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Fermentation performance of the yeast *Saccharomyces cerevisiae* in media with high sugar concentration

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Abstract

High sugar concentration is more preferred in industrial bioethanol production, as it can increase the amount of ethanol produced by the end of fermentation. However, when high sugar concentration is used in the media, yeast cells are exposed to high osmotic stress, which can affect the fermentation performance. The present experiment aimed to investigate the fermentation performance of the yeast *S. cerevisiae* at high sugar concentration, in poor (yeast nitrogen base/YNB) and rich (yeast extract peptone/YEP) media. Growth parameters and sugar utilization were monitored during the fermentations. The results indicate that all three strains used had stuck fermentations, leaving 50 to 60% residual sugar. Therefore, a follow up experiment was conducted by using media of different nutritional value, including YNB with nitrogen supplementation and YEP, as well as different sugars (sucrose or glucose). Yeast cell grown in YEP had better fermentation performance indicated by higher sugar utilization. Addition of ammonium sulphate to YNB media did not change fermentation performance of the yeast cells. In YEP media, cells grown with glucose tended to maintain better viability than cells grown with sucrose. Our study confirmed that nutrient availability is very important for fermentation performance. Comparison of YNB and YEP media indicates that nutritionally insufficient media are not suitable for high sugar concentration fermentation.

Keywords: Fermentation performance, high sugar concentration, nutrition availability

Introduction

In industrial processes, it is common to use feedstocks with high sugar concentrations. A high sugar concentration at the start of fermentation may lead to higher amounts of ethanol produced at the end of the fermentation. This would lower production costs by reducing the water and energy processing requirements for distillation. However, when a high sugar concentration is initially present in fermentation media, yeast cells are exposed to high osmotic stress. This is not ideal for yeast cells, and the fermentation may become stuck (Bafrncová *et al.* 1999). When basal media were used in fermentation experiments, sugar was not fully utilized by yeast cells, leading to high residual sugar at the end of the fermentation (Bafrncová *et al.* 1999; Reddy & Reddy 2006; Thomas, Hynes & Ingledew 1994). Cell viability, sugar uptake and ethanol productivity have been enhanced by supplementation of media with excess assimilable nitrogen in the form of yeast extract, casamino acids and other supplements such as glycine, glycine betaine, proline, finger millet flour, soya flour and yeast cell walls (Bafrncová *et al.* 1999; Reddy & Reddy 2006; Thomas, Hynes & Ingledew 1994; Thomas & Ingledew 1990).

Most experiments with high sugar concentration have used complex media such as yeast extract, peptone, casamino acids, wheat hydrolysate or corn hydrolysate (Bafrncová *et al.* 1999; Reddy & Reddy 2006; Thomas *et al.* 1993; Wang *et al.* 2007). However, these media components include fluorophores, and in the current project it was planned to utilize fluorescence spectroscopy to assess yeast membrane fluidity. Therefore, complex media could not be used as high fluorescence background interferes with the spectroscopic techniques.

The initial experiments in this study were designed to model industrial conditions which use high sugar concentrations, up to 22 % (w/v), with many studies utilizing 16% (w/v) sucrose (Andrietta, Steckelberg & Andrietta 2008; Caylak & Sukan 1998; Cazetta *et al.* 2007). In addition to mimicking industrial conditions, we also proposed to assess yeast plasma membrane fluidity *in situ* during fermentation. Therefore, background fluorescence intensity of the fermentation media had to be considered.

Thus in the present experiment, we examined fermentation performance of the yeast in defined media with different sugar concentrations. The experiments commenced utilizing Yeast Nitrogen Base (YNB) medium containing 16% (w/v) sucrose as the carbon source. It was considered that this medium composition provided sufficient nutrition to sustain fermentation of this level of sugar.

