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# Optimization of Culture Media and Conditions Enhances Mannan Oligosaccharides Production of *Wickerhamomyces anomalus SZ1* Strain

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**A B S T R A C T**

A potential non-Saccharomyces yeast species, identified as *Wickerhamomyces anomalus SZ1* strain ( Gupta, *et al.*, 2018), which gave even higher (33%) mannan oligosaccharides (MOS) than that obtained from the traditionally used *Saccharomyces cerevisiae* strain were selected for optimization of suitable media study for maximum yield of MOS by the one factor at a time (OFAT) method. Mannose was found to the best carbon source for optimum production of MOS, which significantly enhanced the yield by 1.2 folds of MOS at 2% mannose concentration as in place of dextrose in YEPD media. Higher concentration of Mannose cannot significantly ($p<0.05$) enhance the MOS production further. 2% peptone and 1% yeast extract in combination were found to be the best nitrogen source. An initial pH 6.0, temperature $32^0$C and shaking condition at 180 rpm for a period of 96 hours were found significantly favour the MOS production. the result revealing that 5% ($1.05x10^8$cfu/mL) is the optimum inoculum size to attain the maximum MOS yield (701.13±23.23 mg/L at 96 hours incubation) that was 2.0 fold higher than that to incubated at 24 hours and 1.2 fold higher to that 1% ($2.1x10^7$cfu/mL) inoculum density but economically yield was insignificant with period of 72 (656.67±23.12 mg/L) to 96 (701.13±23.23 mg/L) hours incubation. It was concluded that *W. anomalus SZ1* strain can be grown on optimized media up to 72 hours and used as an alternative of *S. cerevisiae* yeast for commercial mass scale MOS production for human food and animal feed industries in future.

## Introduction

Mannan oligosaccharides, a polymer of mannose sugar is a yeast derived natural sugar complex that is used as food grade growth promoters in modern livestock and poultry production and possesses marked immunological properties over the traditionally used antibiotic based growth promoters without posing any adverse effects ((Baurhoo *et al.*, 2009; Yang *et al.*, 2008). Most of its health-promoting properties is present within the yeast cell wall (together with glucan, chitin, and protein) with its properties varying with the fraction of polysaccharides extracted, its degree of polymerization which in most cases depends on the strain type, and its growth conditions

(Aguilar-Uscanga and Francois, 2003; Kim and Yun, 2006; Latge, 2010). Till date, the commercial MOS production depends on *Saccharomyces cerevisiae* with a very little or no significant use of other species even though some have proved their commercial importance (Giovani *et al.*, 2012; Gupta *et al.*, 2018; Hoffman *et al.*, 2015; Legras *et al.*, 2007; Barnett, 2003).

This makes the present work quite significant as the demand of MOS for animal feed is increasing and it may not be possible to meet the requirement of mannan oligosaccharides (MOS) solely from *Saccharomyces spps*. Hence an extensive research is required to find out a non- Saccharomyces species that would be exploited as an alternative of *S. cerevisiae* for commercial MOS production. Additionally, each yeast/ fungal MOS has its own characteristic property based on the degree of polymerization that could contribute to its ability to modulate the host growth and innate immunity ((Podzorski *et al.*, 1990; Jones and Ballou, 1969, Gupta *et al.*, 2020). In our previous study, we conducted a performance feeding trial in Catla (*Catlacatla*) with extracted MOS from *W. anomalus SZ1* (W-MOS) and MOS extracted *S.cerevisiae* (S-MOS) with or without probiotic (*Bacillus subtilis* ATCC 6633). The result exhibited that the extracted MOS from *W.anomalus*is *at par* to the commercial MOS of *S.cerevisiae*to promote animal/fish production. It can be used as sole prebiotic additive or in combination with *Bacillus subtilis* probiotic, the growth and performance of experimental fishes effects are further enhanced without any effect on body composition [Gupta *et al.*, 2020].

*Wickerhamomyces* genera has been indexed in the group of probiotic fungi due to its potentially exploitable physiological and metabolic characteristics like wide metabolic, physiological and nutritional diversity, stress tolerance; enzyme secretion, antimicrobial properties; probiotic effects and production of potential commercial metabolites (Mo *et al.*, 2004; Gupta *et al.*, 2018). Since till now, little attention has been paid to the ability of non- Saccharomyces yeast strains to release cell wall polysaccharides, particularly mannopolymers (Giovani *et al.*, 2012) that exist as covalent mannose complex with protein, and can be released into extracellular medium during yeast growth and autolysis (Alexandre and Guilloux- Benatier, 2006). The present study attempts to optimize production parameters for augmenting the production of MOS with prebiotic nature from a non-Saccharomyces yeast strain *Wickerhamomyces anomalus*. However, the culture medium affects mannan oligosaccharides production is unknown. Therefore, the optimum conditions for the mannan oligosaccharides production were investigated for a cost effective commercial production using the one factor at a time (OFAT) process.

## Materials and Methods

### Microorganism, media and growth conditions

The potential yeast isolate from homemade dahi, identified as *W. anomalus SZ1* (gupta *et al.*, 2018), which gave the highest mannan oligosaccharide (MOS) yield among all isolates was selected for production study. The culture was maintained in Yeast extract peptone dextrose (YEPD) agar (HiMedia laboratories, India) slants at $4^{o}$C before use. One loop of potential strain on YEPD agar slant was rejuvenated separately for 24 h in 50 mL of liquid seed medium containing (per litre) 20 g, glucose; 20 g, peptone; and 10 g, yeast extract at $28^{0}$C at 180 rpm. The cultures were centrifuged at 5000 rpm for 10 minutes and cells were washed twice with sterilized normal saline.

