

Supplementary Figure 2: Southern blot hybridization to visualize the translocant chromosome presence and size in the BAP4-bity3 strain. Appropriate controls were analyzed in parallel.

between YAC and chromosome IV. The reason why we chose the break point in chromosome IV, close to *PAD1* gene, is that *PAD1* gene is known as Phenylacrylic acid decarboxylase and we wanted to use BIT as an advantage in this point [11] to increase *PAD1* gene expression level. Because it has been already shown that overexpression of *PAD1* gene associates with the increased rate of carbon source utilization, increased rate of ethanol production and increased resistance to some aromatic compounds [19].

Expression profiling of genes located at the translocation break- points

It has been previously shown that single translocation event creates too many different effects on cellular morphology, physiology and genome organization level. One of the important effects was that translocation results in different modifications of the gene expression levels around the translocation break point (Nikitin., *et al*. 2008). In this study, this pattern had an important role for the recombinant gene expressions for the strain improvement. For this reason translocation break point was chosen in a way that it was close to the genes that we were interested to increase their expression level.

We used quantitative PCR profiling to analyze the effect of translocation on the expression of the genes around the translocation breakpoints; three cellulase genes (*BGL5, EGC1 and CBH2)* on YAC, and *PAD1* gene on chromosome IV were analyzed. *ACT1* gene was chosen as an internal control of constitutive gene expression. BAP4-y3 and BAP4-bity3 strains were used to compare the recombinant gene expression levels. BAP4-ciry3 strain was used as a reference strain for the fold change of targeted genes. As a result of quantitative PCR, we observed that recombinant yeast strains have different expression profile (Figure 3). BAP4-ciry3 and BAP4-y3 strains showed more or less the same expression profile and as expected, translocant BAP4-bity3 strain showed increased gene expression level compared to other recombinant strains.

Translocant strain showed higher cellulases enzyme activity

To make possible the complete hydrolysis of cellulose to glucose, yeast strains were engineered to secrete cellulases. BAP4-ciry3 is a strain that contains the recombinant circular YAC with three cellulase genes, BAP4-y3 is a strain that contains the recombinant linear YAC with three cellulase genes and BAP4-bity3 is the strain that BIT technology was applied between chromosome IV and recombinant YAC. After obtaining the recombinant yeast strains with cellulases genes, we checked their enzyme activity for the cellulose degradation.

Figure 3: Graphic representation of the quantitative PCR gene expressions pattern for BGL5, EGC1, CBH2 and PAD1 genes on both sides of the translocations breakpoints on chromosome VI and YAC. Their expression levels in BAP4-bity3 and BAP4-y3 strains were compared to BAP4-ciry3 strain. ACT1 gene expression level was used as an internal control. The whole experiment was repeated three times. Student's t-test was used to determine significant differences between groups, where a p-value < 0.05 was considered statistically significant.

Complete hydrolysis of cellulose requires the synergistic reaction of *EGC*, *CBH*, and *BGL* [20]. To determine the activity of these three enzymes, EnzChek Cellulase Substrate from invitrogen was used as a substrate. If there were cellulases in media, it would react with the substrate and degradation could be read at 360 nm spectrophotometrically. With this method, we compared BAP4-y3, BAP4-ciry3 and BAP4-bity3 strains. In parallel, appropriate controls were also run and no activity was detected in the reference yeast strain harboring the empty pYAC3. Taken together, cellulase enzymes were shown to be active in recombinant strains in different levels. After BIT application, enzyme activity and enzyme production were found to be increased (Figure 4). With these results, we saw that BIT technology helped to increase the heterologous enzyme activity. Since it increased the gene expression levels, this also proved that expressed heterologous proteins were secreted and active in media.

Figure 4: Graphic representation of cellulase test. Axis labels represent different strains and Y line shows the absorbance value of the reaction. The error bars indicate the Standard Error. Control + is the commercial cellulase enzyme mixture.

Figure 5: Graphic representation of recombinant chromosome loss. Axis labels represent different strains and Y line shows the percentage of the chromosome loss among the 2500 colonies.

Chromosome stability test

YACs are linear chromosome-like shuttle-vectors that are used for the cloning and manipulation of large DNA inserts in the *S. cerevisiae* [13]. This allows YACs to be important in biotechnological purposes like production of secondary metabolites and heterologous protein. To be stable as a chromosome in yeast cells, YACs have to reach a certain size, which is around 150kb. The instability of short chromosomes can be a restriction of the YAC cloning.

The recombinant YAC produced in this study was around 25kb and after BIT application it became 40 kb, which is still much smaller than 150 kb. Since the recombinant YAC was so small, it was checked for its stability as a chromosome in the yeast cells. We compared the linear YAC carrier strain (BAP4-y3) and the BIT applied YAC carrier strain (BAP4-bity3) for the loss of YAC under non-selective conditions.

We let BAP4-y3 and BAP4-bity3 strains grow in non-selective media, where there was no pressure on YAC to be present in the cells. Later on, we replica plated them onto selective media to calculate the colony loss, which was a verification for the recombinant YAC lost. After checking 2500 cfu per each recombinant strain, as a result we saw that BAP4-bity3 strain had less chromosome loss compare to the BAP4-y3 strain (Figure 5).

Together with this result, we demonstrated that BIT could make recombinant YAC stabilize into the resident yeast genome. Even though that translocant YAC was larger than linear YAC, it was still far from size of 150kb for being stable as a chromosome. Since the translocant chromosome is highly stable, this result proves that BIT is bringing positive effect on the stability of short chromosomes.