Materials and Methods

Yeast Strains and culture conditions

Initial experiments with YNB containing 16% sucrose assessed the yeast strains A12, A14, K7 and PDM. A12 is an ethanol tolerant baker's yeast according to a previous study (Lewis 1993), A14 is a yeast strain used in industrial production of bioethanol, PDM is an industrial wine strain (Mauri Yeast) which usually can produce up to 17% (v/v) ethanol, and K7 is a sake yeast strain (ATCC 26422) that can produce up to 17.5% ethanol. After obtaining variable data in initial experiments (data not shown), it was decided to follow up using only one strain (A12), the best growing strain, to simplify the investigation of fermentation performance with different media and sugar concentrations. For this experiment the following media were used:

1. YEP with 16% Sucrose (YEP16S)
2. YEP with 16% Glucose (YEP16G)
3. YNB with 16% Glucose (YNB16G)
4. YNB with 1.5% ammonium sulphate and 16% glucose (YNBAS16G)
5. YNB with 2% glucose (YNB2G)
6. YNB with 1.5% ammonium sulphate and 2% glucose (YNBAS2G)

Yeast strains were maintained on slopes of a complete medium, yeast extract peptone (YEP), containing (w/v) 0.5% yeast extract, 0.5% bacteriological peptone, 0.3% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, 1% glucose and 1.5% agar. Slopes were stored at 4°C and sub-cultured every 6 months. Master cultures were stored in a Sanyo –80°C freezer.

Aerobic cultures were prepared by aseptically adding YNB media to sterile Erlenmeyer flasks, each sealed with an oxygen-permeable cotton wool bung, and then inoculating to give an initial viable cell number of ~10^6 cells/mL. The ratio of flask size to culture volume was 4:1 to ensure adequate oxygen mixing.

Samples from the cultures were aseptically removed by drawing off with a micro pipette every 6 hours from 0 to 30 hours. Examination of the samples included assessing growth rate by measuring optical density, viable cell numbers, % budding and glucose and ethanol concentrations. Detailed analysis including ethanol tolerance and membrane fluidity was performed at 6 h and 24 h.

Growth rate

Yeast growth was monitored by measuring optical density of the culture at 600 nm (OD_{600nm}) using a Beckman DU 650 spectrophotometer, making dilutions where necessary. Measurements were made using 1 mL (10 mm path length) PMMA cuvettes (Sarstedt).

Viable cell number

Viable cell numbers were assessed using the methylene violet staining method and light microscopy (400× magnification) with a Neubauer-type haemocytometer. Methylene violet staining is proposed as a better method for monitoring yeast cell viability compared to the traditional methylene blue staining method (Smart *et al.* 1999).

An equal volume of the sample was mixed with methylene violet solution (0.01% w/v in 2% sodium citrate solution) (Smart *et al.* 1999). Methylene violet crosses the membrane of all cells but in dead cells is unable to be metabolized, as a consequence dead yeast cells stained violet. Viable cells are able to metabolize methylene violet and as a result are unstained under the microscope.

Percent viable cells

When counting, both live and dead cells were recorded, to give the total cells per mL. The number of viable cells were then divided by the total cells and multiplied by 100 to give the percentage of viable cells.

Percent budding

When counting, both budding and non-budding cells were recorded as an indicator of cell growth rate. The number of budding cells was then divided by the total number cells and multiplied by 100 to give the percentage of budding cells.

Determination of total sugar concentration by the phenol-sulphuric acid method

The sugar concentration was determined by the phenol-sulphuric acid method of Dubois *et al.* (1956).

A standard curve was prepared as a series of glucose solutions with concentrations from 0 to 100 µg/mL. A volume of 150 µL of either standard or sample solution was then added to a reaction tube, to which 150 µL of 5% phenol solution in water was added. When necessary, samples were diluted to ensure experimental readings fell within the standard curve. The tubes were then mixed and stood at room temperature for 5 minutes. Then 750 µL of concentrated sulphuric acid was added rapidly; the tubes were then vortexed and placed on a hot plate (100°C) for 10 minutes. After cooling to room temperature, the absorbance of the standards and samples were read at 490 nm with a Beckman DU650 spectrophotometer, using the reagent blank to zero the spectrophotometer. A standard curve was plotted and this was used to calculate the concentration of glucose in each of the samples.

Results and Discussion

Growth parameters of different yeast strains grown in YNB with a high sugar concentration

As the initial objective of the present study was production of high quantities of bioethanol under modeled industrial conditions, a high initial sugar concentration (16% sucrose in YNB) and three yeast strains were assessed.

