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ORIGINAL PAPER

Bioethanol production from rice bran with optimization of parameters by *Bacillus cereus* strain *McR*-3

'Fermentation of rice bran for fuel ethanol production'

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Abstract The potential environmental benefits that can be obtained from replacing petroleum fuels with biofuels derived from renewable biomass sources are the main driving forces for promoting the production and use of biofuels. Due to depletion of fossil fuels, ethanol, which can be obtained via the bioconversion of renewable feedstock, is widely regarded as an efficient alternative for gasoline as transportation fuel. Biomass energy can play an important role in reducing greenhouse gas emissions. Rice bran is a by-product of milling process of rice, and due to its carbohydrate contents, it may serve as good source for bioethanol production. The present study deals with bioethanol production from rice bran and screening of bioethanol-producing bacteria from rice bran. In the screening process, three fermentative bacteria were obtained; they were studied on the basis of morphology, biochemical characteristics and maximum bioethanol production. The maximum bioethanol-producing bacteria was identified by sequencing method. The bacteria thus identified as *Bacillus cereus* strain *McR*-3 is a novel bacteria reported in bioethanol production from rice bran substrate. Different parameters like temperature and pH also affects the production of bioethanol. It was observed that optimum temperature and pH for maximum bioethanol production was 37 °C and 5, respectively.

Keywords Biofuels · Energy · Fermentative bacteria · Parameters

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Introduction

Worldwide interest is increasing in alternative sources of energy due to inevitable depletion of energy supply (Aristidou and Penttila 2000). The increase in the prices of petroleum-based fuels, strict government regulations on exhaust emissions and future depletion of worldwide petroleum reserves encourages studies searching for alternative fuels (Harkin 2000; Howard 1994). The increasing concerns about environmental protection have led to the use of bioethanol as sole fuel, or a blend with gasoline (Tofighi et al. 2010). Biomass energy can play an important role in reducing greenhouse gas emissions because air pollution, especially in mega cities, figures prominently among the main environmental causes which affect human health (Pour et al. 2007). Environmental concerns and the desire to be less dependent on imported fossil fuel have intensified worldwide efforts for production of ethanol from starch- and sugar-producing crops (Kataria and Ghosh 2011). The production of ethanol has two routes: synthetic and biological. The synthetic ethanol production is commonly carried out by a catalytic hydration of ethylene. Biological production of ethanol is from biomass with the involvement of living microorganisms. Alcoholic fermentation has been carried out using a number of sugary materials depending upon their availability and suitability in particular geographic situations. The increasing need for bioethanol as an energy source has stimulated worldwide investigations in search of cheaper substrate for bulk ethanol production. The primary challenge with biofuels use is the availability of suitable feedstock in sufficient quantity for large-scale adoption. As an oxygenated compound, ethanol provides additional oxygen in combustion and hence obtains better combustion efficiency. The main environmental advantages of fuel ethanol are its

sustainability in using a renewable resource as a feedstock, thus promoting independence of fossil fuel and maintaining the level of greenhouse gas (CO_2). Bioethanol, an already well-established fuel mainly in Brazil and the USA, is usually obtained by alcoholic fermentation from starch (cereal grains, such as corn or wheat), sugar (sweet sorghum, sugar cane and sugar beet) and lignocellulosic feedstocks (Miranda et al. 2012). In an effort to combat climate change, to aid energy independence and to counteract diminishing supplies of fossil fuels, there has been a resurgence of research on renewable and carbon–neutral energy sources. Biofuels production captures the energy of the sun as chemical energy in the bonds of biologically produced materials. There are some routes to convert renewable resources into energy-rich, fuel-like molecules or fuel precursors: first, direct production by photosynthetic organisms, such as plants and algae; second, fermentative or nonfermentative production by heterotrophic microorganisms, such as bacteria, yeast or fungi; and third, chemical conversion of biomass to fuels (Rude and Schirmer 2009). Traditionally, the yeast *Saccharomyces cerevisiae* has been used all over the world as the major ethanol-fermenting microorganism. The larger size, thicker cell wall, better growth at low pH, less stringent nutritional requirement and greater resistance to contamination give yeast advantages over bacteria for commercial fermentation (Jeffries 2006; Narendranath et al. 2000). Among bacteria, the most promising microorganism is *Zymomonas mobilis* which has a low energy efficiency resulting in a higher ethanol yield (Sanchez and Cardona 2008).