Direct fermentation of cellulose to glucose

All recombinant strains were tested for their growth abilities in cellulosic medium. In parallel, the reference yeast strain harboring the empty YAC was also run as a control, in which insignificant growth was detected. We inoculated $1x10^5$ cells/ml of each strains in YPCMC and followed their growth for a week by counting the cells under the microscope. As shown in figure 6, all recombinant strains grew on cellulosic media, with faster growth exhibited by BAP4-bity3 strain as compared to BAP4-ciry3 and BAP4-y3 strains.

BIT-YAC system is potentially applicable to any host that can undergo homologous recombination. Our study has demonstrated a success in the development of a yeast strain as a new host for heterologous enzyme production for the cellulose degradation. BIT positive effect on strain improvement for cellulose degradation was once more proved with this growth curve analysis.

Figure 6: Growth curve of the BAP4, BAP4-y3, BAP4-ciry3 and BAP4bity3 strains in YPCMC media. BAP4 is a wild type strain that carries empty YAC. Axis labels shows the days and Y values are the number of the cells counted at the time point.

Discussion

Cellulose, the most abundant renewable carbon source, is the main part of the plant cell wall and it can be hydrolyzed enzymatically for the ethanol production. The main restrictions for the cellulose-based ethanol are the cost of the process and the efficiency of the degradation. Therefore, engineering of a microorganism that can convert all the sugars of cellulosic substrate into ethanol is an attractive approach in bioethanol production studies. Complete hydrolysis of cellulose requires the synergetic reaction of *EGC*, *CBH* and *BGL* [20]. In this study, BIT technology and YAC recombineering were used to engineer *S. cerevisiae* strains (Figure 1) and the results showed us that the recombinant yeast strains were able to hydrolyze cellulose to glucose better with BAP4-bity3 strain as compared to BAP4-y3 and BAP4-ciry3 strain.

Three cellulase genes of fungal origins were successfully expressed in *S. cerevisiae*. All three genes were fused to the *MFa1* secretion signal sequence [21] and expressed under the constitutive promoters. To begin with, we first cloned these three genes into different plasmids that contain different constitutive promoters to eliminate the recombination between the same promoters. Later than, whole constructs (promoter + secretion signal + *GENE* + terminator) were extracted from plasmids to be cloned into pYAC3 plasmid. This step is the first novel step in our project for the reason that recombinant YAC can be transform to any other organisms and in this case it can be used as a carrier for cellulases.

After YAC engineering, BIT technology was used to improve the recombinant yeast strain for the cellulose degradation. BIT method involves the use of a PCR-derived artificial DNA construct in which a selective marker is flanked by two regions of homology targeted to different loci in the genome [10]. We targeted YAC and chromosome IV to obtain a translocant chromosome that contains YAC on the left side and chromosome IV on the right side. Once we had the translocant strain, we marked severe alterations at both genomic and transcriptomic level as it is shown in previous translocation studies [11].

In previous BIT studies, microarray analysis results showed that genes, which are responsible of metabolism and stress response are the ones that undergoing differential regulation after translocation [11]. Hence, the cells re-gain a homeostasis after the BIT event, in the levels of regulation of gene expression, quite different than the wild type cells. Therefore, best translocant strain that we were interested in, was chosen among the other differently regulated translocants by the application of right selective pressure.

As a result, we can say that we gained some advantages by the application of BIT. One of them was to make YAC stabilize into the yeast genome. To be stable as a chromosome, size of the YAC has to be at least 150 kb. Our recombinant YAC with cellulases genes was around 25 kb and after BIT application it became 40 kb, which was still really far from being size of 150 kb for the chromosome stability. When we checked the stability of YAC before and after BIT event, it was seen that YAC gained more stability in the cells under non-selection conditions by the application of BIT. Before the BIT application, almost 12% of the cells lost the recombinant YAC. On the contrary, after the BIT application, this number decreased enormously and 0.6% of the cells lost the translocant recombinant YAC. This result showed that short chromosomes could be more stable by BIT application. The other advantages that we gained after BIT were related to the gene expression levels and the genome re-organisation. It was shown in previous studies that around the break point of the translocation, gene expression level is increasing comparing

to wild type [11]. We wanted to use this BIT effect as an advantage for the heterologous gene expression and the translocation break point was chosen close to cellulases genes and *PAD1* gene in chromosome IV. The reason why we chose break point in chromosome IV, close to *PAD1* gene, is that it has been already shown that overexpression of *PAD1* gene demonstrates the increased rate of carbon source utilization, increased rate of ethanol production and increased resistance to some aromatic compounds [19]. Therefore, increased *PAD1* expression level could help to strain improvement of bioethanol producing yeast cells. For that reason, we compared the BAP4-bity3, BAP4-ciry3 and BAP4-y3 strains' gene expression profiles where we saw that in BAP4-bity3 strain, cellulases genes and *PAD1* gene had increased expression levels compared to BAP4-y3 and BAP4-ciry3 strains. We don't know the mechanism of BIT on gene expression level however in this case it was a very useful tool for the strain improvement.

In our study, we utilized two novel technologies (YAC recombineering and BIT) to introduce new, multi-factorial, complex genetic traits into yeast strains. Selection strategies were implemented to obtain novel genetic regulation that achieved the final phenotype originally desired with the high cellulose degradation and high ethanol producing features.

Conclusion

This work describes the successful recombinant translocant yeast that is able to efficiently utilize the cellulosic material as a carbon source with highly stable recombinant translocant chromosome and has high level of cellulases capacity. BIT-YAC system can also be used for the expression and production of other recombinant proteins for different purposes and for different kind of strain improvements.

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