BRAZILIAN JOURNAL OF MICROBIOLOGY

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Industrial Microbiology

Staphylococcus xylosus fermentation of pork fatty waste: raw material for biodiesel production

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ARTICLE INFO

Article history:
Received 8 October 2015
Accepted 17 February 2016
Available online 21 April 2016
Associate Editor: Solange Ines Mussatto

Keywords:
Sustainability
Microorganism
Staphylococcus xylosus
Waste recovery
Slaughterhouse waste

ABSTRACT

The need for cleaner sources of energy has stirred research into utilising alternate fuel sources with favourable emission and sustainability such as biodiesel. However, there are technical constraints that hinder the widespread use of some of the low cost raw materials such as pork fatty wastes. Currently available technology permits the use of lipolytic microorganisms to sustainably produce energy from fat sources; and several microorganisms and their metabolites are being investigated as potential energy sources. Thus, the aim of this study was to characterise the process of *Staphylococcus xylosus* mediated fermentation of pork fatty waste. We also wanted to explore the possibility of fermentation effecting a modification in the lipid carbon chain to reduce its melting point and thereby act directly on one of the main technical barriers to obtaining biodiesel from this abundant source of lipids. Pork fatty waste was obtained from slaughterhouses in southern Brazil during evisceration of the carcasses and the kidney casing of slaughtered animals was used as feedstock. Fermentation was performed in BHI broth with different concentrations of fatty waste and for different time periods which enabled evaluation of the effect of fermentation time on the melting point of swine fat. The lowest melting point was observed around 46 °C, indicating that these chemical and biological reactions can occur under milder conditions, and that such pre-treatment may further facilitate production of biodiesel from fatty animal waste.

Introduction

Contemporary society relies on the use of various forms of energy to meet and achieve the best quality of life; and the products and processes being used to that end essentially rely on an uninterrupted supply of energy which also needs to be of high quality, be competitively priced and leave the least possible impact on the environment. The global energy matrix largely depends on the use of fossil fuels and is thus

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http://dx.doi.org/10.1016/j.bjm.2016.04.018

non-renewable. This is worrisome as international agencies monitoring energy sources predict a 60% reduction in oil and coal supply within the next 15 years, which will in turn lead to a phenomenal rise in extraction, product and process costs.[1–3] This predicted decrease in energy resources, constitutes one of the greatest challenges of our society and has resulted in research into and improvement of alternate technologies that ensure energy sustainability. Biofuels are a pertinent example which while being obtained from renewable raw materials such as biomass, effluents and fatty waste, are also less polluting because their combustion results in much lower emission levels of carbon monoxide, sulphur compounds, particulate matter and aromatic hydrocarbons.[4–8] Therefore, the development of biofuels from agro-industrial waste constitutes an important alternative as these can be further transformed into second-generation biofuels that do not compete with agriculture for resources, have negligible cost compared to vegetable sources and are plentiful in countries with agricultural potency.[9–13] Technological problems continue to prevent the economic, attractive and sustainable use of pork fatty waste for the production of biodiesel, one of them is the high cold filter plugging point, which precludes its use in vehicles operating in regions with temperatures below 15 °C due to crystal formation, leading to low or none viscosity.[14–16] The high melting point of pork fat (>70 °C) demands significant energy consumption to liquefy this waste, which contributes to an increase in the final price of biodiesel from such sources. Thus, even though the raw material has a lower cost compared to vegetable oils, improvements in the technology to transform pork fatty waste into biodiesel are required to make this an economically competitive fuel source.[17]

Biofuel production now increasingly relies on the use of microorganisms, either for the direct or for the assisted use of lipids as biofuels.[18–22] Therefore, a process that uses lipolytic microorganisms for both treatment and recovery of animal fatty waste show promise as a workable strategy that uses agro-industrial waste for biodiesel production with desirable quality characteristics and under economically feasible conditions.[23–26] *Staphylococcus xylosus*, a Gram-positive coccus, is a microorganism that fits these requirements as it has GRAS certification (Generally Recognised As Safe), known to have proteolytic and lipolytic activity, and regularly used in the food industry especially for fermentation of meat products as well as for the production and intensification of flavour, colour stabilisation and peroxide decomposition.[27–29] Importantly, apart from these *in vivo* applications, *S. xylosus* and other bacteria from the same genus also secrete exoenzymes that catalyse lipid-based reactions.[22,30–35] A useful example of such secreted enzymes is the serine acetyltransferase (E.C.3.1.1.3), which is capable of catalysing alcoholysis, esterification, transesterification and hydrolysis, among others.[36,37] Additionally, the ease with which *S. xylosus* can be isolated and cultured makes it a potential enhancement agent in the use of pork fatty wastes for biodiesel production.[38]

Given these favourable characteristics, the main aim of this study was to investigate the process characteristics of pork fatty waste fermentation by *S. xylosus*. We also wanted to explore the possibility of this fermentation effecting a change in lipid carbon chains to reduce its melting point and thus act directly on one of the main technical barriers to obtaining biodiesel from this abundant source of lipids.