Figure 1 shows growth parameters of PDM, A12 and A14, cultured in YNB medium with 16% glucose. Either OD_{600nm} or total cell counts indicated that among these three strains, PDM had the highest cell numbers (Figure 1 (A) and (B)), while A12 and A14 were similar. Respiro-fermentative (exponential) phase growth was observed during the first 24 hours, however growth and viability decreased after this time, and ferments seemed to have stuck at around 8% to 12% sucrose (Figure 2). The sugar was rapidly taken up by all strains during the first 24 hours. A14 maintained a sugar utilization rate relatively higher than the other strains up to 72 hours. After the rapid sugar utilization, the level of sugar in the medium was relatively constant until the end of the experiment. The residual sugar concentrations at 168 hours were 6.9, 7.7 and 6.0% (w/v) for PDM, A12 and A14, respectively.

The growth parameters of the three yeast strains were similar, with exponential growth up to 24 hours and thereafter loss of cell viability. A12 showed the highest rate of cell viability decline followed by PDM and A14. This result indicates that even though the total cell number of A14 was lower than PDM, its viability was better. This property is required for lengthy fermentations, as the cells could maintain their activity for a longer period. This property might be an advantage for high sugar concentration fermentation which requires long fermentation times. Further investigation comparing yeast cell viability and fermentation performance of different yeast strains is required to test this hypothesis.

While A14 achieved the highest viable cell number at 48 hours, PDM and A12 showed more rapid growth earlier, with the highest viable cell numbers at 8 hours (Figure 1 (C) and (D)). It is also noteworthy that the total viable cell number of A14 was similar at the 8 h time point to A12, while PDM has the highest total viable number. All strains showed their highest cell viability at 8 hours, and at this time point A14 had the highest cell viability of all strains. This initial experiment utilized YNB medium with 16% glucose. YNB contains 0.5% ammonium sulphate as the primary nitrogen source. On determination that all ferments were becoming stuck at high residual sugar concentrations and cells were dying off, we considered that the level of a key nutrient may have been insufficient. While this medium has been used successfully in the past for ferments with moderate sugar levels (e.g. 2 to 5%), its nutritional value seems to be limited for high sugar ferments. Nitrogen insufficiency is a common cause of stuck ferments, so we investigated whether increasing the level of bioavailable nitrogen could resolve this problem. Therefore, we added an additional 1% ammonium sulphate to provide a final concentration of 1.5% (w/v), as has been applied in previous studies (Chi, Kohlwein & Paltauf 1999).

Growth of the A12 strain in different media

Similar to the findings with high glucose levels in YNB, when A12 was grown in YNB with high sucrose concentration the growth and fermentation performance (as assessed by sugar utilization) was poor (Figures 3 and 4). However, when the YNB was brought up to 1.5% (w/v) ammonium sulphate, cells were still unable to completely assimilate 16% glucose and ferments still stopped at high residual sugar levels. In order to confirm the feasibility of high gravity (16% sugar) ferments with the chosen yeast strains, we investigated the nutritionally richer (although compositionally relatively undefined) YEP-based media, as have been applied in previous studies (Thomas & Ingledew 1990).

Based on OD data, yeast grown in YNB media, whether or not supplemented with an additional 1% ammonium sulphate, showed respiro-fermentative (exponential) growth up to 8 hours, and started to slow at 24 hours. In contrast, yeast grown in YEP media still showed high cell growth rates for up to 96 hours. OD data clearly indicate that yeasts grown in YEP can reach higher cell densities. Yeast grown in YEP with glucose showed the highest OD followed by cells grown in YEP with sucrose.

In Figure 3 it can be seen that yeast cells grown in YEP with glucose showed the highest total cell number, confirming the OD data. However, the total cell number for yeast cells grown in YEP with sucrose was not different to that of yeast grown in YNB media. All cultures grown in YNB media showed a similar pattern. During the first 8 hours, cells grown in YNB or YEP with sucrose showed respiro-fermentative (exponential) growth, with growth starting to decline at 24 hours. However, cell counts of yeast grown in YEP with sucrose (or glucose, to a lesser extent) were moderately increased again at about 120 hours, indicating a small secondary fermentation.