The cells were suspended in the sterilized normal saline, after which the optical density (OD) of the culture was adjusted to approximately 1.17 at 600 nm, corresponding to a density of 2.1x $10^9$ cfu/ml [16].

## Mannan oligosaccharide extraction and purification

Aliquot of 1 ml of inoculum of *W. anomalus SZ1* yeast strain at a cell density of 2.1x $10^9$ cfu/ml (i.e. 2.1x$10^7$cfu/ml in 100 mL) were transferred to 250 ml of Erlenmeyer flasks containing 100 ml defined medium prepared by replacing one at a time carbon source and nitrogen source respectively. Additionally the influence of pH, temperature, aeration and inoculum size on the growth of the organisms in medium was studied. Incubation of all experimental media and control were performed at RT for 96 h on rotary shaker at 180 rpm. While the yeast cell biomass was harvested every 24 h to assess its mannan oligosaccharide yield using modified Peat method (Peat *et al.*, 1961; Nakajima and Ballou, 1974). 1 g cell paste (wet weight) was suspended in 5 mL of 0.02M citrate buffer (pH 7.0), and the mixture was autoclaved at 125$^o$C for 90 min.

After cooling, the gelatinous solid was centrifuged and supernatant was collected. The paste was re-suspended once again in 7.5 mL of citrate buffer and the same procedure was followed as mentioned above. The two supernatants were combined and an equal volume of Fehling's solution was added and stirred for 2 h. The precipitate of mannan copper complex was allowed to settle at the bottom and the major part of the liquid poured off. The copper complex of mannan was converted to mannan oligosaccharides by hydrolysis using 6 mL of 3N hydrochloric acid. The resulting green colour solution was poured off slowly into 10 mL mixture of methanol and acetic acid (8:1 v/v) and the precipitate of mannan oligosaccharide was left for several hours to settle, after which it was dried and weight of precipitated mannan oligosaccharide recorded. The green colour supernatant aftermath was decanted carefully into fresh methanol-acetic acid mixture and precipitated again. This washing procedure was repeated till the supernatant was colourless. All the precipitates were then collected on a sintered glass funnel, washed thoroughly with methanol and finally with a little ethyl ether, and dried at room temperature and estimated by Dubois method (Dubois *et al.,* 1958) and expressed mannan oligosaccharide yield in mg per litre.

## Optimization of carbon substrate for enhanced mannan oligosaccharides yield

The experimental basal media (YEPD without carbon source) containing 1% yeast extract and 2% peptone pH 6.0 was prepared and the carbon source was supplied by addition of 2% various sugars selected from the representative of different types of carbon groups like mannose, dextrose, fructose, mannitol, glycerol to assess its effect on the mannan oligosaccharides (MOS) production.

A control flask containing no carbon was also run during the experiment. 250 ml of Erlenmeyer flasks containing 100 ml of media were inoculated with 1 ml (1%) of *W. anomalus SZ1* at a cell density 2.1x$10^9$cells/ ml and incubated at RT on a rotary shaker. An aliquot was harvested every 24 hours over a period of 96 hours and its cell biomass analysed for its MOS yield (Vasylkovska *et al.,* 2015).

## Effect of concentration of mannose

The experimental media containing 1% yeast extract and 2% peptone pH 6.0 was supplemented with different concentration of optimized carbon source i.e. mannose ranging

from 2 to 6% to enable the study of its effect on MOS production. The defined medium with no sugars was set up as a control.

### Effect of nitrogen sources

The experimental media containing 2% mannose as optimized carbon source at pH 6.0 with different nitrogen sources were prepared. The nitrogen source was supplied individually as well as in combination from the representative of different types of nitrogen sources like peptone, malt extract, beef extract and yeast extract; to assess its effect on the mannan oliogosaccharide (MOS) production (Table 1). No nitrogen source was provided in the control media (Costa *et al.,* 2002; Tremaine and Miller, 1956).

### Effect of PH

The experimental media containing 2% mannose as carbon source and optimized nitrogen sources i.e.1% yeast extract and 2% peptone was used to study the effect of pH variation on MOS yield. The medium pH was adjusted using 1N NaOH or1N HCl to cover a range from 3.0 to 8.0 (All adjustments were made before sterilization) and then the media was autoclaved (Arroyo-López *et al.,* 2009; Liu *et al.,* 2015).

### Effect of temperature and aeration

Optimized experimental media (100 ml in 250 Erlenmeyer flask) supplemented with 2% mannose, 1% yeast extract and 2% peptone at pH 6 was used to study the effect of temperatures and aeration on mannan-oligosaccharide production. For the study, two sets of the production media were prepared, one set was incubated under static condition and another set under shaker condition (180 rpm). Each set was incubated at RT, $32^0$C and $37^0$C thereof on a rotary shaker at 180 rpm over a period of 96 hours.

