Efficient production of isomalto-oligosaccharides by cell associated transglucosidase of isolated strain *Debaryomyces hansenii* using microfiltration membrane

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Abstract

An efficient recycling method of microfiltration membrane was used to develop the continuous production of isomalto-oligosacharides (IMOs) by cell associated transglucosidase (TG) of a novel strain, *D.hansenii* from maltose. Transglucosidase synthesized seventy types of microbial strains were screened, isolated and investigated the efficiency of IMOs production. The most potent transglucosidase producer was identified as *Debaryomyces hansenii* using 18s rRNA sequencing. Media optimization preliminary studies were performed to maximize the synthesize of transglucosidase and biomass yield (54 g L^{-1}). Parameters optimization studies were investigated using whole cells of *D.hansenii* (~4023 units L^{-1} α - glucosidase activity) from 10 L fermenter to increase the transglucosidase activity through biotransformation. IMOs was continuously synthesised by reusing the cell biomass (6%, w/v) in a 3 L bioreactor using microfiltration membrane system with 30% (w/v) maltose concentration under controlled temperature of 34°C in 9 to 18 h for 5 cycles. The isolated yeast strain, *D.hansenii* was found to utilize more than 98% maltose with higher conversion efficiency for production of IMOs with 65% purity, 85% yield and productivity of 48.8 g L^{-1}.h which confirmed by HPLC.

KEYWORDS: Biotransformation, *Debaryomyces hansenii*, Isomalto-oligosaccharides (IMOs), Microfiltration membrane system, Transglucosidase

1 | Introduction

Isomalto-oligosaccharides (IMOs) are one of the most promising prebiotics among all oligosaccharides that can stimulate the growth of colonic *Bifidobacterium* and *Lactobacillus* in rats and humans (**Madsen L R et al., 2017**). Due to outnumbered growth of beneficial bacteria by this prebiotic, it prevents the growth of pathogenic flora (**Mussatto S I et al., 2007**) by decreasing the pH by formation of short chain fatty-acids (SCFAs). IMOs are considered as glucose oligomers which connects with α-D-(1,6)-linkages or with α-D-(1,4)-linkages (**Yun J W et al., 1994**) and the most prominent components in IMOs (**Goffin D et al., 2010**) are isomaltose (α-D-Glc p(1,6)-α-D-Glc p), panose (α-D-Glc p(1,6)-α-D-Glc p-(1,4)-α-D-Glc p), isomaltotriose (α-D-Glc p(1,6)-α-D-Glc p-(1,6)-D-Glc p). The enzyme responsible for the production of IMOs is transglucosidase or α-glucosidase or glucosyl transferase and accelerates both hydrolytic and transfer reactions on react with higher concentration of maltose and the process called transglucosylation. In the primary reaction, maltose yields panose by transferring glucosyl residue whereas in secondary reaction, D-glucose yields isomaltose (**Lee W C et al., 2005**).

α-glucosidase is ubiquitously present in nature and is classified into three categories viz. Type I glucosidase acts on sucrose and aryl glucosidase, more efficiently than maltose, Type II glucosidase catalyzes mostly maltose and isomaltose, Type III glucosidase has same specificity as Type II, but also hydrolyses on starch and oligosaccharides (**Frandsen T P et al., 1998**). IMOs are being considerably used in food, pharmaceutical and cosmetic applications because of their distinctive properties like prebiotic efficiency, non-digestible, low calorific value, low glycaemic index and resistance to crystallization. The most important property is its prebiotic effects and responsible for their beneficial effects including cholesterol regulation, mineral absorption, better intestinal health, immunity, prevention and resistance to various diseases caused by gut microbiota (**Florowska A et al., 2016**). Enzymatic synthesis from disaccharides of oligosaccharides is being considered as alternative and promising method preferably to chemical synthesis due to their selectivity and efficiency (**Mala S et al., 1999**). The enzyme membrane reactor has developed to immobilize the glucosidase packed between sheets of ultrafiltration membrane by sandwich method to produce IMOs (**Zhang L et al., 2010**). We have demonstrated in our process that more than 98% maltose (with

higher substrate concentration) conversion to IMOs with the purity of >65% and yield of >85% using the free cells of *Debaryomyces hansenii* in number of recycles. Among all enzymes, α - glucosidases are prominent enzyme with a high capable for the production of higher oligosaccharides. The specificity of donor/acceptor and the linkage type are the main elements that dominate the type of product formed. Very few research articles have reported the use of yeasts to produce IMOs. Therefore it is necessity to have single effective process to convert oligosaccharides from disaccharide using yeast. Whole cell biocatalyst is an emerging area in biotransformation of substrate to product and involves use of free cells. This proposal has been used in our previous studies for the synthesis of (i) galacto-oligosaccharides by *Sporobolomyces singularis* and *yeast isolate* (**Saravanan R et al., 2016**), (ii) fructo-oligosaccharides by *Aureobasidium pullulans* and *Pachysolen tannophilus* (**Gote M et al., 2014**).