Material and methods

Collection of samples

The fatty waste was collected from freshly slaughtered animals prior to the washing of the carcasses from a slaughterhouse in southern Brazil. In order to collect the fat with the least blood contamination as possible, the fatty kidney sheaths of 25 carcasses, each weighing approximately 0.6 kg were removed, collected into sterile bags, transported on ice, and immediately subjected to fermentation. All analyses were performed in triplicate.

Bacterial cultures and growth

S. xylosus NRRL B-14776 was cultured in Brain-Hearth Infusion broth (BHI – Acumedia – Neogen Corporation® 7116A, Brazil) as previously described by Mauriello et al.[27] until required initial cell mass concentration (8.0 log CFU mL^{-1}), after which a loop of bacterium was transferred to the BHI broth and incubated at 35 ± 0.1 °C overnight.

Pork fatty waste fermentation

The experiment followed a fully randomised two-factorial design with three repetitions wherein the two factors were fermentation time (1.5, 3.0, 4.5, 6.0 and 7.5 h) and fat concentration of the BHI broth (2, 4, 6, 8, 10, 50, 60, 70, 80 and 90%).

The fermentation was carried out under semi-solid-state conditions and consisted of pork fatty waste as the water insoluble component that provided nutrients (primarily carbon) and anchorage for microorganisms, apart from BHI broth and the inoculum (1%, v/v). The fatty waste was melted, dried, filtered (filter paper Whatman 12.5 cm), ground and sieved through a 0.5 mm sieve (32 mesh). Next, the fat was sterilised by membrane filtration and aseptically weighed in sterile plastic bags. After the addition of the required volume of BHI broth with the necessary initial cell concentration of both the strains of *S. xylosus*, the bags were placed in a "Stomacher" homogeniser (SP Labour – model SP-190-type) for 2 min and then transferred to sterile Erlenmeyer flasks; the total fermentation volume was 200 mL. The flasks were kept in a shaker incubator (35 ± 0.1° C, buffer pH 7.0, 100 rpm) for initiation of fermentation, and for maintenance of aerobic conditions in the reactor. Dissolved oxygen was measured with a portable oximeter (LUTRON DO-5519) and dissolved oxygen saturation was maintained at 100% throughout fermentation. The choice of growth conditions and culture medium used were based on previously reported optimal growth conditions for *S. xylosus*.[27] Fermentation was interrupted at the mentioned target analytical points, which corresponded to ten different concentrations of testing; and samples of approximately 1 g were aseptically collected from each Erlenmeyer flask and subjected to melting point analysis.

Melting point analysis

Test tubes containing approximately 1 g of sample were partially immersed in a volumetric flask with water and kept on a heating mantle that slowly increased the temperature from 30 °C to 80 °C.[39] This method detects three temperatures that characterise the melting points of three different fatty acids as described by Douare et al.[40] As Knothe and Dunn[41] have reported that initial and final melting temperatures are affected by the quantity of the sample being analysed, only core temperature of the sample was considered. Melting point of the non-fermented fatty waste was determined according to AOCS methodology.[39]

Statistical analysis

The normality of data was tested using Shapiro-Wilk's test, homoscedasticity was tested with the Hartley test, and residue independence was tested using graphical analysis. After ensuring that the assumptions were met, analysis of variance (ANOVA) was performed using *F* test on values with $p < 0.05$. Experimental data were analysed using response surface methodology to fit a second-order polynomial equation that was generated using regression. The response was initially fit to factors using multiple regression and the initial model selection was based on: (a) low residuals; (b) low *p*-value; (c) low standard deviation; and (d) high R^2. A second order polynomial equation was then fitted to the response data (Eq. (1)).

$$y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} \cdot x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

where y is the response factor (fatty waste melting point), x_i is the first independent factor (fermentation time), x_j is the second independent factor (fat concentration), β_0 is the intercept, β_i and β_j are the first-order model coefficients, β_{ii} and β_{jj} are the quadratic coefficients for the factors *i* and *j* and β_{ij} is the linear model coefficient of the interaction between factors *i* and *j*. All data were analysed using The R Project software (version 3.1.0, *The R Foundation*®). Statistical parameters showing a *p-value* lower than 0.05 were considered significant.

Results

Melting point

The estimated values for the various factors are presented in Table 1, and the *p*-values indicated that most of the coefficients were significant and that error in model design was minimised when all of these coefficients were considered together. Additionally, the final estimated response of the model adequately represented real values and the estimated values of these factors.

The final response model equation was derived (Eq. (2)) and the response surface for this equation (Fig. 1) shows that the melting point tends to decrease when intermediate fermentation times were reached and formed a cell point between four and five hours of the fermentation process. At this time-point, it also appears that the fat concentration of the medium significantly contributes (linearly) to the observed phenomena as the level curves at this point have a near null slope.