Viable cell numbers and proportions showed similar patterns in all medium compositions. The number of viable cells increased rapidly during the first 8 hours. Yeast cells grown in YEP maintained a higher viable cell number and % viability than cells grown in YNB.

The growth pattern of the yeast varied depending on the medium. In YEP media the yeast grew more rapidly compared to the YNB media.

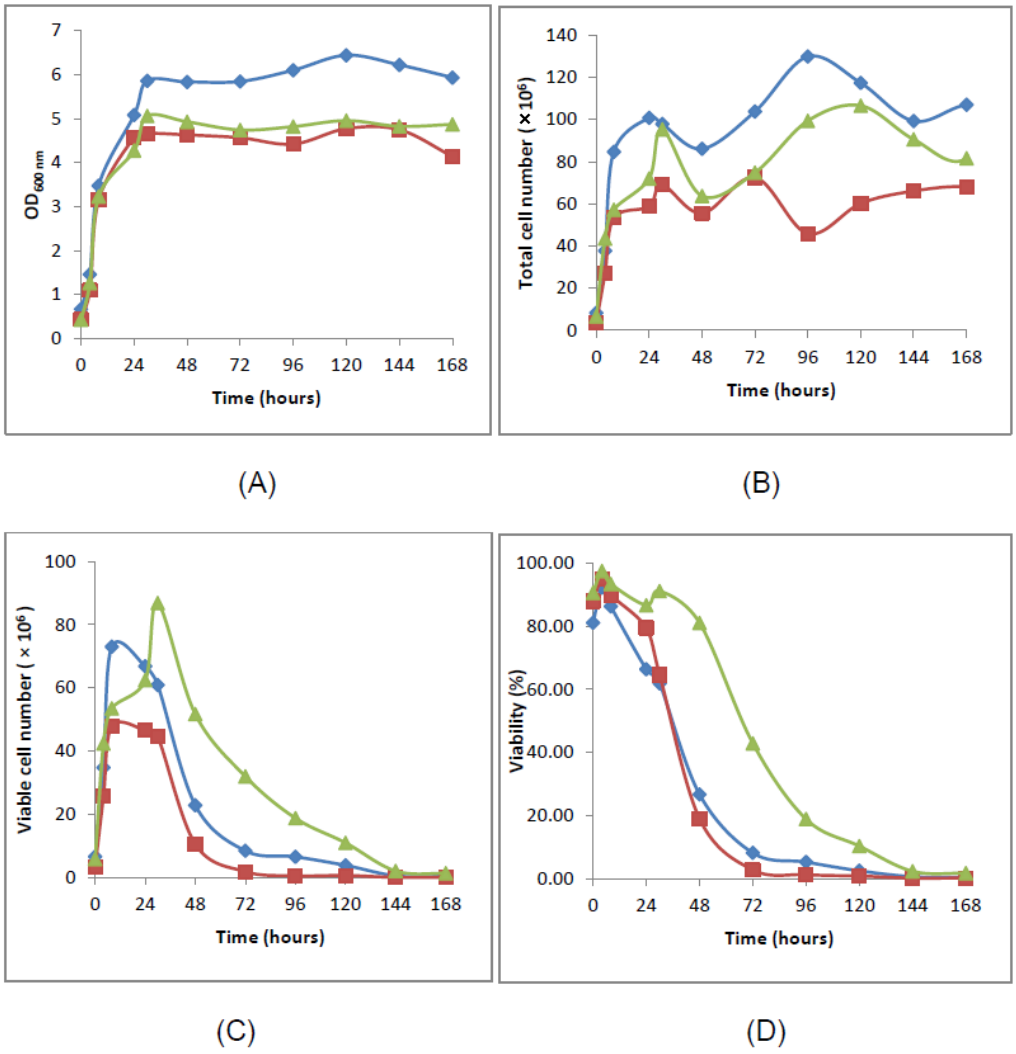


Figure 1. Growth parameters of PDM (♦), A12 (■) and A14 (▲) yeast strains grown on YNB media with 16% sucrose. (A) OD_{600nm} (B) total cell number (C) viable cell number (D) cell viability.