In the current study, a potent yeast strain that can produce IMOs with maximum conversion from maltose was isolated and explored the α-glucosidase activity of the yeast strain by altering the various parameters during biotransformation. This is the first report to explore the potential use in transglucosylation reactions in continuous synthesis of prebiotic oligosaccharides with higher purity and by whole cell biomass in a single pot bio-reaction using hollow fibre microfiltration membrane technique.

2 | MATERIALS AND METHODS

2.1 | Collection of soil samples and materials

Samples were collected from the sugarcane field soil samples and collected from a village named Bhugaon near Pune, Maharashtra, India in sterile sample collection bags, sealed and stored in cold room. All chemicals, media components and other reagents were purchased from Sdfine, HiMedia Laboratories (India), Merck (India).

2.2 | Pre-treatment of soil samples

Sweet potato (500 g) was boiled in salt water, smashed and mixed in the soil sample and maltose solution (3% w/v) was sprayed on the soil containing smashed sweet potato for 120 h with 24 h interval followed by inoculating in 180 mL of sterile yeast malt (MGYP) medium containing 3% maltose for enrichment and incubated at 30^{o}C for 48 h. To avoid the growth of fastidious bacteria,

antibiotic streptomycin (1 µg/mL) was used in modified yeast malt medium (MGYP enriched with 3% Maltose).

2.3 | Screening, isolation, biochemical characterization and identification of maltose utilizing isolated strains through bioconversion

The isolated colonies from the plates were sub-cultured to obtain pure culture in the plates and liquid medium containing modified media containing maltose (3% w/v). After 96 h of incubation, the culture supernatants of each tube were injected in HPLC to check the maltose utilizing isolates. Biochemical characterization of maltose utilizing selected yeast strains (SB112, SB111, SB107, SB103, SB109, SB104, SB102, SCY204, SCY208 and SCY210) with different carbohydrates was studied in the carbohydrate kit (HiCarbo Kit from Himedia, India) according to manufacturer's instructions. Kits were kept for incubation at 30^{o}C for 24 to72 h.

2.4 | Identification of transglucosidase producing microbial strain

The hydrolytic and transfer reaction activity of the isolated strains (SB112, SB111, SB107, SB103, SB109, SB104, SB102, SCY204, SCY208 and SCY210) was determined through bioconversion with maltose. Flasks with the volume of 100 mL of 10% wet weight (w/w) pelleted biomass from each isolate and 25% (w/v) maltose solution in water (pH 6.0) were taken and kept in shaker for incubation at 30^{o}C for 48 h and samples were analysed in HPLC. Results were plotted average of triplicate values obtained from triplicate experiments.

2.5 | Morphology characterization and Identification of isolated strain

Scanning electron microscopy (SEM) has been widely used to characterize the surface structure of various microbes. SCY204 strain was treated with washing, fixation, drying followed by sputtering Gold nano films (20-30 nm thickness) on yeast specimen using an ion sputter and the images were obtained using a SEM device (**Perevedentseva E et al., 2007**). The isolated strain SCY204 was identified using ITS region of gene (rRNA) sequencing and phylogenetic analysis. The evolutionary distances were computed using the Maximum Composite Likelihood method. There were a total of 1218 positions in the final dataset and evolutionary analyses were conducted in MEGA6.

2.6 | Analytical methods for determining composition

The α - glucosidase activity assay (MAK123, Sigma-Aldrich, Steinheim, Germany) was determined using *p*NPαG as a substrate according to the manufacturer's instructions. One unit of enzyme that catalyzes the hydrolysis of 1.0 µmole substrate per minute at pH 7.0. The product determination and quantification of saccharides composition was analysed using high performance liquid chromatography system (HPLC) equipped with RI detector (Jasco RI 2031 plus), column (Shodex Asahipak NH2P 50 4E) along with guard column (Shodex Asahipak NH2P-50G 4A) and mobile phase (Acetonitrile: water (70:30) with the following parameters; flow rate: 1.5 mL/min, injection volume: 10 µl, column temperature: 30^{o}C and time: 30 min run. Standards such as glucose and maltose were purchased from Sigma and isomaltose, isomaltotriose, panose and isomaltotetraose from carbosynth. The saccharides (mono-, di-, oligo-saccharides) content of the samples were determined from the peak areas with respective retention time by comparing with the relevant standards.

2.7 | Effect of Media

The effect of media on strain SCY204 was carried out in shaker flask where maltose used as an inducer in all flask and different concentrations of nitrogen source, salts and metal ions were used. Various media were prepared as: **M1** – 3 g L^{-1} Malt extract, 10 g L^{-1} Glucose, 3 g L^{-1} Yeast extract, 5 g L^{-1} Peptone, 30 g L^{-1} Maltose; **M2** – 24 g L^{-1} Potato dextrose broth, 30 g L^{-1} Maltose; **M3** – 3 g L^{-1} Malt extract, 5 g L^{-1} Glucose, 3 g L^{-1} Yeast extract, 5 g L^{-1} Peptone, 30 g L^{-1} Maltose, 0.5 g L^{-1} $MgSO_4$, 0.01 g L^{-1} $FeSO_4$; **M4** – 20 g L^{-1} Yeast extract, 30 g L^{-1} Maltose, 0.5 g L^{-1} $MgSO_4$, 5 g L^{-1} K_2HPO_4; **M5** – 5 g L^{-1} Glucose, 7.5 g L^{-1} Yeast extract, 30 g L^{-1} Maltose, 0.5 g L^{-1} $MgSO_4$, 7.5 g L^{-1} CSL; **M6** – 5 g L^{-1} Yeast extract, 5 g L^{-1} Peptone, 30 g L^{-1} Maltose, 0.5 g L^{-1} $MgSO_4$, 0.01 g L^{-1} $FeSO_4$, 0.5 g L^{-1} K_2HPO_4, 0.5 g L^{-1} KCl, 3 g L^{-1} $NaNO_3$, 70 g L^{-1} NaCl. Growth was performed at 30^{o}C with shaking at 180 rpm for 36 h. Results were plotted average of triplicate values obtained from triplicate experiments.