### Effect of inoculum size

Optimized experimental media (100 ml in 250 Erlenmeyer flask) supplemented with 2% mannose, 1% yeast extract and 2% peptone at pH6.0 was used to study the effect of inoculum size on MOS production. The flasks were inoculated with inoculum range from 1% to 5% of *W. anomalus* of cell density $2.1x10^9$ cells/ ml. The flasks were incubated under optimized shaker condition at 180 rpm at $32^0$C (Vasylkovska *et al.,* 2015).

### Statistical analysis

The data was statistically analysed using the statistical package SPSS version 13 in which data was subjected to two-way ANOVA and Turkey's multiple range test was used to determine the significant difference between the mean..

## Results and Discussion

The commercial acceptability of prebiotic oligosaccharides from yeasts would be determined by economic factors. Environmental factors and specific culture conditions can dramatically impact cell wall oligosaccharide production in terms of yield as well as the size and chemical composition of the saccharides being formed. Thus optimization of critical parameters for the maximum production of mannan oligosaccharide like carbon and nitrogen sources, temperature and pH optima and inoculum sizes [25] needs to be targeted for the large scale production.

### Optimization of production parameters for enhanced mos yield

### Carbon source

*W. anomalus* SZ1 strain was grown to different carbon sources at the 2% level and

results are given in Fig. 1. The highest MOS yield obtained with 2% mannose supplemented media was 632.33 mg/L within 96 hours, which was 2.45 fold more than that obtained within the first 24 hours and followed by dextrose supplemented media from 198.25 mg/l at 24 hours to 602.12 mg/L at 96 hours and fructose supplemented media from 215.26 mg/l at 24 hours to 524.24mg/L at 96 hours respectively.

The MOS yield in mannitol and glycerol supplemented media showed a poor yield ranging from 45.25 to 54.25 mg/L at 24 hours and 89.75 to 124.25 mg/L at 96 hours whereas the control gave the lowest MOS yield from 25.2 (24hrs) to 51.2 mg/L (96 hrs). The two-way analysis ANOVA revealed the interaction of different carbon sources with incubation periods. A highly significant ($p<0.05$) differences was observed in the MOS yields among the specified carbon sources whereas the MOS yield was not significantly increased from 72 to 96 hours of incubation periods. The result supported that addition of mannose in place of dextrose in YEPD media would significantly enhance 1.2 folds of MOS yield over a period of 96 hrs. The carbon studies, as expected, showed the highest yields of mannan oligosaccharides with mannose sugar containing media proving it to be a suitable substrate for enhancement of MOS production.

Our result is an agreement of Aguilar-Uscanga and Francois (2003), they grew the yeast culture on different carbon sources like glucose, mannan, sucrose, galactose, maltose and ethanol, which were known to influence their growth behaviour. The interesting finding of their result was that the ratio of β-glucan to mannan was lower with mannose sugar supplemented media. This finding indicated that efficiency for MOS production was high with mannose in compared to other sugars.

Hence, *W. anomalus SZ1* showed better growth with fermentable sugars (glucose, mannose and fructose) in comparison non fermentable sugars (mannitol and glycerol). Hence yeast cells from non-fermentable carbon sources were found to be having less growth and yield of MOS thereof.

## Concentration of mannose

They are polymer of α-D-mannose i.e. α-D-Mannans, which are built of α-(1,2)- and α-(1,3)- D-mannose branches which are attached to a backbone of α-(1,6)-D-mannose chains [26]. Since mannose sugar is precursor of biosynthesis of mannan oligosaccharides, as expected, mannose as carbon source offered the highest growth rate and MOS yield among other carbon sources tested. Thus MOS yield was assessed with increasing concentration of mannose sugar and results are given in Fig. 2. The two-way ANOVA analysis revealed a statistically insignificant interaction between the concentration of mannose sugars and period of incubation in relation to MOS production. Thus supplementation of mannose sugar at 2% gave an optimal MOS yield while at higher concentration, the culture became more flocculent and hence MOS production was not further boosted.

Similarly, Aguilar-Uscanga and Francois (2003) reported that that higher concentration of mannose was not advisable for attaining growth and mannan yield. Martins *et al.*, (2014) grew *Pichia anomalus* on yeast malt broth, containing dextrose 10% at pH 6.0±0.2 and reported growth as flocculent within the media along with high amount of bioethanol and glycerol indicating that the higher concentration of carbon sources might be utilized for formation of fermentable products and not for cell wall polysaccharides biosynthesis. Similarly Li and Cai (2007) aslo reported that high concentration of sugar

substrate supported reduced growth rate due to the formation of flocculent in the culture broth media of yeast and thus recommended less than 5% concentration of sugar substrate for cell wall polysaccharides formation.

## Effect of nitrogen source

Nitrogen sources play a vital role to influence growth of microorganisms (Pavlova *et al.*, 2004). *W. anomalus* SZ1 strain was grown in different nitrogen sources and results are given in Fig. 3. The highest yield of MOS obtained with treatment C, containing 2% peptone with 1% yeast extract in media wherein MOS yield of 245.98±17.17 mg/l at 24 hours and 632.23±67.72 mg/L at 96 hours were obtained, which was 1.9 fold more than in which 3% peptone was supplemented. The lowest MOS yield as expected, was reported with no nitrogen sources i.e. 78.28±12.2 at 24 hours and 101.12±18.23mg/L. The two-way ANOVA analysis revealed a statistically significant interaction between the specified nitrogen sources and period of incubation in relation to MOS production. The carbon nitrogen sources studies showed that along with peptone and mannose, yeast extract must be an essential media ingredient similar to YEPD for growth and optimum MOS yield obtained from of *W. anomalus* SZ1 strain.