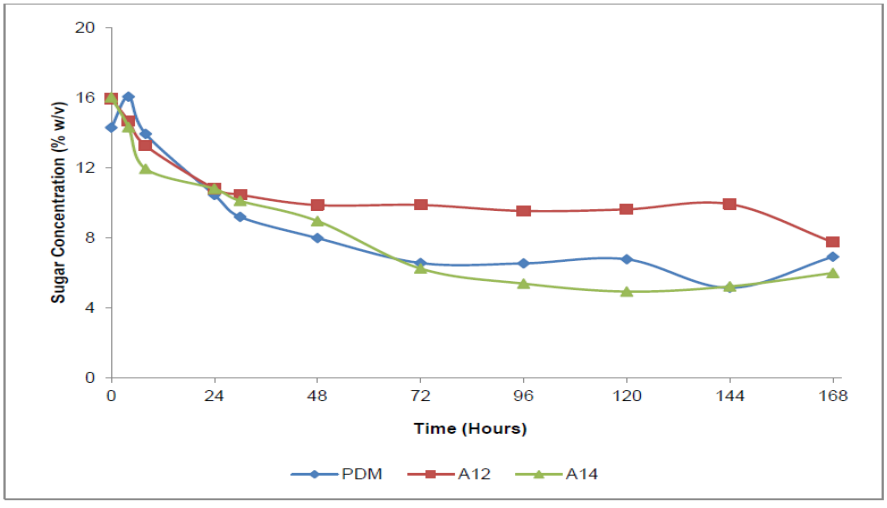


Figure 2. Sugar utilization by the three different yeast strains. Cultures were grown in YNB medium with 16% (w/v) glucose as carbon source under aerobic conditions at 30°C.

The highest cell numbers were achieved for yeast grown in YEP with glucose as the carbon source as can be seen in Figure 3 (A) and (B). However, in maintaining the cell viability, sucrose tended to give a better result as can be seen in Figure 3 (C) and (D) only due to secondary ferments at later time points. In general, viability decreased faster with sucrose compared to glucose in YEP-based media. The higher nutrition in YEP appeared to promote cell growth as evidenced by higher OD and total cell numbers.

In the last part of the fermentation process it can be seen that viable cell numbers were increased after 72 hours for yeasts grown in YEP medium either with sucrose or glucose as the carbon source (Figure 3(C)). However, the viable cell number dropped again after 120 and 144 hours for YEP cultures with sucrose and glucose, respectively. Cells grown in YEP also showed better viability at the end of fermentation. This result indicates that YEP is better for maintaining viability of the yeast cells compared to YNB.

When the performance of cultures grown in media with only 2% sugar were analysed, a different pattern was observed. Yeast grown in YNB tended to have higher viability (Figure 3(D)). Addition of ammonium sulphate to the YNB, seemed to marginally change the growth pattern, however YNB alone is sufficient to sustain complete fermentation of 2% glucose.