2.8 | Fermentation

Batch fermentations were conducted using 10 L of Bioflo115 fermenter (New Brunswick Scientific). Optimized media was prepared and inoculated from late log phase flask seed culture at 10% (500 mL) of working volume. Fermentations were conducted at 30^{o}C with stirring of 350 rpm and aeration 1

vvm. Sampling was done at regular intervals to check the parameters like Optical density (OD), pH for growth kinetics. Also, samples were injected in HPLC to check the maltose utilization.

2.9 | Optimization studies of transglucosidase producing strain

2.9.1 | Effect of pH

The optimum pH for α – glucosidase activity was determined with 5% Maltose solution in 200 mM of various pH (3.0 to 10.0) buffers at 50^{o}C for 60 minutes. Effect of pH on stability was calculated by pre-incubating the cell biomass in 200 mM of various pH (3.0 to 10.0) buffers at 50^{o}C for 3 h and bio-reaction was carried out with 5% Maltose solution. Samples were taken at different intervals and injected in HPLC. Results were plotted average of triplicate values obtained from triplicate experiments.

2.9.2 | Effect of Temperature

Determination of the optimum temperature for α – glucosidase activity was performed with 5% Maltose solution in 200 mM of sodium citrate buffer (pH 4.0) at various temperatures (20 - 80^{o}C) for 60 min. Effect of temperature on stability was calculated by pre-incubating the cell biomass in 200 mM of citrate buffer (pH 4.0) at various temperatures (30 - 80^{o}C) for 3 h and bio-reaction was carried out with 5% Maltose solution. Studies were also carried out to determine the product formation through transfer reaction using higher maltose concentration up to 25% (w/v) with cell associated α – glucosidase in 200 mM citrate buffer medium (pH 4.0) and kept in various temperatures ranging from 27^{o}C to 40^{o}C. Bioconversion was performed for 24 h; samples were taken at regular intervals and injected in HPLC. Results were plotted average of triplicate values obtained from triplicate experiments.

2.9.3 | Effect of Substrate concentration

The effect of substrate concentration was determined through bioconversion of strain SCY204 with various concentrations of maltose (10% to 50%, w/v) in 200 mM citrate buffer of pH 4.0 in the shaker flask, incubated at 34^{o}C for 24 h in the orbitek shaker for 180 rpm. The purpose of this study was to evaluate the hydrolysis / transglycosylation activities at different substrate concentrations. Aliquots were taken at different time intervals (0, 6, 12, 18 and 24 h respectively) for HPLC analysis. Results were plotted average of triplicate values obtained from triplicate experiments.

2.9.4 | Effect of Biomass concentration

The rate of hydrolytic / transglucosidic activity was examined in shaker flask using different concentrations of strain SCY204 with 25% maltose concentration (in 200 mM citrate buffer of pH 4.0) followed by incubation at 34^{o}C for 24 h in the orbitek shaker for 180 rpm. Aliquots were removed from the reaction mixture at different timings for HPLC analysis. Results were plotted average of triplicate values obtained from triplicate experiments.

2.9.5 | Effect of Metal ions

Effect of metal ions / addition of salts ($CaCl_2$, NaCl, KCl, $MgCl_2$, $CoCl_2$, LiCl, $MgSO_4$, $FeSO_4$, NH_4Cl, $BaCl_2$, $FeCl_3$, $CuSO_4$, $ZnCl_2$, $ZnSO_4$ and $MnCl_2$) were performed in the bioconversion for better substrate conversion and product yield. Strain SCY204 cell pellet (10%, w/v) was added with maltose solution (10%) in 200 mM of citrate buffer (pH 4.0) with various metal ions / salts (0.05 M) and incubated at 50^{o}C for 60 min. Samples were taken at regular intervals and injected in HPLC. Results were plotted average of triplicate values obtained from triplicate experiments.

2.10 | Continuous production of Isomalto-oligosaccharides (IMOs) by cell associated transglucosidase of *Debaryomyces hansenii*

IMOs synthesis was investigated in a 3 L bioreactor with 2 L working volume using free cells of *Debaryomyces hansenii* (6%, w/v) and maltose as sole substrate (30%, w/v) in 200 mM Citrate buffer (pH 4.0). The reaction mixture was then incubated at 34^{o}C and the sample taken at regular intervals up to 24 h. Parameters were adjusted as aeration to 0.5 vvm and agitation to 300 rpm during bioconversion to maintain positive pressure and better mixing throughout the reaction. After achieving desired product purity by HPLC, the reaction mixture was then passed through 0.2 µ microfiltration membrane (MF) to separate IMOs and cell biomass. Once 50% of the product (1 L) was collected in a clean container, 1 L of prepared maltose (30%, w/v) solution was passed through microfiltration (MF) to the bioreactor and suspended with the reaction mixture. The experiment was repeated for 5 cycles and the biomass was reused where no extra biomass was used. The time profile of the bioconversion for the IMOs production was carried out. Results were plotted average of triplicate values obtained from triplicate experiments.