Batista *et al.*, (2013) used extruded bean as nitrogen source in the culture medium and recommended 1% extruded bean and 1% yeast extract or 1% yeast extract and 1% peptone present in medium gave comparable growth to the commercial YED medium for *S. cerevisiae* and *P. pastoris GS115* strains. Martins *et al.*, [16] used peptic digestion of animal tissues as nitrogen source in place of peptone for *P. anomalus*CE009 and reported that the growth was at par of peptone. Xiao *et al.*, (2014) reported that organic nitrogen source gave rise to maximum production of exopolysaccharides.

They also found that supplementation of yeast extract with peptone stimulated exopolysacchrides yield (De Vuyst and Degeest, 1999). These studies revealed that peptone can be replaced with other nitrogen sources while 1% yeast extract is the most essential ingredient of yeast cells for attaining optimum growth.

## Effect of pH

The pH of a cell's surrounding environment affects intracellular pH, which in turn alters the enzymatic activity within cells, leading to cell growth. *W. anomalus* SZ1 strain was grown at different pH ranging from 3 to 8 and result is given in Fig. 4. The highest MOS yield obtained with the media having pH 6.0 was 257.65±8.9 mg/l at 24 hours and 635.56±23.23mg/L at 96 hours, followed by 215.26±9.8 at 24 hours to 423.9±23.23mg/L at 96 hours with media having pH 5.0 and 87.65±5.15 at 24 hours to 356.23±21.21mg/L at 96 hours with media having pH 4.0. The lowest MOS yield was reported with media having pH 3.0 i.e. 25.2±2.21 mg/l at 24 hours and 48.2±2.67mg/L at 96 hours.

When the pH of media increased from 6 to 8, yield decreased significantly from 124.25±3.65 to 89.75±6.21 mg/L over a period of 96 hours. The two-way analysis thus revealed that the interaction of different pH with incubation periods shows a significant ($p<0.05$) differences in the MOS yields, with pH of mannose supplemented defined media of 6.0 best supporting the growth and optimum MOS yield from *W. anomalus* SZ1 strain.

Wang and Lu (2004) observed that the initial medium pH is a critical factor associated with the growth and exopolysaccharides biosynthesis. They studied the effect of different pH on exomannan production by marine yeasts and found the optimum initial

pH of the basal medium should not less than 5.6. The results also showed that when the initial pH was lower than 5.6, MOS production decreased, indicating that yeast strain was very sensitive to initial pH (Heald and Kristiansen, 1985; Adami and Cavazzoni, 1990; Elinv, 1992). Similarly Tao *et al.*, (2011) studied the effects of pH on the *P. anomalus* growth and reported that the growth decreased pH ranged from 3.0 to 4.5 while the medium pH fluctuation between 5.0 to 6.0 did not affect the growth rate though within the range from 6.5 to 7.5, it underwent a remarkable decreased in growth. Thus they recommended the initial optimum pH for *P. anomalus* is 5.0 and found a tolerance limit from 4.5 to 6.0.

## Effect of temperature and aeration

The effect of temperature and aeration on MOS yields was presented in Fig. 5. The results clearly reflected a significant ($p<0.05$) difference that showed the effect of temperature and aeration on growth and MOS yield. The highest yield of MOS obtained from the *W. anomalus* SZ1 strain cultured at $32^0$C within shaker flask conditions at 180 rpm was 257.65±9.78 mg/l at 24 hours and 654.12±19.76 mg/L at 96 hours, which was 1.2 fold more than that obtained without shaking of flasks. The lowest MOS yield was reported with room temperature without shaking the flask i.e. 167.66±7.56 at 24 hours and 423.9±17.12mg/L.

There exists a highly significant ($p<0.05$) differences in the average MOS yields among the different temperature and aeration condition with incubation periods. The rest of the temperature like RT and $37^0$C with or with the shaking of flask poorly supported the growth of *W. anomalus* SZ1 strain hence yield was reported in the range of 167.66 to 201.12 mg/l at 24 hours and 345.24 and 412.23 mg/L at 96 hours respectively.

The two-way ANOVA interaction between temperature and aeration along with incubation showed a significant ($p<0.05$) difference. The result revealed that the optimum temperature was $32^0$C with aeration for optimum MOS yield.

The temperature and aeration are important in growth of microorganisms and enhancing their productivity for commercially important products like alcohol, organic acids, alkaloid, flavonoid, polysaccharides and its oligosaccharides, single cell proteins, essential amino acids, vitamins and secondary metabolites was used for human and animal food and feed industries. Tao *et al.*, (2011)reported that *P. anomalus* viable cell counts increased as temperature was increased from 25 to $30^0$C after which it declined sharply when the temperature increased from 35 to $45^0$C, indicating that $32^0$C was the optimum temperature and $40^0$C and above temperature might be lethal for *P. anomalus.* Martins *et al.*, (2014) reported that the optimum growth of *P. anomalus* CE009 was reached at the temperature ranging from 25 to $30^0$C. Similarly, Hanneh *et al.*, (2014) found that mannan content increased linearly, attaining the maximum yield (95.447± 8.8 mg/ 100 ml) at $32^0$C under aeration. Similarly Liu *et al.*, (2009) studied the effect of temperature on mannan production and reported a maximum yield (71.25 mg/ 100ml) at $32^0$C and thereafter a significant decrease in exomannan production was seen at higher temperature. This was nearly similar to our findings and supported by several previously reports, concerning the optimum temperature and aeration of exopolysaccharides (Cho *et al.*, 2001; Heald and Kristiansen, 1985; Adami and Cavazzoni, 1990; Elinov *et al.*, 1992).

