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Supplement article

Parametric optimization of xylitol production from xylose by fermentation

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ABSTRACT: Environmental parameters play a major role on the optimum production of bio-chemicals by bio-chemical pathways of a micro-organism. The effect of different environmental parameters like initial pH, temperature and initial xylose concentration were found for xylitol production using the micro-organism *candida parapsilosis* NCIM-3323, that belongs to the genus *candida* and species yeast. Maximum values of xylitol concentration, xylitol yield, xylitol volumetric productivity, xylitol specific productivity were found to be 28.14 g/l, 0.469 g/g xylose, 0.7 g/l-h, 0.0116 g/g-h at an initial pH of 3.5, temperature of 30 °C and initial xylose concentration of 60 g/l respectively. Optimum initial pH, temperature and initial xylose concentration for highest xylitol productivity were found to be 3.5, 30 °C, 60 g/l respectively. © 2012 Curtin University of Technology and John Wiley & Sons, Ltd.

KEYWORDS: fermentation; microorganism; parametric optimization; volumetric productivity; specific productivity; yield

INTRODUCTION

Xylitol is a natural substance found in fibrous vegetables and fruits as well as in corncobs. But, extraction of xylitol from the above materials is uneconomical due to its low concentration. It is a natural, intermediate product, which regularly occurs in the glucose metabolism of man, other animals as well as in the metabolism of several plants and microorganisms. Alternate methods of xylitol production are chemical and microbial reduction of D-xylose or hydrolysates of xylan rich hemicellulose materials. Certain moulds, yeasts and bacteria are capable of reducing xylose to xylitol as a first step in metabolism and yeasts are considered to be more efficient producers than bacteria and fungi. The best productivities have been so far achieved using yeasts belonging to the genus *candida*.[1,2]

Xylitol can be chemically characterized as a five-carbon sugar alcohol, which has many properties similar as that of sucrose.[3] It readily dissolves in water and is as sweet as sucrose. It is anti-microbial and prevents the growth of bacteria. Sugar is acid forming, xylitol is alkaline enhancing. It finds application in pharmaceutical, health care and food industries. It can be used as a sugar substitute for diabetic patients, has anti-cariogenic properties[4] (prevents tooth decay) and has skin smoothing properties. It is used as a sweetener in food products such as chewing gum, candy, soft drinks and personal health products like mouth wash and tooth paste. Adhesive property of xylitol has been reported adequate to replace phenolic resin for plywood bonding.[5,6]

Microbial production of xylitol is an alternative to its chemical production. Microbial processes can be carried out at mild conditions of temperature and pressure and hence require low energy. Bioconversion is highly specific, thus resulting in high yields, low purification costs and cleaner effluents.

Xylitol is produced from D-xylose as a metabolic intermediate in many xylose utilizing micro-organisms by converting D-xylose directly to xylitol by Nicotinamide Adenine Dinucleotide Phosphate (NADPH) dependent xylose reductase. Several bacteria, filamentous fungi and yeasts are known to convert xylose to xylitol. Among these yeasts, especially genera *candida* is considered to be the best xylitol producers. The conversion of D-xylose to xylitol by microorganisms is important for industrial production and has been studied extensively with yeasts.

The first three enzymes in xylose metabolizing pathway in yeasts are D-xylose reductase (XR), xylitol dehydrogenase (XDH) and xylulokinase (XK)[7](lachke). Inside the yeast cell, D-xylose is reduced to xylitol by either NADH or NADPH dependent XR. Xylitol is either secreted from the cell or oxidized to xylulose by NAD or NADP dependent XDH which is phosphorylated to xylulose 5-phosphate and then channeled through the pentose phosphate pathway and then be converted to ethanol via glycolysis or further metabolized via the tricarboxylic acid cycle and respiratory pathway.[8] Under anaerobic conditions or at very low oxygen transfer rates, Nicotinamide Adenine Dinucleotide (NAD) linked xylitol dehydrogenase is considerably inhibited, thus leading to xylitol accumulation rather than the conversion of D-xylose to xylulose.[9]

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Xylose concentration was found to be critical for yeast growth and fermentation. In the absence of D-xylose, xylitol formation does not occur. Together with aeration, D-xylose concentration affects xylitol formation the most. D-xylose is required for the induction of xylose reductase and xylitol dehydrogenase activities in yeasts. High xylose concentration induces xylitol formation in yeasts.