3 | RESULTS AND DISCUSSION

3.1 | Screening and isolation of transglucosidase producers

In the present work, we investigated a yeast strain isolated from the pre-treated soil source that produces an oligosaccharides using maltose as carbon source. After pretreatment of soil sample with sweet potato and maltose solution, the screening was carried out based on maltose utilizing capability. Initially screened 70 isolated yeasts strains and inoculated in maltose enriched media for substrate adaptation and better enrichment. Among all isolates, 10 yeast strains (SB102, SB104, SB109, SB112, SCY204, SCY210, SB111, SB107, SB103 and SCY208) were selected in the initial screening using HPLC analysis through its maltose utilizing capability. The strains utilized maltose completely during 72 h incubation / fermentation in the maltose enriched MGYP media. Similar screening method was reported in our previous work (**Saravanan R et al., 2016**) where lactose was added in the media for adaptation & better enrichment during growth and isolated galacto-oligosaccharides (GOS) producing yeast strain that was confirmed by HPLC. Among those isolated strains, SCY204 strain utilized maltose at 24 h whereas other strains utilized in later hours. SCY204 showed maximum substrate conversion in short duration time which was confirmed by HPLC analysis. This result demonstrated that strain SCY204 can able to utilize maltose efficiently.

3.2 | Biochemical characterization of Isolated strains with different carbohydrates

The cultural characteristics, such as microbial growth along with its pattern and sugar utilization were studied on biochemical kits and their results are summarized in **Table 1**. All the isolates utilized carbohydrates and leads to change in colour. In case of positive, it changes to yellow due to acid production and remains red in case of negative. All the isolates utilized particularly maltose in 24 h to 36 h where SCY204 utilized maltose in 18 h. The sugar utilization characteristic was determined by biochemical characterization method where isolated strain SCY204 utilized major carbohydrates for their growth including maltose. Similar biochemical characterization method (**Shajahan S et al., 2017**) was determined for highest cellulose producing isolate using biochemical kits.

3.3 | Identification of Maltose utilizing isolated strains through bioconversion

Furthermore study helped to identify the maltose conversion to product through bioconversion of screened 10 isolates. The samples of aliquot containing cell pellet of selected 10 isolated strains (SB102, SB104, SB109, SB112, SCY204, SCY210, SB111, SB107, SB103 and SCY208) with higher

concentration of maltose (25%, w/v) were incubated and samples were injected in HPLC to check the efficiency of cell associated isolates to produce oligosaccharides. Among all isolates, yeast strain SCY204 converted maltose into IMOs with the purity of 64% where the most abundant saccharide present was isomaltose, panose and isomaltotetroase (**Fig. 1A**). This formation was due to strain grown in shaker flask. Apart from that, strain SCY204 produced lesser by-products like glucose and other polyols during bioconversion when compared to other isolates.

3.4 | Identification of Transglucosidase producer

SEM image (**Fig. 1B**) of strain SCY204 reveals to be a spheroidal to short - ovoidal shaped yeast cells of 1-3 µm size; the gene (18s rRNA) sequence of strain SCY204 exhibited a high similarity with sequences of the genus *Debaryomyces*. The close phylogenetic relationship (**Fig. 1C**) between strain SCY204 and *Debaryomyces hansenii* of >99% similarity was found using multiple sequence alignment. Similar identification method was reported (**Saravanan R et al., 2016**) with highest similarity of *S.singularis*. Furthermore, there have not been any reports the production of oligosaccharides using *Debaryomyces hansenii*.

3.5 | Effect of Media

The cultural characteristics such as microbial growth along with the activity of strain *D.hansenii* in various media components were studied and the biomass was incubated with maltose (25%, w/v) found that strain *D.hansenii* grown in M4 media (Yeast extract, Maltose, $MgSO_4$ and K_2HPO_4) produced oligosaccharides (66% purity) through hydrolysis and transfer reaction of maltose at 24 h (**Fig. 2A**). The components obtained among IMOs saccharides were isomaltose, panose, isomaltotetraose and isomaltotriose. The biomass yield (g L^{-1}) was also determined and confirmed that strain *D.hansenii* grown in M4 media produced 36 g $L^{-1}$when compared to other media.