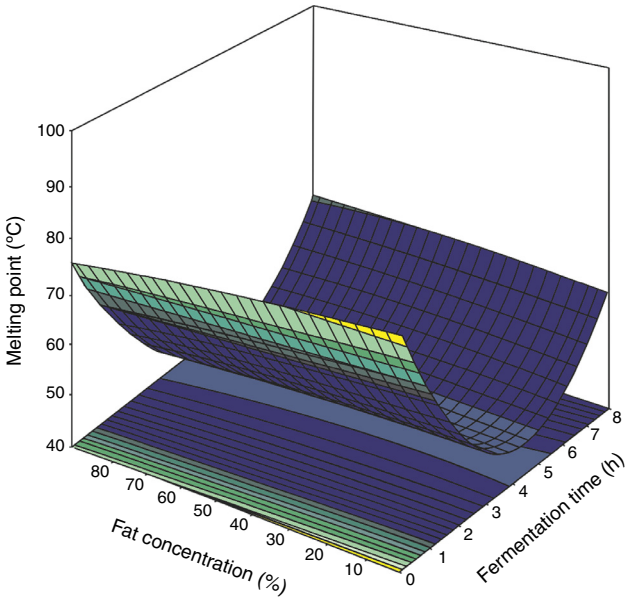

Fig. 1 – Response surface plot representing the effects of fat concentration, fermentation time and their reciprocal interaction on pork fatty waste melting point.

Table 1 – Estimative response model regression coefficients, standard error, t value and significance of the response surface model for the melting point behaviour of pork fatty waste obtained through *S. xylosus* fermentation.

Factor*	Parameter estimate	DF	Standard error	*F* value* *t* value**	$p > t$
General model	–	5	2.817	155.854*	<0.001
Intercept	81.873	5	1.283	63.836**	<0.001
X_1	−15.650	5	0.574	−27.279**	<0.001
X_2	−0.128	5	0.036	−3.607*	<0.001
X_1^2	1.648	5	0.061	26.978**	<0.001
X_2^2	0.0005	5	<0.0001	1.452**	0.149
X_1X_2	0.022	5	0.0032	6.680**	<0.001

X_1: fermentation time (h); X_2: fat concentration (%); $R^2 = 0.844$; R^2adj = 0.839.

$$Y = 81.873 - 15.650X_1 - 0.128X_2 + 1.648X_1^2 + 0.0005X_2^2 + 0.022X_1X_2 \quad (2)$$

Discussion

The response surface plot of the fermentation process shows that the initial estimated melting point was 81.87 °C ($X_1 = 0$; $X_2 = 0$), which significantly declined as fat concentration and fermentation time increased and the maximum decrease in estimated melting temperature ($Y = 44.8$ °C) occurred between 4 and 5 hours of fermentation and at 38–42% fat concentration. Further, during this period, the melting point appears to be influenced neither by fermentation time nor by fat concentration of the medium, indicating a stabilisation in bacterial activity which in turn led to the observed decrease in physicochemical parameters. Fat concentration of the medium did not individually influence metabolic activity as the melting point showed a non-significant variation over the range of fat concentration used in the experiments. However, Fig. 1 also shows that there is a possibility of an interaction between the two process variables (i.e., fat concentration and time), with fermentation time being the most prominent factor capable of effecting a modification in pork fatty waste. These process characteristics are especially promising because a relatively cheap feedstock was used for fermentation. We found that after reaching the lowest estimated melting point, the fat waste then exhibited an increase in melting point as both fermentation time and fat concentration increased; similar process characteristics have been previously reported.[42]

Zhou et al.[43] have reported that sudden changes in the operational conditions of industrial and laboratory processes involving microorganisms causes disturbances in their metabolism and physiological activity which in turn accelerate or halt microbial growth. This reflects a period of adaptation that microbes require to adjust DNA synthesis and RNA replication to suit the changes in culture medium and to resume their growth.

Accordingly, our data suggest that optimal operating conditions were achieved at fat concentrations between 55% and 62% and between 3.8 and 4.5 hours of fermentation, as these conditions reduced the melting point to approximately 45–47 °C. These inferences are based on the assumptions that the best process conditions are those that result in the greatest decrease in melting point, use the highest concentration of fatty waste possible and require the shortest fermentation time so as to increase the economic feasibility of the overall process.

Conclusion

Based on the results presented herein, it is possible to conclude that semi-solid fermentation of pork fatty wastes by *S. xylosus* causes a drop up to 36 °C in melting point under ideal process conditions. This implies that a liquid state fermentation process, which is closer to ambient temperature, may facilitate a reduction in reaction costs and also produce an increase in reaction yield. Further, process enhancements such as flow conditions during fermentation, would ensure that milder process conditions are sufficient to achieve efficiency. Therefore, pre-treatment of the fatty waste can help optimise the production of biodiesel from such agro-industrial wastes in a process that is both economically attractive and sustainable.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors thank the Coordination for the Improvement of Higher Education (CAPES – *from Portuguese*) for the financial support given to this research.

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