Numerous substrates are used for bioethanol production worldwide. Recently, research on the production of ethanol from waste has been accelerating for both ecological and economical reasons. Le man et al. (2010) used full-factorial central composite design that was employed to optimize the parameters of ethanol production from Korean food waste leachate. Tiwari et al. (2010) studied the effect of temperature variation in the bioethanol-production process. Tiwari et al. (2011) used some cereals as barley, maize, oat and sugar beet for bioethanol production. Tiwari et al. (2012) worked on Jatropha oil cake for production of fuel ethanol and studied optimum incubation period for bioethanol production. Pandey et al. (2013) used *azolla* as a source of bioethanol. Beliya et al. (2013) used deoiled rice bran for bioethanol production. Tiwari et al. (2014) produced fuel ethanol from waste fruits. Substrate that is neither a feedstock nor is of economic importance is good choice for bioethanol production. Currently, the second-generation bio-products such as bioethanol, biodiesel, biohydrogen and methane from lignocelluloses biomass are increasingly been produced from wastes rather than from energy crops. Chhattisgarh is known as "Bowl of rice" due to its huge production of rice. The rice bran refers to the coating removed from rice during the process of milling (Gupta 1989). Rice bran is a by-product of rice milling obtained during polishing of rice. It is the part between a paddy husk and endosperm. The contents of rice bran are as follows: 12–25 % fat, 10–16 % protein, 10–20 % starch, 3–8 % reducing sugar, 8–11 % hemicelluloses, 10–12 % cellulose, 6–15 % crude fiber and 6.5–10 % ash content (Sharma et al. 2004). The bran contains a significant amount of sugars such as residual starch, cellulose and hemicelluloses, which could be converted to ethanol and enhance efficiency of production (Beaugrand et al. 2004).

This work is part of Ph.D. thesis and was carried out at School of Studies in Biotechnology, Pt. Ravishankar Shukla University, Raipur (Chhattisgarh), India, between 2010 and 2013. The aim of this study was screening of bioethanol producing bacteria from rice bran substrate and morphological and biochemical characterization of isolated bacteria. Identification and molecular characterization of maximum bioethanol-producing bacteria was also an aspect of this study. This investigation also deals with optimization of important parameters, temperature and pH, on bioethanol production from rice bran.

Materials and methods

The present study deals with the bioethanol production from rice bran (waste rice material), screening of bioethanol-producing bacteria and characterization of selected bacterial species on the basis of morphological, biochemical and molecular characters. Materials and methods used in this study were as follows:

Collection of substrate

Rice bran was chosen as a raw material in this work because it is the most widely grown cereal in Chhattisgarh State, India. Rice bran was collected from Sita rice mill, Raipur, Chhattisgarh State, India. Rice is a major food commodity throughout the world. India is the second largest producer of rice in the world and Chhattisgarh State is largest contributor of rice production in India. Chhattisgarh is known as "Bowl of rice" due to huge production of rice. The total rice productivity in Chhattisgarh was 1,257 kg/ha in 2008, 1,201 kg/ha in 2009 and 1,751 kg/ha in 2010. Since rice bran does not compete with food market, easy availability and low cost, it is economically good choice of substrate in Chhattisgarh for bioethanol production. Rice bran is waste product of rice after milling process. Rice bran contains high carbohydrate; hence, it was found quite appropriate for the application of fermentation technology in order to produce bioethanol.

Pretreatment of biomass

Pretreatment plays a key role in the overall efficiency of the hydrolysis and fermentation steps since the purpose of pretreatment is to remove structural and compositional impediments to hydrolysis. With the appropriate pretreatment technology, the enzymatic hydrolysis rate and the yield of fermentable sugars will significantly increase (Mosier et al. 2005). Biomass was not pretreated with different methods, but it was physically pretreated by steam explosion method by autoclaving process. Steam explosion seems the best suitable physical pretreatment of straw as it partially hydrolyzes hemicelluloses and increases its enzymatic digestibility in the biomass residue (Kristensen et al. 2008).

Isolation of bacteria from rice bran

Rice bran substrate was dipped in distilled water (20 g in 200 ml) for screening of bacteria and kept for 48 h at 37 °C in incubator. Then, the fermented sap was poured on plates containing nutrient agar medium (NAM). After 48 h of incubation period, different bacterial colonies were observed on nutrient agar plates. These bacterial colonies were pure cultured through the streak plate method in NAM.