3.6 | Fermentation trials at 10L level

To determine the hydrolytic / transglucosidic activity of strain *D.hansenii* in different optimal conditions and to check the growth trend, strain *D.hansenii* was inoculated in eppendorf (New Brunswik Scientific - Bioflo 115) fermenter with 5 L working volume (**Fig. 2B**) of modified media where maltose used as major carbon source. Parameters were maintained during fermentation like temperature 30^{o}C, aeration 1 vvm and agitation 350 rpm. Samples were taken at regular intervals for

24 h to check the growth trend (**Fig. 2C**). The maltose and glucose assimilation trend was analysed by HPLC and it showed maltose utilization was started after complete assimilation of glucose, which clearly showed the presence of α-glucosidase. Maximum biomass yield of 54 g $L^{-1}$was achieved in the fermenter whereas 36 g L^{-1} yield was obtained in shaker flask. The obtained biomass from the fermentation was used to determine the hydrolytic / transglucosidic activity of strain *D.hansenii* under various conditions.

3.7 | Optimization studies through bioconversion

3.7.1 | Effect of pH

During the transglycosylation steps of IMOs production, *D.hansenii* strain can be used to hydrolyse maltose into glucose residues and transferring the glucose residues with residual maltose to form trisaccharides, tetrasaccharides, etc., however, the bio-reaction requires optimum temperature, pH, substrate and biomass concentration. Studies have shown that the optimum pH on activity (**Fig. 3A**) to carry out bioconversion in terms of maltose conversion to product and by-products was pH 4.0 (200 mM citrate buffer) at 50^{o}C for 1 h. i.e. loss in enzyme activity below pH 4.0 and above pH 6.0. The optimum pH of 6.0 – 7.0 of activity with sucrose by α-glucosidase was investigated (**Nimpiboon P et al., 2011)**. It was little acidophilic condition for the enzyme reaction using α-glucosidase and optimum pH of 4.0-7.0 was reported, where *Oryza sativa cv* of pH 4.5 (**Iwata H et al., 2003**) and *Chaetomium thermophilum* of pH 7.0 (**Jorge J A et al., 2006**). pH on stability (**Fig. 3B**) was also determined and found that the pH was more stable at 6.0 (200 mM sodium phosphate buffer) at 50^{o}C for 3 h and pH was unstable above 7.0.

3.7.2 | Effect of Temperature

Effect of temperature was determined with respect to activity and stability of *D.hansenii* with maltose to determine the rate of the reaction at various temperatures and it has been found that the cell associated α - glucosidase on activity (**Fig. 3C**) attain higher enzyme activity at 60^{o}C in terms of maltose conversion to product and by-products at 1 h. The rate of bio-reaction decreases when temperature decreases below 60^{o}C and increases above 60^{o}C. Commercially available transglucosidase have reported as optimum temperature of 55^{o}C (**Niu D et al., 2017**). The bio-reaction on stability (**Fig. 3D**) shows stable up to 50^{o}C over an time period. At certain temperatures (70^{o}C and

80^{o}C), a drop in activity was observed with 15 and 10 min respectively. The results obtained by increasing the maltose concentration from 10% to 50% (**Fig. 4A**) were quite interesting. The higher product purity of 57% in short duration (24 h) with lesser by-products was obtained at 34^{o}C at 24 h. The rate of reaction was faster when temperature increases and glucose concentration also increased during bioconversion. As a result of glucose accumulation, the rate of reaction remains slow and inhibits at certain duration and produces only 55% product. In a tranglycosylation reaction, α - glucosidase first hydrolyse maltose and formed glucose molecules and then maltose acts as an acceptor and acts on either glucose or maltose to form panose, isomaltose, isomaltotriose, isomaltotetraose or higher oligosaccharides (**Ojha S et al., 2015**). Hence product synthesis reaction (Biotransformation) was carried out hereafter at 34^{o}C. The stable temperature of 45^{o}C was observed using cell pellet of *Microbacterium sp* containing α – glucosidase and drop in activity in 50 and 55^{o}C and the synthesis reaction was carried out at 40^{o}C (**Ojha S et al., 2015**).

3.7.3 | Effect of Substrate concentration

The cell associated α – glucosidase showed dependence of maltose concentration (shown in **Fig. 4B**) and showed that the reaction was kinetically regulated. The purity formation in the transglucosylation reaction is dependent on the correlation between water and maltose for the glycosyl-enzyme intermediate. If water activity is more, the enzyme acts as hydrolase and if water activity is less by increasing the maltose concentration, the enzyme acts as transglucosidase and performs both hydrolytic and transfer reactions. Lower substrate concentrations (10%) reduced the transfer reactions and increased the hydrolytic activity. Higher substrate concentrations reduced the hydrolytic activity and increased the transfer reactions and require high substrate concentration to perform sufficient transglycosylation activity (**Mala S et al., 1999**). As shown in **Fig 4B**, the purity of IMOs (60%) proliferating by increasing the maltose concentration up to 30% and slight reduction <55% purity using above 40% maltose concentration. In this study, maximum product formation (63% on dry basis) was obtained using 30% at 18 h, whereas using 40% and 50% maltose concentration, the IMOs purity obtained was 55% and 49% respectively due to increase in viscosity of the reaction medium with 6% biomass concentration. The result clearly indicates the rate of conversion to IMOs formation was inhibited when the glucose concentration increases above 35% purity (confirmed by HPLC). The

result also clearly stated that using 10% and 20% maltose concentration could lead the reaction only to hydrolytic activity to produce more by-products and fewer products (IMOs). Mostly, bioconversion efficiencies using 20-50% maltose concentration to product have been described using purified α - glucosidase from various microbial sources such as *T.tengcongensis* (**Zhou et al., 2009**), *Aspergillus niger* (**McCleary & Gibson 1989**), *Aspergillus nidulans* (**Kato et al., 2002**), *Aspergillus carbonarious* (**Duan et al., 1994**) and *X.dendrorhous* (**Fernandez-Arrojo et al., 2007**).