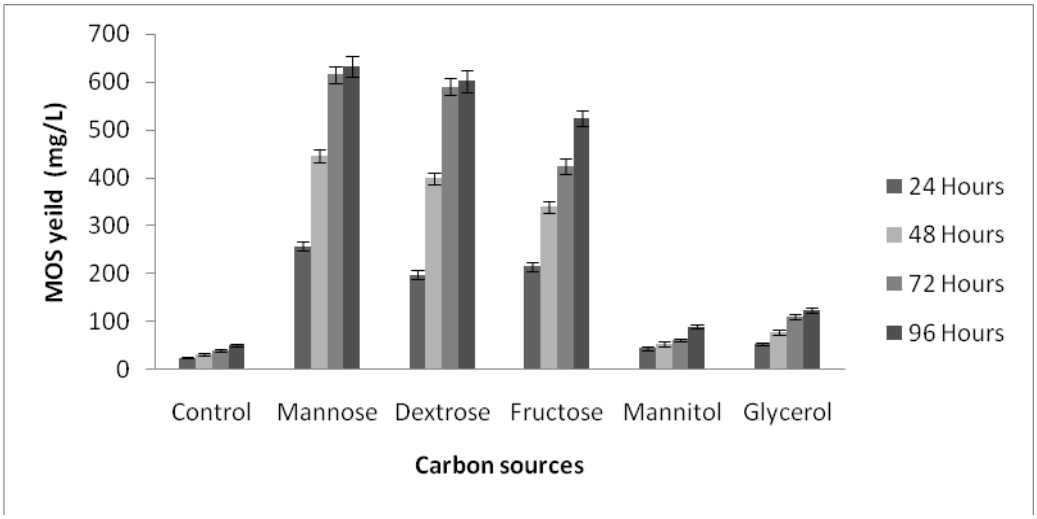
## Effect of inoculum size

The initial inoculum density added to broth for MOS production showed a highly

significant ($p<0.05$) differences on the yield wherein the yield was found to increase with the increase in the period of incubation in all treatments (Fig. 6). The highest MOS yield was reported from 378.15±17.13 at 24 hours to 701.13±23.23 mg/L at 96 hours incubation with inoculum density of 5% ($1.05x10^8$ cfu/mL), followed by 312.15±14.15 to 688.35±22.23 with 4% ($8.4x10^7$ cfu/mL), 276.45±13.13 to 665.78±21.78 with 3% ($6.3x10^7$ cfu/mL), 212.12±13.13 to 645.90±21.21 with 2% ($4.2x10^7$cfu/mL) and 198.25±12.14 to 623.12±19.78 mg/L at 96 hours with 1% ($2.1x10^7$cfu/mL) incubation respectively. The two-way ANOVA interaction between inoculum density and MOS yields showed a highly significant ($p<0.05$) differences with the result revealing that 5% ($1.05x10^8$ cfu/mL) is the optimum inoculum size to attain the maximum MOS yield of 1.2 fold higher to that 1% ($2.1x10^7$ cfu/mL) inoculum density whereas there was not a significant increase in the MOS production from 72 to 96 hours. The incubation up to 72 hours with in optimized condition will be more economically practical for mass scale production of MOS by *W. anomalus* SZ1 strain.

**Table.1** Different nitrogen sources added to modified YEPD media

| Flask | Nitrogen source |
|---|---|
| **A** | 2% peptone |
| **B** | 3% Peptone |
| **C** | 2% peptone + 1% yeast extract |
| **D** | 2% peptone + 1% Beef extract |
| **E** | 2% peptone + 1% Malt extract |
| **F** | No nitrogen source |