Selection of fermentative bacteria

During screening process, on the basis of morphology seven different types of bacterial species were observed. They were characterized by their cultural and physiological characteristics. By fermentation test, it was confirmed whether they are fermentative bacteria or not. In this method, fermentation broth was prepared; composition of fermentation broth medium was the following: peptone 10 g, sodium chloride 15 g, carbohydrate 5 g, phenol red 0.018 g, distilled water 1,000 ml and pH 7.3 (Prescott 2002).

Microscopic study of bacteria

For microscopic study, Gram's staining, acid-fast staining, endospore staining and motility test were performed.

Biochemical study of bacteria (Prescott 2002)

For biochemical characterization of selected bacteria, IMViC test (indole test, methyl red test, Voges-Proskauer test, citrate utilization test), hydrogen sulfide production test, oxidation-fermentation test, fermentation of carbohydrate test, nitrate reduction test, amylase production test, cellulase production test, urease test and catalase test were performed.

Estimation of bioethanol

Qualitative estimation

Bioethanol production was examined by Jones reagent ($K_2Cr_2O_7 + H_2SO_4$; Jones 1953). One milliliter of $K_2Cr_2O_7$ (2 %), 5 ml of H_2SO_4 (concentrated) and 3 ml of sample were added to Jones reagent. Ethanol was oxidized into acetic acid with potassium dichromate in the presence of sulfuric acid and gave blue-green color. Green color indicates positive test (Caputi et al. 1968).

Quantitative estimation

Substrate solution was distilled in alcohol distillation unit for quantitative estimation of bioethanol. For the quantity estimation of bioethanol, specific gravity method was applied; the method was as follows:

Twenty-five milliliters of fermented sample was mixed with 150 ml of distilled water, and distillation was performed; 90 ml of distillate was collected, 100 ml of distilled water was added, and the resulting mixture was poured to 25-ml specific gravity bottle (Pharmacopoeia of India 1985). The percent of ethanol was calculated using following formula:

$$\rho^{t0} = \frac{W3 - W1}{W2 - W1} \times \text{Density of water at t°C}$$

where ρ^{t0} = specific gravity, W1 = weight of empty specific gravity bottle, W2 = weight of empty bottle + distilled water, W3 = weight of empty bottle + fermented liquid (Yadav 2003).

Molecular characterization of selected bacteria

It was observed that bacterial species I gave maximum production of bioethanol followed by bacterial species II and bacterial species III. Bacterial species I was sequenced and identified at molecular level.

- DNA quality was evaluated on 1.2 % agarose gel, and a single band of high-molecular-weight DNA has been observed.
- Fragment of 16S rDNA gene was amplified by PCR from the above isolated DNA. A single discrete PCR amplicon band of 1,500 bp was observed when resolved on agarose gel.
- The PCR amplicon was purified to remove contaminants.
- Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl genetic analyzer.

- Consensus sequence of 1305-bp 16S rDNA gene was generated from forward and reverse sequence data using aligner software.
- The 16S rDNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI GenBank database. Based on maximum identity score, the first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database, and the phylogenetic tree was constructed using MEGA4.

Effect of temperature variations on bioethanol production

Temperature exerts a profound effect on all aspects of growth, metabolism, survival of fermenting organism and fermentation. Firstly, inoculated sample was kept at 37 °C because it is optimum temperature for growth of mesophilic bacteria. To study the effect of temperature on bioethanol production, rice bran substrate was inoculated with *Bacillus cereus* strain *McR*-3 and was kept at different range of temperatures as 19, 22, 25, 28, 31, 34, 40, 43, 46, 49, 52, 55, 58 and 61 °C.

Effect of pH variation on bioethanol production

pH also affects the rate of fermentation. To study the effect of pH on bioethanol production, rice bran substrate's initial pH was recorded 5, *B. cereus* strain *McR*-3 was inoculated and kept for optimization of pH, and it was adjusted at 2, 3,4,6,7 and 8.

Results and discussion

Screening of bacteria from rice bran and fermentation test

Microbial screening from rice bran was done. For this, rice bran was dipped in distilled water (20 g in 200 ml). The sap of rice bran was poured into Petri plate containing NAM for culture of bacteria, and it was kept for incubation at 37 °C for 24 h. Several colonies grew on NAM plate. They were isolated in the form of pure culture. After morphological study of these colonies, seven bacterial species were observed. By the fermentation test, it was checked whether bacteria are fermentative or not. For this, bacteria were inoculated in fermentation broth containing phenol red indicator. Fermentative bacteria changes red color of broth into yellow color due to production of acid, which shows positive test (Prescott 2002). Out of seven, three bacteria were found to be fermentative. Selected bacteria were named as bacterial species I, II and III.