The most suitable temperature for xylitol production in yeasts was shown to be 30 °C. Small temperature variations above this temperature, do not significantly affect xylitol production in *candida tropicalis DSM* 7524. The xylitol yield was temperature independent when the yeast was cultured in the temperature range 30 – 37 °C but above 37 °C the xylitol yield decreased sharply.[10] The xylitol formation in *candida guilliermondii FTI* 20037 was same at both 30 and 35 °C, but decreased when the temperature increased to 40 °C.[1] At temperatures of 45 °C and above, the conversion was sharply reduced. This is due to loss of activities of both NADPH and NADH – dependent xylose reductase as the temperature is increased.

Investigating the effect of temperature on production of ethanol and xylitol, Du Preez *et al*.[11] found that at higher temperatures, production of xylitol is favored over that of ethanol. xylitol production by *candida. shehatae CSIR*-Y492 increased eight fold as the temperature increased from 22 to 36 °C.

If uncontrolled, pH drops during the fermentation and hence under such conditions the initial pH values have to be higher than under controlled conditions. The optimum initial pH value for best xylitol yield in *Candida boidinii* was 7,[12] whereas under controlled conditions a pH of 5.5 was better.[9] The yeasts are generally cultivated at pH values between 4 and 6 *candida guilliermondii* NRC 5578[13] were grown at 6, *candida. mogii* ATCC 18364[12] and *pichia stipitis* NRRL Y-7124 at pH 5 and 5.5 respectively, while pH of 4 was optimum for *candida tropicalis* IFO 0618.[14] In contrast Da Silva and Afschar[10] reported that *candida tropicalis* DSM 7524 was not very sensitive to pH and attained a maximum xylitol yield at pH of 2.5. Increasing the pH from 2.5 to 4 led to an increase in xylitol productivity but a decrease in xylitol yield.

The objective of the study is to find the optimal environmental parameters for higher production of xylitol by fermenting D-xylose with *candida parapsilosis NCIM-3323*. The study has practical importance as the process involves microbial production, which requires less separation cost, and leads to more purified products and also due to the fact that xylitol has several applications as cited above. Also the available literature on xylitol production is mostly with reference to organisms other than *candida parapsilosis*, while the work using *candida parapsilosis* is very less. It is not very well studied with this microorganism, especially at low volumes. Hence the present work is concentrated on detailed study of xylitol production varying different parameters using *candida parapsilosis*.

MATERIALS AND METHODS

Culture preparation

Candida parapsilosis NCIM-3323 obtained from National Chemical Laboratory, Pune, India, on agar slants, was preserved in a refrigerator at a temperature of 4 °C by periodic sub culture on agar slants.

Preparation of stock cultures

The yeast was sub cultured once in a month by preparing slants using agar. The following chemicals are required per 100 ml of distilled water, for slant preparation. Chemicals required for slants preparation are malt extract 0.3 g/l, glucose 1.0 g/l, yeast extract 0.3 g/l, peptone 0.5 g/l, agar 2 g/l. All the chemicals were mixed and sterilized in an autoclave for 15 minutes at a temperature of 120 °C and a pressure of 15 psi. The medium for slant preparation was poured up to one-third of the test tube, which was already sterilized and kept at an angle of 30 ^{o} and cooled to solidify the medium. By doing so, more surface area of the nutrients will be available for the growth of microorganism. After the slants have reached room temperature, they were exposed to ultraviolet (UV) light with an Alternating current (AC) supply of 250 volts in a laminar hood using a UV lamp for 40 minutes. UV light exposure will enable the total destruction of any other microorganism, which is undesirable. Then the slants were inoculated with the strain of yeast. The slants were incubated at 30 °C for a period of 2 days. After the colonies have developed the slants were stored at 4 °C.

Inoculum preparation

Inoculum for fermentation was prepared from the prepared slants. Medium composition for inoculum is D. xylose 10 g/l, yeast extract 2 g/l, $MgSO_4.7H_2O$ 0.4 g/l, KH_2PO_4 5 g/l, $(NH_4)_2SO_4$ 2g/l. To prevent the reaction with xylose, $(NH_4)_2SO_4$ was sterilized separately in an autoclave for 15 minutes at 15 psi and 120 °C to kill the undesirable micro-organisms. It was exposed to UV light with an AC supply of 250 volts in a laminar hood using a UV lamp for 40 minutes to kill the remaining undesirable microorganisms. A Loopful of organisms are used to inoculate the medium. Microorganism was grown in 100 ml of the medium in a 250 ml erlenmeyer flask at 100 RPM in an incubator shaker for 24 h at 30 °C.