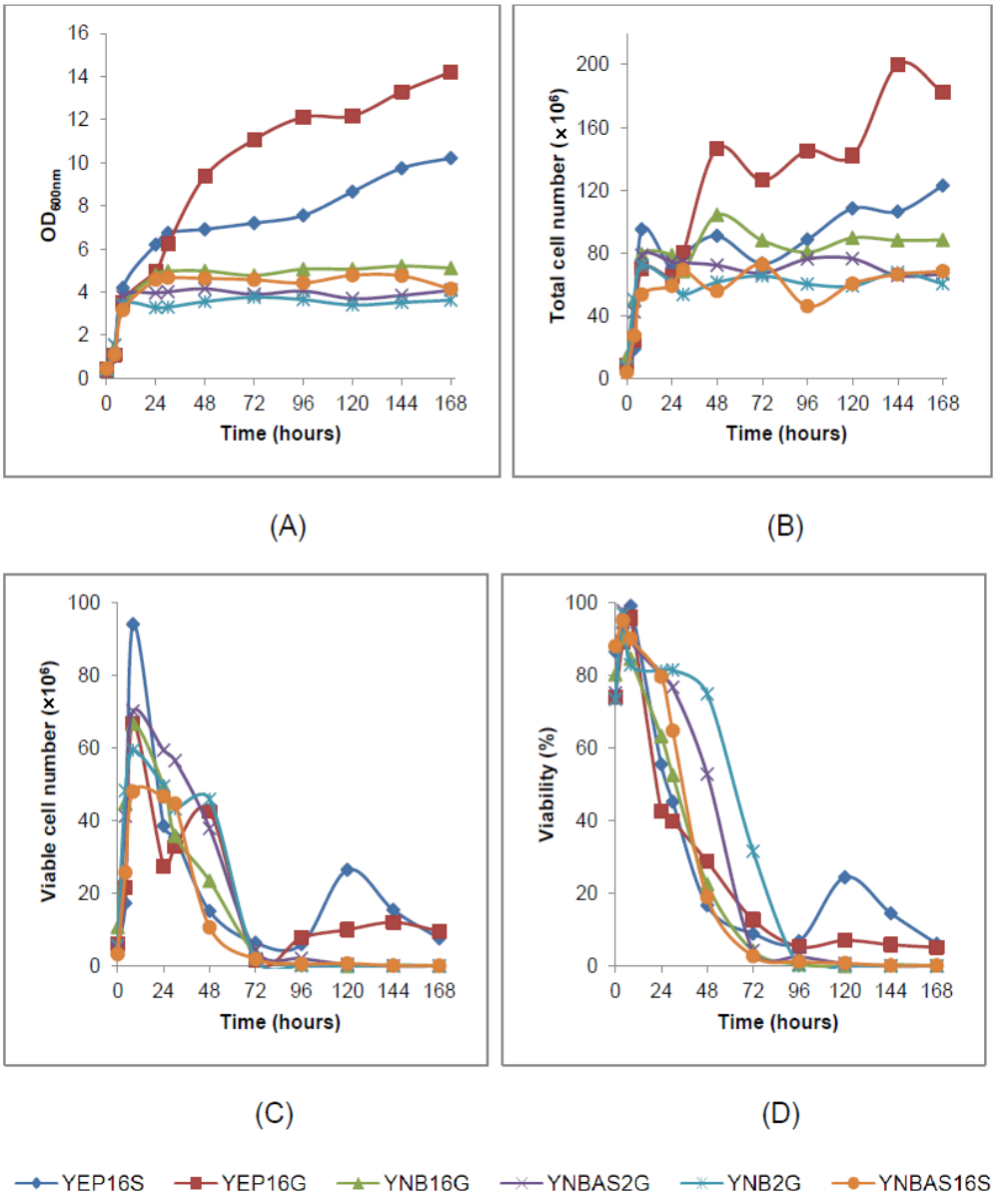
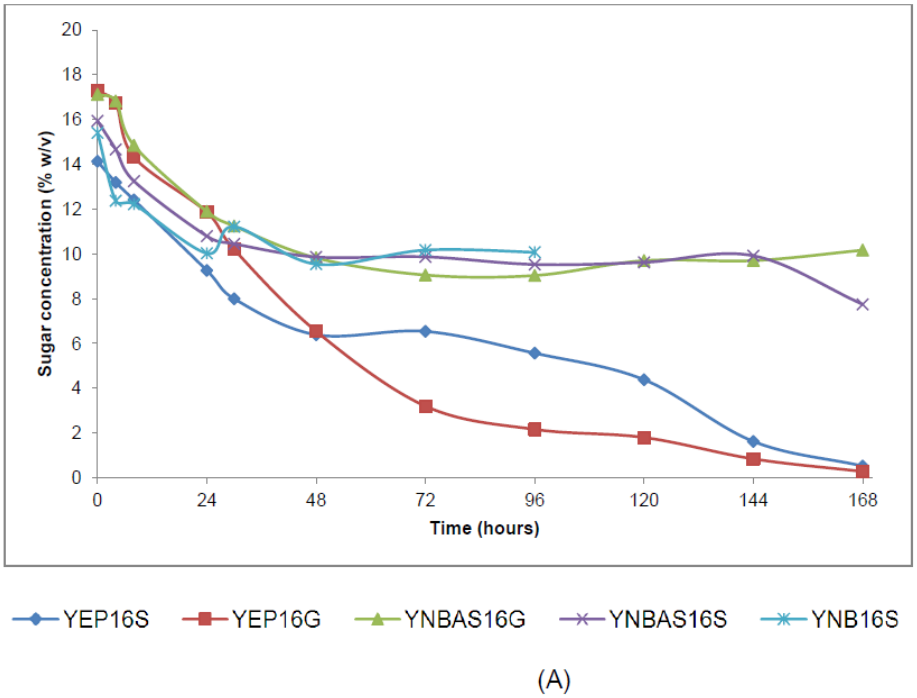
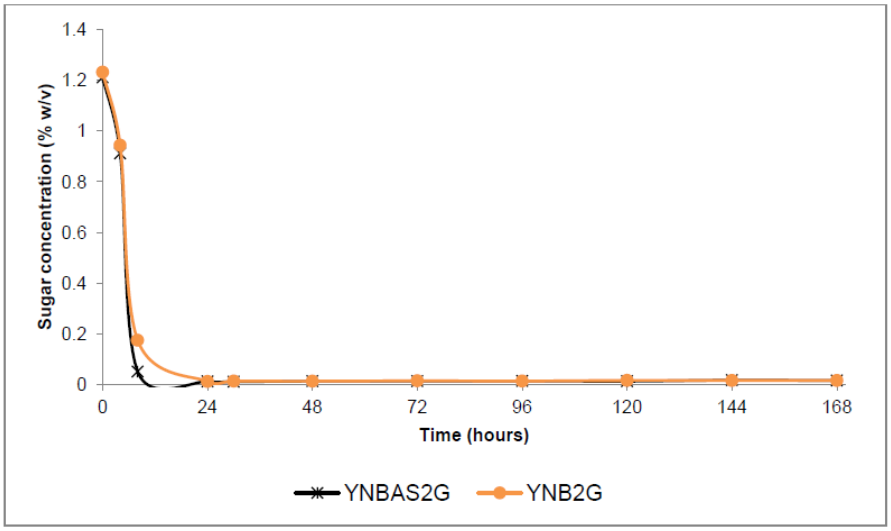