Characteristics of bacteria

Microbial screening is the most important step of understanding the activity of microorganism. Seven different types of bacteria were isolated from rice bran, and they were tested for fermentation test. Three were found to be fermentative in nature and selected for the further studies. The colony characteristics of all three selected bacterial species were irregular shaped, large, flat elevated and creamish in color, but having difference in their colony margin and optical characteristics. The colony of bacterial species I had entire margin, while bacterial species II and III had undulated margin. The colonies of bacterial species I and III were translucent, and of bacterial species II was opaque in optical character. After cultural characteristics of bacteria, microscopic study was performed with different types of staining techniques such as Gram's staining, acid-fast staining and endospore staining. It was found that all the bacteria were rod shaped, gram positive, acid-fast staining negative, motile and without endospore. Many researchers also studied the microorganism and their characterization. Gold et al. (1996) have also constructed a series of gram-positive strains for ethanol production. Talarico et al. (2005) constructed an operon for expression of ethanol production in gram-positive bacteria. Senthil Kumar and Gunasekaran (2005) stated that the gram-positive bacteria *Clostridium cellulolyticum*, *Lactobacillus casei* and several yeast strains have been engineered for bioethanol production from cellulosic substrate. Tiwari et al. (2011) explained that gram-positive bacteria can produce bioethanol from some carbohydrate substrates.

Biochemical activities of bacteria

After characterization of fermentative bacterial species, it was found that bacterial species I, II and III were negative for indole test, methyl red test and Voges-Proskauer test and that all three gave positive test for citrate utilization. All bacterial species I, II and III were negative for hydrogen sulfide production test and cellulase production test. All bacterial species showed positive results for oxidation-fermentation test, fermentation of carbohydrate test, nitrate reduction test, urease test and catalase test. Bacterial species I was positive for amylase production test, but bacterial species II and III were negative for this test. All selected bacteria were studied according to biochemical characteristics. Different tests such as indole test, methyl red test and Voges-Proskauer test (IMViC test), citrate utilization test, hydrogen sulfide production test, cellulase production test, oxidation-fermentation test, fermentation

of carbohydrate test, nitrate reduction test, urease test, catalase test and amylase production test were performed for studying the biochemical activity of bacteria.

Bioethanol production and estimation

All three selected bacterial species I, II and III were inoculated into sample rice bran for bioethanol production by the process of fermentation. Bioethanol was produced by fermentation and distilled by distillation unit, and amount of ethanol was calculated by specific gravity method. The bioethanol content of the fermented broth was determined by measuring specific gravity of the distillate according to the procedure described by Amerine and Ough (1984). All three bacteria were inoculated in rice bran substrate, and incubation period was also observed. In quantification of bioethanol, bacterial species I was most efficient and produced maximum amount of bioethanol on the fifth day of incubation. Therefore, bacterial species I was selected for the further studies for molecular identification (Table 1).

Molecular characterization of selected bacterial species I

All three selected test bacteria were used for bioethanol production. They were inoculated in rice bran sample and kept for incubation. After process of distillation, amount of bioethanol was estimated with specific gravity method and it was found that bacterial species I was most efficient for bioethanol production in comparison with two other bacterial species. So bacterial species I was used for further study and identified at molecular level. After the sequencing and phylogenetic analysis, bacterial species I was identified as *B. cereus* strain *McR*-3 (GenBank Accession Number: JF894159.1) based on nucleotide homology and phylogenetic analysis. Information about other close homologs for the microbe can be found from the Alignment View table (Table 2). The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987). The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and were in the units of the number of base substitutions per site. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 1,305 positions in the final dataset. Phylogenetic analysis was conducted in MEGA4 (Fig. 1; Tamura et al. 2007).