3.7.4 | Effect of Biomass concentration

Effect of increasing the cell biomass (~4023 units/L α-glucosidase activity) associated α - glucosidase of *D.hansenii* was evaluated by adding the substrate concentration at 300 g L^{-1}. **Fig. 4C** showed that the maximum substrate conversion of more than 97% was observed at 21 h using 10% (w/v) cell biomass concentration. The formation of glucose and product gets saturated while increasing in biomass concentration above 4%. However, moderate conversion was also observed using 6 and 8%. The result also showed that more formation of by-product by increasing the biomass concentration (above 6%) during bioconversion. From the economic point of view, 6% biomass concentration was chosen for pilot scale preparation of IMOs. The production of isomaltose and isomaltotriose using immobilized neopullulanase / amylase from *Bacillus subtilis* (**Kuriki T et al., 1993**) and dextranase from *Penicillium lilacinum* (**Aslan Y et al., 2007**) were reported besides α – glucosidases. The product yield of 60% was increased by combined these enzymes when compared with 45% using conventional methods. In our study, more than 60% product purity was obtained using free cells of *D.hansenii* and the process shows economically viable as the yeast cells can be cultivated in modified media with cheaper ingredients and no undesirable products was obtained.

3.7.5 | Effect of Metal ions / Salts

To suppress the water activity, high concentration of salts was used in an aqueous system. Thus, the effect of addition of metal ions / salts was evaluated through bioconversion to determine the inducing / inhibiting the product formation. Each cation was used to 0.05 M in the reaction mixture containing cell biomass and maltose. It was observed (**Fig. 4D**) that there was no significant increase in product purity by adding metal ions / salts using 10% maltose as the initial substrate for 60 min. Among those, the addition of $ZnSO_4$, $CuSO_4$, $MnCl_2$, $ZnCl_2$, $BaCl_2$ exhibited same levels of glucose and IMOs

production (76-80% purity) and close to control (without salts) 83% purity. Using $CaCl_2$ and NaCl, the purity of the product was moderately reduced to 62-69%. But major inhibition and drastic change in purity was observed in addition of $FeSO_4$ to 36% and $CoCl_2$ to 55% in the reaction mixture when compared to control (without addition of salts). The increased in product yield by ~15% - 70 g L^{-1} was reported by adding LiCl with the concentration of 0.2-0.5 M in the reaction (**Ojha S et al., 2015**). Salts of many cations Mg^{2+}, Mn^{2+}, Ca^{2+} or CO^{2+} have been added in the bioconversion processes involving β-galactosidases from *Sulfolobus sulfataricus* for the production of lactulose and reported as these ions exhibited the same level when compared with control (without adding ions), while using only Fe^{2+} ion increased the lactulose production by 10% (**Oh D K et al., 2006**). The addition of Mn^{2+} that induced the enzyme with the increase of 15% in GOS production (**Oh D K et al., 2005**) and no effect on GOS production with the addition of Mg^{2+}, Ba^{2+} and Ca^{2+}.

3.8 | Continuous production of Isomalto-oligosaccharides (IMOs) by cell associated transglucosidase of *Debaryomyces hansenii*

The schematic representation of the synthesis of IMOs syrup using isolated strain in a bioreactor was shown in **Fig. 5**. The method of reusing the cell biomass of transglucosidase producer allows reuse and easy filtration / separation of product from the reaction mixture and easy downstream processing. Free cells of *D.hansenii* were incubated with maltose solution and reused in the bioreactor for IMOs synthesis was investigated. **Fig. 6A** shows the continuous production of IMO (confirmed by HPLC) at 300 g L^{-1} of initial maltose by recycling the *D.hansenii* cell pellet in number of cycles and kinetics profile (**Fig. 6B**) of IMOs production (% HPLC area) in different time course of the maltose transglycosidation. The IMOs production was reported using immobilized endodextranase in an enzyme reactor with addition of dextran T40 substrate with controlled pH, temperature, flow rate and concentration (**Chalane S et al., 2017**).

The results shown in **Fig. 6A** that the purity (concentration) of total IMOs was reached >60% in all the cycles between 9 to 18 h reaction time and the yield achieved was 85%. With 6% w/v cell biomass, the 30% maltose (w/v) was suspended in the buffer medium (pH 4.0) at 34^{o}C and formed oligosaccharides and by-product such as glucose, polyols. In a reactor, the product concentration formed up to certain extent time of 18h and remains constant due to feedback inhibition by glucose.