Fermentation conditions

The batch fermentation was performed in 500 ml erlenmeyer flask in an incubator shaker at 100 RPM for 5 days by transferring 10 ml of inoculum into the

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fermentation medium. The medium for fermentation was prepared by taking medium composition as mentioned above except xylose concentration. Batch fermentation studies were performed by varying the temperature, initial xylose concentration (carbon source) and initial pH of the medium.

Methods of analysis

Dry cell weight was estimated using 0.22-micron filter paper by taking the difference of initial and final weights of filter paper. The residual concentration of xylose was estimated using Miller's reducing sugar method[15], taking 1 ml of filtrate from fermentation broth and measuring the absorbance of the sample at 540 nm wave length using UV-visible spectrophotometer. Xylose concentration of the sample was found from the standard graph prepared. Xylitol concentrations were estimated according to the method given by European pharmacopea supplement 2000 (p.1237). 0.5 M iodine solution is used for titration to estimate the amount of xylitol in the product. The method was validated by using standard solutions of xylitol

PROCEDURE

Work was carried out to find the most favorable conditions for the maximum productivity of xylitol. Initial pH was varied from 3 to 6. Temperature was varied from 25 to 35 °C. Initial xylose concentration was varied from 50 to 100 g/l. The culture medium was sterilized in an autoclave at 120 °C and 15 psi pressure for 15 minutes, subsequently UV sterilized in a laminar hood using a UV lamp with an AC supply of 250 volts for 40 minutes. All experiments were carried out by submerged fermentation in erlenmeyer flasks, in an orbital incubator shaker provided with temperature and revolutions per minute (RPM) control. RPM of the incubator shaker was maintained at 100. Two replicates of the samples were taken for all the experiments and the mean values are repoted.

RESULTS AND DISCUSSION

All biological processes are very sensitive to reactor operating conditions. The rate of reaction changes significantly with change in any of these parameters. Hence it is very much required to optimize the parameters of biological processes.

In the present study, the effect of initial pH, temperature and the initial xylose concentration on xylitol production was studied in detail to find out the favorable conditions for maximum xylitol production by the microorganism. In order to optimize the parameters, several experiments were performed by changing the parameters and the favorable conditions are reported.

Effect of initial medium pH

The pH of fermentation medium effects both microbial growth rate and its activity. Hence xylitol production is effected by the pH of the medium. Optimum value of pH for growth may be different from that of product formation.

The initial medium pH for xylitol production was studied using *candida parapsilosis* NCIM-3323 for fermentation. Experiments were conducted at different initial medium pH ranging from 3–6 with initial xylose concentration at 50 g/l at 30 °C with a batch volume of 350 ml.

From the experimental results obtained it is observed that, for different initial medium pH values studied, xylitol concentration raised gradually from zero to a maximum value. Afterwards it is observed that it remained constant. In the range of pH values studied, it is noticed that xylitol concentration was maximum at pH of 3.5 with an initial xylose concentration of 50 g/l at 30 °C. Xylitol concentration after reaching a maximum value remained more or less constant may be due to conversion of xylitol to other products in the metabolic pathway of the microorganism or due to depletion of essential nutrients for the microorganism growth and multiplication. Figs. 1 and 2 show the effect of pH on xylitol productivity and its yield. In the figures solid lines represent xylitol concentration, xylitol volumetric productivity and broken lines represent xylitol yield, xylitol specific productivity respectively.

Effect of temperature

Another factor affecting xylitol production is temperature. As per Arrhenius law, the reaction rate increases with increase in temperature, but since microorganisms

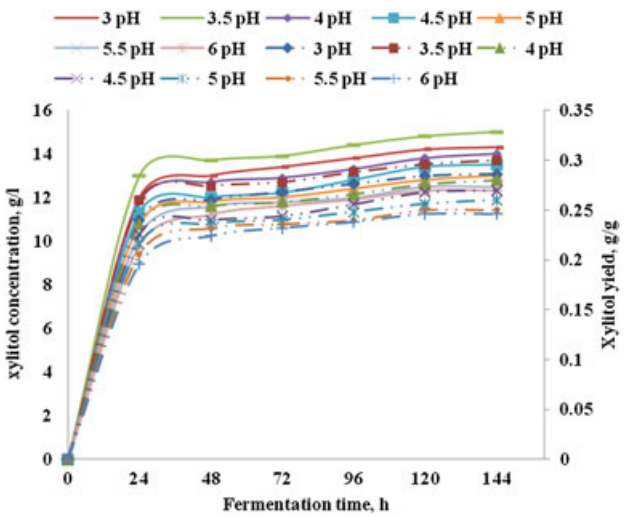

Figure 1. Effect of initial pH on xylitol concentration and yield.