Effect of temperature variations on bioethanol production

Bacillus cereus strain *McR*-3 was inoculated in rice bran sample and incubated for 48 h. Then, temperature of

Table 1 Amount of bioethanol (%) at 37 °C from selected bacterial species (I, II &III)

Bacterial species	Day 3	Day 4	Day 5	Day 6
Bacterial species I	6.15 ± 1.13	7.69 ± 0.48	10.21 ± 0.43	9.36 ± 0.44
Bacterial species II	5.86 ± 0.65	7.62 ± 0.59	8.30 ± 0.77	5.68 ± 0.77
Bacterial species III	5.75 ± 1.15	7.60 ± 0.56	9.06 ± 0.08	7.50 ± 0.45

Table 2 Sequence producing significant alignments for bacterial species I identification

Accession	Description	Max score	Total score	Query coverage (%)	E value	Maximum identity (%)
JF947357.1	*Bacillus thuringiensis* strain 2110	2,410	2,410	100	0.0	100
JF895480.1	*B. cereus* strain KU4	2,410	2,410	100	0.0	100
JF901711.1	*Bacillus* species A-BT-nw	2,410	2,410	100	0.0	100
JF901703.1	*Bacillus* species B-AS-16	2,410	2,410	100	0.0	100
JF701945.1	*Bacillus* species YXA3-34	2,410	2,410	100	0.0	100
JF894159.1	*B. cereus* strain McR-3	2,410	2,410	100	0.0	100
JF900020.1	*Bacillus* species A2-18	2,410	2,410	100	0.0	100
JF900010.1	*Bacillus* species A1-8	2,410	2,410	100	0.0	100
JF825991.1	*Bacillus* species BM3(2011)	2,410	2,410	100	0.0	100
JF820118.1	*Bacillus* species PG-5-5	2,410	2,410	100	0.0	100

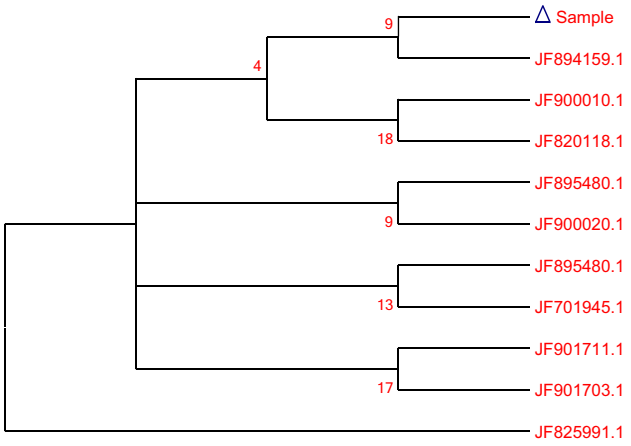

Fig. 1 Phylogenetic tree of bacterial species I and evolutionary relationships of 11 taxa

incubator was increased and decreased from 37 °C to see the effect of temperature on it (Fig. 2). Results showed that at 37 °C temperature, bioethanol production was maximum 10.50 ± 0.10 % and that variation in temperature caused decreased production of bioethanol. Hughes et al. (1984) also worked on the kinetics of ethanol formation from glucose in batch culture by thermotolerant yeast *K. marixianus* and reported over the temperature range 30 °C and 48 °C. Perego et al. (1985) explained the influence of temperature, dilution rate and sugar concentration on ethanol fermentation of molasses They found that at 27 °C, the system attained a steady state and high ethanol yield. Steady state was never reached at 37 °C even at relatively low ethanol concentration. At 32 °C, the system response depend on the values of the dilution rate and sugar concentration. Bajpai and Margaritis (1986) also studied the effect of temperature of medium on the ethanol production by the immobilized *Zymomonas mobilis* cells during continuous fermentation and found maximum ethanol productivity and activity at 37 °C. Rousseau et al. (1992) explained the effect of temperature on fermentation kinetics of waste sulfite liquor by *S. cerevisiae* for bioethanol production and found that the fastest consumption of substrate resulting in the shortest fermentation times of 13 and 45 h was achieved at 35 and 30 °C for synthetic medium. Cazetta et al. (2007) stated the effect of temperature and sugar concentration on ethanol production by molasses and found that the temperature 30 °C was the most favorable for production process. Neelakandan and Usharani (2009) produced bioethanol from cashew apple juice using immobilized yeast. They found maximum production of ethanol at 32 °C and 14 h of incubation. Periyasamy et al. (2009) observed bioethanol production from sugar molasses using *S. cerevisiae* and reported the maximum bioethanol yield of 53 % at temperature 35 °C. Ado et al. (2009) explained ethanol production from cassava starch using co-culture of *Aspergillus niger* and *S. cerevisiae* in a simultaneous saccharification and achieved the maximum ethanol at 35 °C. Tiwari et al. (2010) studied the effects of temperature variation in the bioethanol-production process from some cereals and obtained highest bioethanol production at 40 °C. Tahir et al. (2010) studied the effect of cultural conditions on ethanol production by locally isolated *S. cerevisiae Bio-07* and found that 30 °C was optimum temperature for ethanol production. Banerjee et al. (2009) studied effect of temperature on bioethanol production from rice husk and found maximum production at 30 °C. Beliya et al. (2013) found 25 °C was optimum temperature for bioethanol production from deoiled rice bran.