The product concentration for 1st cycle at 18 h was 189 g L^{-1} which corresponded to nearly 63% purity where concentration of total IMOs were constant up to 24 h and the productivity in the bioreactor was 10.5 g L^{-1}.h. After harvesting 50% of volume containing IMOs product through microfiltration, the cycle continued with the addition of maltose solution (150 g L^{-1} of reaction mixture) with the existing mixture and observed there was no product inhibition happened. The purity was increased and reached 64% purity in 11 h of cycle 2. The further harvesting and adding the maltose solution in cycle 3 produced maximum purity of 67%, it clearly indicated that the cells were active while reusing and no drop or reduction in activity or more conversion or higher concentration of product formation was observed. Similar study was reported using consortium of enzymes of *T. maritima* glucanotransferase and *B. stearothermophilus* maltogenic amylase, the produced IMOs yield was 68% (**J. Kaulpiboon et al., 2015**). In the chromatogram of glycosylated products presented in **Fig. 7**, seven peaks exactly matches with standard saccharides corrsponds to their retention times (RT) and the most rich components are isomaltose and panose while isomaltotriose, isomaltotetraose are present in lower concentration but significant quantities. Isomaltose, panose, isomaltotriose and maltose are more abundant in IMOs where kojibiose, kojitriose, nigeraose and isomaltotetroase are present in lower concentrations **(Goffin D et al., 2010**).

Cumulatively for 5 cycles, the product concentration at 11.6 h was 565.5 g L^{-1} which related to nearly 64.6% purity. The productivity chart of IMOs production by reusing the biomass was clearly defined in **Table 2**. The volumetric productivity of 48.8 g L^{-1}.h and yield of 85% were achieved in the bioreactor. The maximum IMO purity reported by using mutant *Aspergillus niger* C-6181 was 188.6 g L^{-1} (**Chen G G et al., 2011**) of total sugar in product was reported. The kinetics of the bio-reaction in different time course was well defined in **Fig. 6B** where the rate of conversion from maltose to IMOs and glucose was much faster at optimized conditions without any drop in purity during 18 h. The distribution of IMOs individual saccharide composition was mentioned in **Table 3**. Among those saccharides, isomaltose (36%) and panose content (19%) produced more than isomaltotriose and isomaltotetraose. Maltose was known to produce panose at shorter reaction period, when the reaction continuous to produce glucose at higher concentrations, isomaltose formed. Similar findings were done by **Casa-Villegas M et al., 2017**. The most increased percentage of trisaccharides and

tetrasaccharide were 20% and 7.4% at 200 g $L^{-1}$and 28.1% and 3.6% at 525 g L^{-1} maltose respectively was confirmed (**Fernandez-Arrojo L et al., 2007**).

In our previous work, the microbial whole cells were reused for the production of galacto-oligosaccharides with cross flow hollow fiber microfiltration system (**Avalakki U K et al., 2015**). The production of IMOs by immobilized cells in a packed bed bioreactor where the product concentration and productivity were 24 g $L^{-1}$and 4 g L^{-1}.h respectively (**Ojha S et al., 2015**). Highest productivity of 42.95 mmol L^{-1}.h of IMOs was reported using *L. mesenteroides* NRRL B-512F (**Rabelo M C et al., 2006**). In our study, >98% maltose conversion to IMOs (65% purity) and by products were achieved with the yield of >85%. This study proves the possibility of reusing the free cells of *D.hansenii* for continuous production of IMOs through biotransformation and eliminates purification costs using enzymes by sophisticated techniques.

4 | CONCLUSIONS

Isomalto-oligosaccharides are important saccharides for its potential use as prebiotics and functional foods. This study could explore the possibility of reusing cell associated α - glucosidase of *D.hansenii* for IMOs synthesis. In the present study, cell pellet of all isolates after centrifuging the broth were treated with high concentration of maltose individually and found that SCY204 strain converts maltose to IMOs with the conversion rate of more than 80%. The isolated strain SCY204 was identified as *Debaryomyces hansenii* with >99% similarity based on nucleotide homology and phylogenetic analysis. The reaction was further accelerated by optimizing the media during fermentation and optimizing the parameters (pH, temperature, metal ions, substrate and biomass concentration) during biotransformation to obtain high efficient production of IMOs with more than >98% maltose conversion rate. The cell associated transglucosidase was efficiently converted maltose into prebiotic oligomers, where isomaltose and panose (as identified by HPLC) produced as major product with the efficient production of 83% content, where remaining comprised of isomaltotriose and isomaltotretraose among IMOs individual components. Higher maltose concentration (500 g L^{-1}) was also effectively converted to IMOs. By optimizing the media components and maltose as inducers, the biomass yield was increased to 54 g L^{-1} from 36 g L^{-1} (control media). With optimal pH and temperature, the maltose conversion to product was faster and the reaction hours reduced to 18 h

from 24 h. Maximum transglucosidase activity in terms of IMOs formation (>65%) was observed using 30% maltose concentration at 18 h and observed gradual decrease in conversion rate by increasing substrate concentration. This study also suggested that the yield and productivity was increased through continuous production of IMOs by reusing the cell biomass to number of times. Hence, the proposed optimized process can be used as commercially viable for the production of IMOs in a bioreactor.

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Figure 1:

(A) Identification of maltose utilizing strains through bioconversion with 25% (w/v) maltose solution at 30°C: Residual maltose (%); Glucose and other by-products (%); IMOs (%). Data shown are averages of three experiments ± standard deviation.