Figure 3. Growth parameters of the A12 yeast strain grown on different media as indicated. Cultures were grown under aerobic conditions at 30°C (A) OD_{600nm}, (B) total cell number, (C) viable cell number, (D) cell viability.



(A)



(B)

Figure 4 Sugar utilization of A12 strain grown in different media. Cultures were grown in different media as indicated on figure legend. Cultures were grown under aerobic conditions at 30°C. Cells grown in YNB16S were only monitored for up to 96 h, as this experiment was performed at the initial stage of experimentation, and as a result it was decided to follow cultures for a longer time in subsequent experiments.

Sugar utilization by the A12 yeast strain in different media

Sugar was rapidly assimilated during the first 24 hours of fermentation (Figure 4 A) in all media (either YEP or YNB-based media, and either sucrose or glucose as sugar source). After 24 hours, yeast grown in YNB-based media seemed to stop utilizing sugar, as indicated by relatively constant sugar levels throughout the remainder of the experiment. Supplementation of YNB containing 16% glucose with 1.5% ammonium sulphate did not ameliorate the stuck fermentation, indicating that something additional to nitrogen nutrition was problematic. However, yeast grown in the nutritionally rich YEP media still assimilated the sugars up to 168 hours of fermentation and therefore the sugar was almost completely utilized, with only 0.5% (w/v) sucrose or

0.3% (w/v) glucose remaining at 168 hours. In contrast to the results with 16% sugar, in Figure 4 (B) it can be seen that YNB, with or without added 1% ammonium sulphate, was nutritionally sufficient to sustain complete utilization of 2% glucose.

The findings in this study indicate that for high sugar fermentation, the nutritionally rich but relatively undefined medium YEP is preferred over the defined medium YNB, as YEP can promote high sugar utilization and therefore increase fermentation performance. Furthermore, YNB cannot be used as a medium for fermentation of high levels of sugar, therefore alternative strategies will need to be developed to assess membrane fluidity under these circumstances.

Conclusions

The present study confirmed that nutrient availability is very important for fermentation performance. Comparison of YNB and YEP media indicates that nutritionally insufficient media are not suitable for high sugar fermentation. When fermentation was performed in YEP media, rapid and complete sugar utilization was observed. Therefore, we recommend a nutritionally rich medium such as YEP for high sugar fermentations.

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