**Interaction of period**

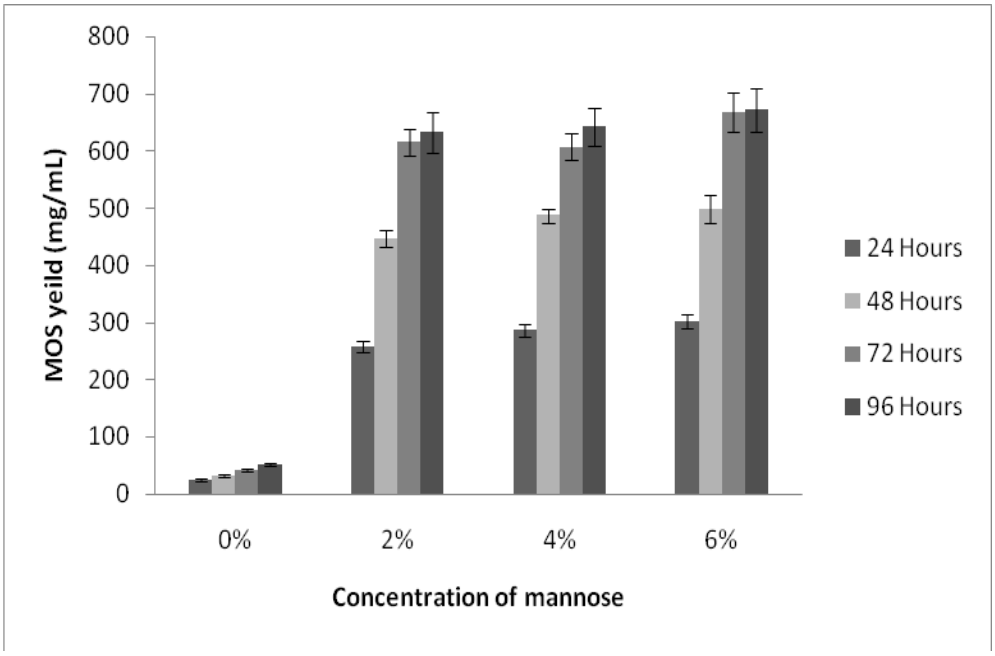
| Time (Hours) | MOS Yield |
|---|---|
| **24** | $132.66^{c}$ |
| **48** | $224.99^{b}$ |
| **72** | $307.79^{a}$ |
| **96** | $337.33^{a}$ |
| **SEM** | 0.76 |
| **P value** | **0.00** |

Interaction of carbon sources

| Control | Mannose | Dextrose | Fructose | Mannitol | Glycerol | SEM | P Value |
|---|---|---|---|---|---|---|---|
| $37.67^{E}$ | $488.05^{A}$ | $447.84^{B}$ | $375.65^{C}$ | $62.96^{D}$ | $91.98^{D}$ | 9.40 | 0.00 |

Data is presented as Mean±SE (n=3) values with different superscripts in the same column differ significantly ($p < 0.05$)

**Fig.1** Effect of different carbon sources on mannan oligosaccharides yield




Interaction of period

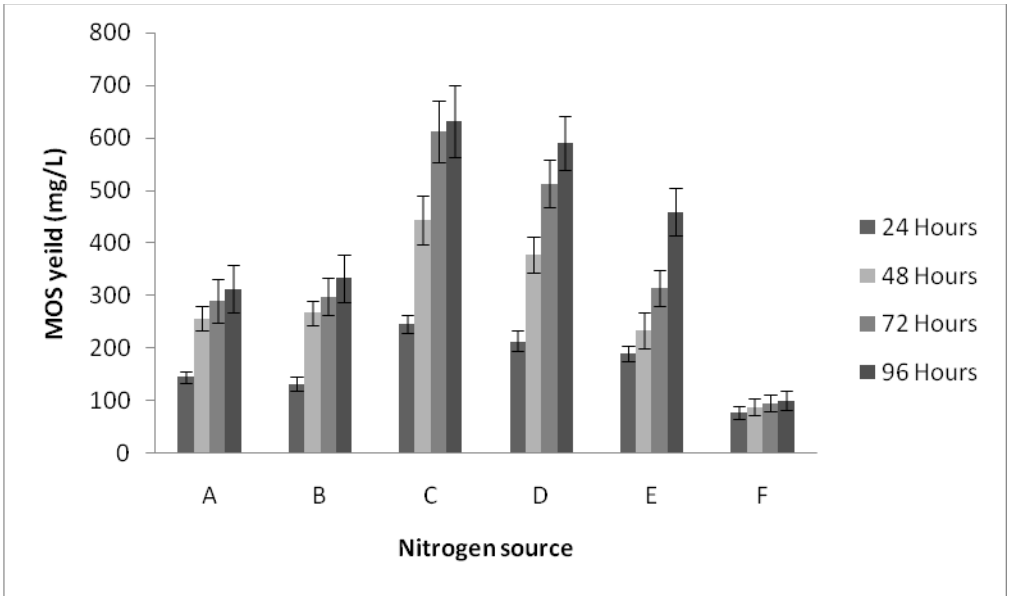
| Time (Hours) | MOS Yield |
|---|---|
| 24 | 217.56$^{d}$ |
| 48 | 366.09$^{c}$ |
| 72 | 482.96$^{b}$ |
| 96 | 497.56$^{a}$ |
| SEM | 5.35 |
| P value | 0.00 |

Interaction of Mannose concentration

| 0% | 2% | 4% | 6% | SEM | P Value |
|---|---|---|---|---|---|
| 37.642$^{B}$ | 497.762$^{A}$ | 506.960$^{A}$ | 531.829$^{A}$ | 5.35 | 0.00 |

Data is presented as Mean±SE (n=3) values with different superscripts in the same column differ significantly ($p < 0.05$)