(B) Morphology of strain *D.hansenii* visualized by scanning electron microscope.

(C) Phylogenetic relationship of the isolated strain SCY204 and some related yeast species based on 18s rDNA sequences obtained from NCBI gene bank database. The scale bar 0.005 denotes the substitutions of each nucleotide position.

Figure 2:

(A) Effect of various media components on the production of IMOs by cell pellet of *D.hansenii*: Residual maltose (%); Glucose and other by-products (%); IMOs (%). Data shown are averages of three experiments ± standard deviation.

(B) Fermentation (Growth) of *D.hansenii* using optimized media in 10L Fermenter.

(C) Growth kinetics study of *D.hansenii* in different time intervals reveals OD (Optical density) A_{600nm}; Glucose utilization (HPLC area %); Maltose utilization (HPLC area %) during fermentation. Data shown are averages of three experiments ± standard deviation.

Figure 3:

Effect of pH on activity (A) and stability (B) of cell associated *D.hansenii* with 5% maltose solution (w/v) in various buffers at 50°C for 60 min. Buffers of 200 mM concentration: pH 3.0 (Glycine/HCl); pH 4.0 and 5.0 (Citric acid); pH 6.0, 7.0 and 8.0 (Sodium phosphate); pH 9.0 (Tris-HCl); pH 10.0 (Glycine-NaOH).

Effect of Temperature on activity (C) and stability (D) of cell associated *D.hansenii* with 5% maltose solution (w/v) at various temperatures for activity (20 to 80°C) and stability (30 to 80°C) for 60 min: Residual maltose (%); Glucose and products (%). Data shown are averages of three experiments ± standard deviation.

Figure 4:

(A) Effect of temperature on IMOs synthesis using *D.hansenii* cell pellet (w/v) with 25% maltose solution (w/v): Residual maltose (%); Glucose and other by-products (%); IMOs (%).

(B) Effect of maltose concentration (10% to 50%, w/v) on cell pellet (w/v) of *D.hansenii* at 34°C: Residual maltose (%); Glucose and other by-products (%); IMOs (%).

(C) Effect of biomass concentration (1 to 10%, w/v) of *D.hansenii* with 30% maltose solution (w/v) at 34°C: Residual maltose (%); Glucose, IMOs and other by-products.

(D) Effect of different metal ions on 6% cell pellet (w/v) of *D.hansenii* with 10% maltose solution (w/v) at 50°C for 30 min: Residual maltose (%); Glucose, IMOs and other by-products. Data shown are averages of three experiments ± standard deviation.

Figure 5: Schematic representation of IMOs production using free cells of isolated strain *D.hansenii* with maltose.

Figure 6:

(A) Continuous production of isomalto-oligosaccharides (IMOs) in a 3L bioreactor by recycle process using whole cells of *D.hansenii* (6% w/v) with 30% maltose solution (w/v) under optimized conditions. IMOs (%) after bioconversion; Bio reaction time (h).

(B) Kinetics profile of IMOs production (% HPLC area) in different time course of the maltose transglycosidation. Residual maltose (%); Glucose and other by-products (%); IMOs (%). Data shown are averages of three experiments ± standard deviation.

Figure 7: HPLC chromatogram of IMOs generated during the transglycosylation reaction of *D.hansenii* cell pellet incubate with 30% (w/v) maltose solution at 34 °C after 18 h and the saccharides recovered in the supernatant fraction were analyzed by HPLC. The peaks in the chromatogram of the standards correspond to: (1) Polyol (Arabitol), (2) Unknown saccharide, (3)

Glucose, (4) Unknown saccharide, (5) Maltose, (6) Isomaltose, (7) Panose, (8) Isomaltotriose, (9) Isomaltotetraose.

APPENDICES

NH_4Cl	Ammonium chloride
$BaCl_2\,2H_2O$	Barium chloride
$CaCl_2$	Calcium chloride
CSL	Corn Steep Liquor
$CoCl_2\,6H_2O$	Cobaltous chloride hexahydrate
$CuSO_4\,5H_2O$	Copper (II) sulfate pentahydrate
K_2HPO_4	Dipotassium hydrogen ortho phosphate
$FeCl_3$	Iron (II) chloride anhydrous
$FeSO_4\ \ 7H_2O$	Iron (II) sulfate heptahydrate
IMOs	Isomalto-oligosaccharides
LiCl	Lithium chloride anhydrous
$MgCl_2\,6H_2O$	Magnesium chloride hexahydrate
$MgSO_4\,7H_2O$	Magnesium sulfate heptahydrate
MGYP	Malt extract, Glucose, Yeast extract and Peptone media
$MnCl_2\,4H_2O$	Manganous chloride
MF	Microfiltration (0.2µ) membrane
pNPαG	p-nitrophenyl-α-D-glucopyranoside
PCR	Polymerase chain reaction
KCl	Potassium chloride
RI	Refractive index
SEM	Scanning electron microscope
NaCl	Sodium chloride
$NaNO_3$	Sodium nitrate
VVM	Vessel volume per minute (Volume of air/Volume of medium)
$ZnCl_2$	Zinc chloride
$ZnSO_4\ \ 7H_2O$	Zinc sulphate heptahydrate