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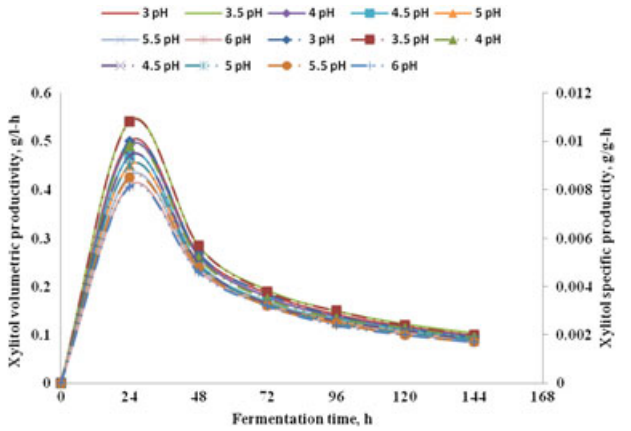

Figure 2. Effect of initial pH on xylitol volumetic and specific productivity.

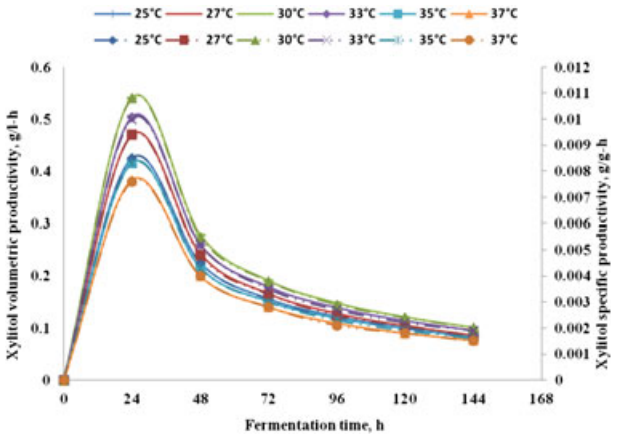

Figure 4. Effect of temperature on xylitol volumetic and specific productivity.

are sensitive to temperature, the activity of microorganisms changes with temperature. Hence the microbial production of xylitol changes with temperature. Optimization of temperature is necessary for maximum production of xylitol.

Experiments were performed in the temperature range 25-35°C with initial pH maintained at 3.5 and initial xylose concentration of 50 g/l with a batch volume of 350 ml. Figs. 3 and 4 indicate the effect of temperature on xylitol productivity and its yield. In the figures solid lines represent xylitol concentration, xylitol volumetric productivity and broken lines represent xylitol yield, xylitol specific productivity respectively. It is seen that the optimum temperature for xylitol production is 30°C.

Effect of initial xylose concentration on xylitol production

Xylose induces xylose reductase and xylitol hydrogenase activities in the yeast. Hence a study on effect of initial xylose concentration on xylitol production has been made. Initial xylose concentration of 60 g/l with initial medium pH of 3.5 and a temperature of 30 °C was found to be congenial for xylitol production in the range studied (50 – 100 g/l) with a batch volume of 350 ml. There is no substantial increase in xylitol production above this concentration. With increase in xylose concentration up to 60 g/l an increase in xylitol production was observed. It can be concluded that xylose concentration of 60 g/l is most suitable with respect to maximum xylitol production. The results of optimum values of xylitol productivity are shown in Figs. 5 and 6. In the figures solid lines represent xylitol concentration, xylitol volumetric productivity and broken lines represent xylitol yield, xylitol specific productivity respectively.