**Fig.2** Effect of mannose concentration on Mannan oligosaccharides Yield




Interaction of period

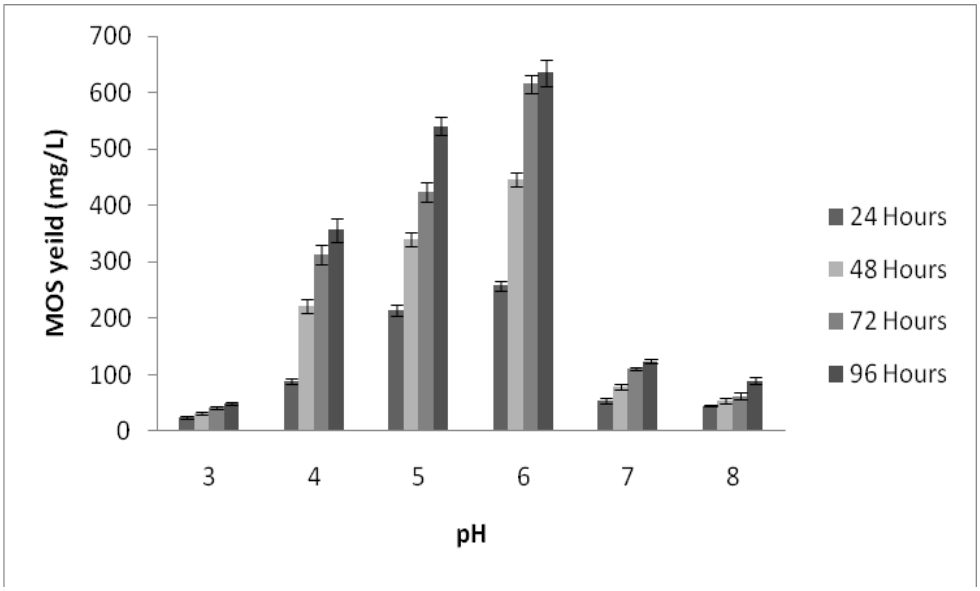
| Time (Hours) | MOS Yield |
|---|---|
| 24 | 167.47$^{d}$ |
| 48 | 278.21$^{c}$ |
| 72 | 353.83$^{b}$ |
| 96 | 380.39$^{a}$ |
| SEM | 15.37 |
| P value | 0.00 |

Interaction of Nitrogen Source

| A | B | C | D | E | F | SEM | P value |
|---|---|---|---|---|---|---|---|
| 251.02$^{C}$ | 257.87$^{C}$ | 483.64$^{A}$ | 423.72$^{B}$ | 262.89$^{C}$ | 90.71$^{D}$ | 15.56 | 0.00 |

Data is presented as Mean±SE (n=3) values with different superscripts in the same column differ significantly ($p < 0.05$)

**Fig.3** Effect of nitrogen source on mannan oligosaccharides yield




Interaction of period

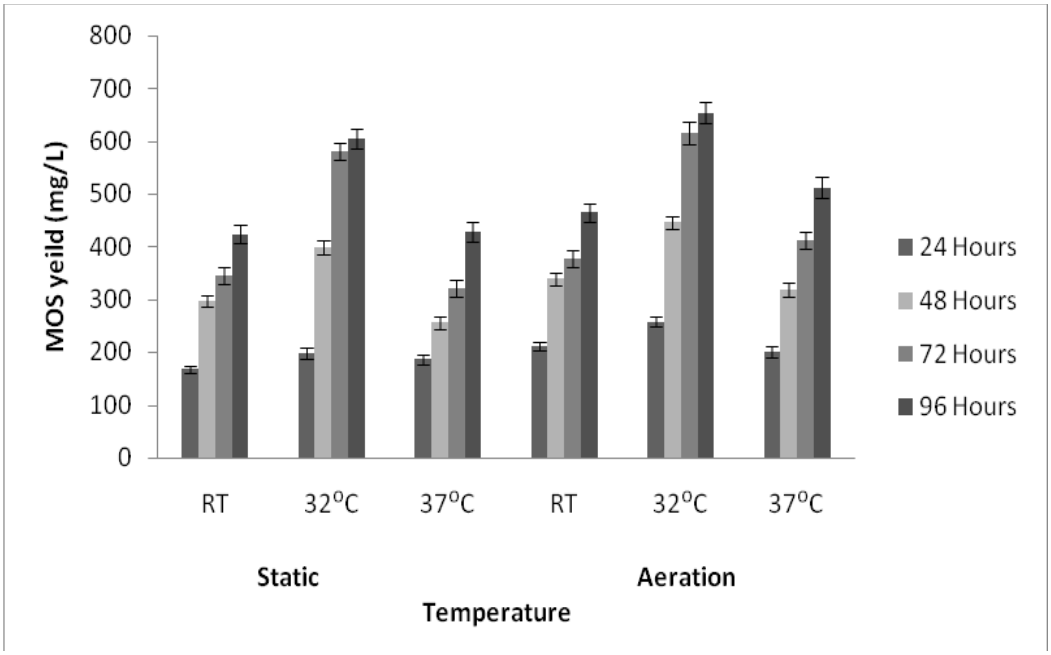
| Time (Hours) | MOS Yield |
|---|---|
| 24 | 114.21$^{c}$ |
| 48 | 195.33$^{b}$ |
| 72 | 261.25$^{a}$ |
| 96 | 299.01$^{a}$ |
| SEM | 9.76 |
| P value | 0.00 |

Interaction of different pH media

| 3 | 4 | 5 | 6 | 7 | 8 | SEM | P value |
|---|---|---|---|---|---|---|---|
| 36.88$^{F}$ | 244.41$^{C}$ | 379.63$^{B}$ | 488.84$^{A}$ | 92.00$^{D}$ | 62.95$^{E}$ | 11.34 | 0.00 |

Data is presented as Mean±SE (n=3) values with different superscripts in the same column differ significantly ($p < 0.05$)

**Fig.4** Effects of pH on mannan oligosaccharides yield




Interaction of period

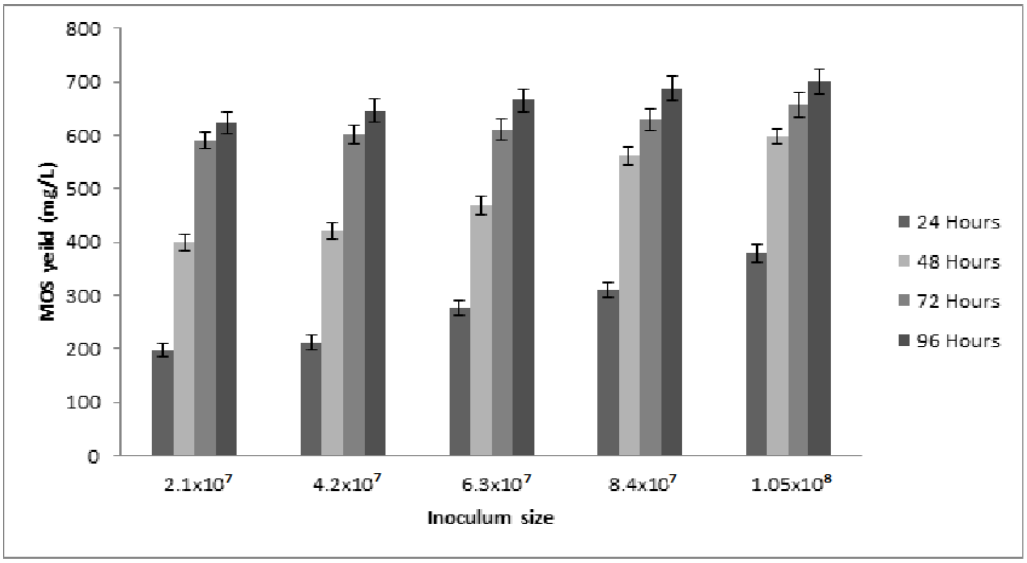
| Time (Hours) | MOS Yield |
|---|---|
| 24 | 203.98$^{d}$ |
| 48 | 343.03$^{c}$ |
| 72 | 442.16$^{b}$ |
| 96 | 514.92$^{a}$ |
| SEM | 14.82 |
| P value | 0.00 |

Interaction of Temperature and aeration

| Static condition | | | Aeration condition | | | SEM | P value |
|---|---|---|---|---|---|---|---|
| RT | 32$^{0C}$ | 37$^{0C}$ | RT | 32$^{0C}$ | 37$^{0C}$ | | |
| 308.59$^{B}$ | 445.87$^{A}$ | 298.2$^{C}$ | 348.77$^{B}$ | 493.48$^{A}$ | 361.2$^{B}$ | 15.47 | 0.00 |

Data is presented as Mean±SE (n=3) values with different superscripts in the same column differ significantly ($p < 0.05$)

**Fig.5** Effect of temperature and aeration on mannan oligosaccharides yield




Interaction of period

| Time (Hours) | MOS Yield |
|---|---|
| 24 | 275.42$^{c}$ |
| 48 | 489.89$^{b}$ |
| 72 | 617.93$^{a}$ |
| 96 | 664.86$^{a}$ |
| SEM | 15.66 |
| P value | 0.00 |

Interaction of Inoculum size

| $2.1x10^{7}$ | $4.2x10^{7}$ | $6.3x10^{7}$ | $8.4x10^{7}$ | $1.05x10^{8}$ | SEM | P value |
|---|---|---|---|---|---|---|
| 452.843$^{D}$ | 470.155$^{D}$ | 505.753$^{C}$ | 547.850$^{B}$ | 583.545$^{A}$ | 17.25 | 0.00 |

Data is presented as Mean±SE (n=3) values with different superscripts in the same column differ significantly ($p < 0.05$)

**Fig.6** Effects of inoculum size on mannan oligosaccharides Yield

Tao *et al.*, (2011) observed the effect of inoculum size of *P. anomalus* on the fermentation process. The biomass increased steadily, when the inoculum varied from 3 to 5%. There was a moderate decrease, when inoculum varied from 5 to 6%. In contrast a notable decrease was observed between inoculum of 6 to 7%. Tao *et al.*, (2011) findings supported that 5% inoculum size was the optimum for attaining maximum growth and mannan oligosaccharides yield thereof. Consequently, a sound understanding of growth parameters is essential to achieve optimum production of mannan oligosaccharides. The present study demonstrated that the optimum culture condition for *Wickerhamomyces anomalus* SZ1 was a temperature of $32^{0}$C, pH 6.0 and inoculum size of 5% in defined media containing 2% mannose sugar, 1% yeast extract and 2% peptone gave maximum yield of 701.13±23.23 mg/L mannan oligosaccharides. The Two-Way ANOVA analysis revealed that there was no significant economical yield benefit of MOS from 72 to 96 hours under optimization condition. Under these optimum conditions, *W. anomalus*SZ1 straincan be exploited as an alternative of *Saccharomyces cerevisiae* yeast strain for the mass production of MOS as dietary prebiotic supplement for promoting better growth and innate immune performances of terrestrial animals and fishes.

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