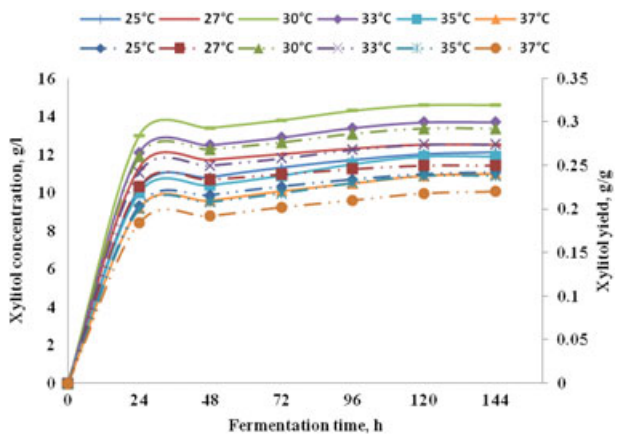

Figure 3. Effect of temperature on xylitol concentration and yield.

CONCLUSIONS

The microbial production of xylitol from D-xylose is influenced by fermentation conditions and stain used.

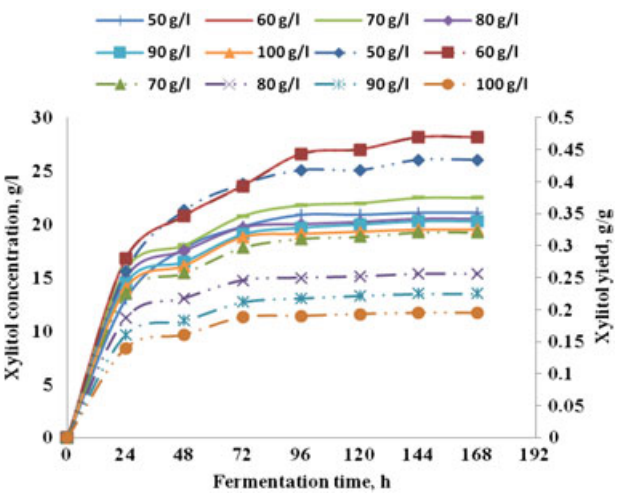

Figure 5. Effect of initial xylose concentration on xylitol concentration and yield.

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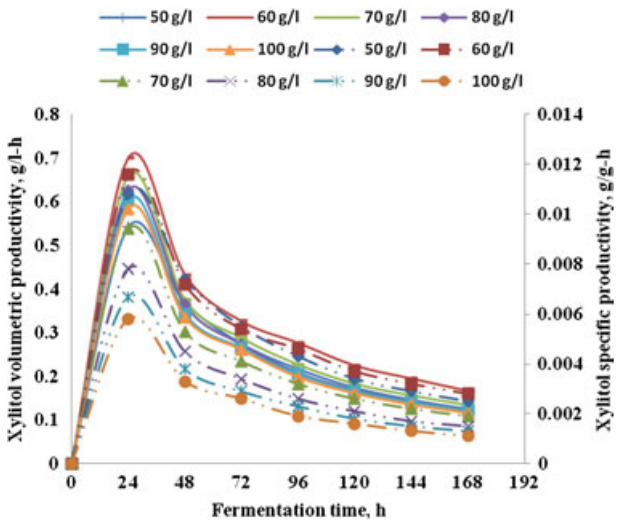

Figure 6. Effect of initial xylose concentration on xylitol volumetic and specific productivity.

The production of xylitol from D-xylose by *candida parapsilosis* NCIM-3323 was carried out by batch fermentation in 500 ml erlenmeyer flasks with 350 ml culture medium. An effort was made to find the optimum operating conditions for higher xylitol production by varying the parameters like medium pH, temperature and initial xylose concentration of medium. In the range of initial pH studied, it is observed that xylitol productivities increased up to an initial pH of 3.5. Above an initial pH of 3.5 a decrease in xylitol productivity is observed. In the range of temperatures studied, it is observed that xylitol productivities increased up to 30°C. Above 30 °C a decrease in xylitol productivities is noticed. In the range of initial xylose concentrations studied, an increase in xylitol productivities is observed up to an initial xylose concentration of 60 g/l. above an initial xylose concentration of 60g/l, a decrease in xylitol productivities is observed. The optimum values of initial pH, temperature and initial xylose concentration for maximum xylitol production are found to be 3.5, 30 °C and 60 g/l respectively. The maximum values of xylitol concentration, xylitol yield, xylitol volumetric productivity and xylitol specific productivity are found to be 28.14 g/l, 0.469 g/g xylose, 0.7 g/l-h and 0.0116 g/g-h respectively. The present study gives an insight to the performance characteristics of *candida parapsilosis* NCIM-3323 for the production of xylitol from D-xylose. The effect of parameters like initial concentration of nutrients, inoculum size, inoculum age, revolutions per minute of the incubator shaker on xylitol production can be studied.

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