Effect of pH variations on bioethanol production

Bacillus cereus strain *McR*-3 was inoculated in rice bran sample and incubated at optimum temperature. Initial pH of sample was 5; for optimization, it was adjusted below 5 and above 5 (Fig. 3). Results indicate that the amount

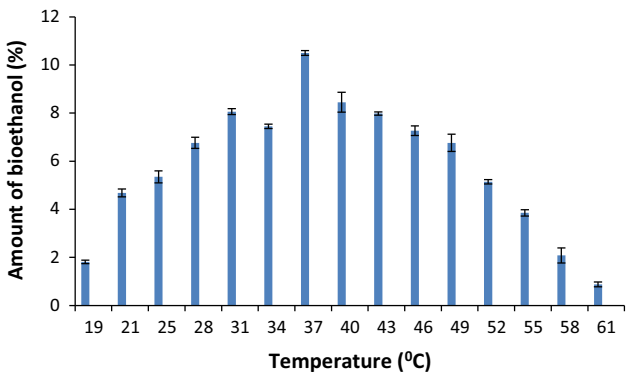

Fig. 2 Effect of different temperatures on bioethanol production from *B. cereus* strain *McR*-3

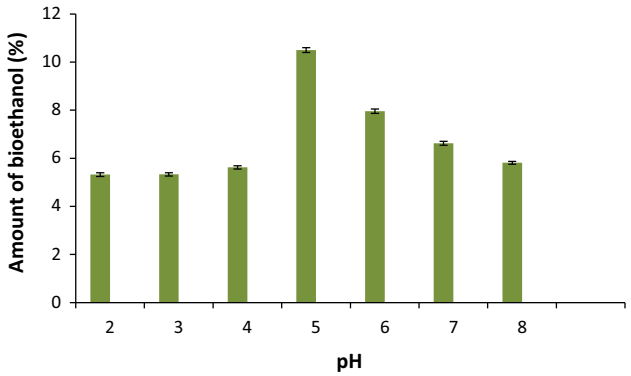

Fig. 3 Effect of different pH on bioethanol production from *B. cereus* strain *McR*-3

of bioethanol was maximum at pH 5 and that pH more than 5 and less than 5 was not helpful to increase the production of bioethanol. Nimbkar et al. (1989) reported maximum alcohol concentration at pH 4.5 from the fermentation of sweet sorghum juice. Marakis and Marakis (1996) obtained maximum alcohol concentration of 5.8 % at pH 4.5 from aqueous carob pod extract after 120 h of incubation. Srivastava et al. (1997) showed that the optimum, initial pH of guava pulp medium was 5 and achieved maximum yield of 5.8 % of ethanol at that pH. Periyasamy et al. (2009) obtained the maximum bioethanol at pH 4 from sugar molasses using *S. cerevisiae*. Ado et al. (2009) studied bioconversion of cassava starch into ethanol and found maximum yield of ethanol at pH 5. Spitzer et al. (2009) also used pH as parameter to characterize bioethanol. Neelakandan and Usharani (2009) produced bioethanol from cashew apple juice using immobilized yeast. They also studied the effect of incubation period, temperature and pH. They found maximum production of bioethanol at pH 6. Asli (2010) studied efficient parameters in batch fermentation of ethanol using *S. cerevisiae* in red grapes substrate, and maximum concentration of bioethanol at pH 4.5 was recorded. Banerjee et al. (2009) studied effect of pH on bioethanol production from rice husk and found maximum production at pH 5.

Conclusion

The present study indicates that *B. cereus* strain *McR*-3 is a novel bacterium for fuel ethanol production and that no earlier work has reported bioethanol production from this bacterium; so, it may be significant for future studies in bioethanol-production technology. The utilization of rice bran for bioethanol production is a sustainable and eco-friendly approach for renewable biofuel production. Bioethanol can serve as an alternative source of energy and can overcome the problem of energy crisis in future. Results of studies also show that physical parameters played an important role in the process of fermentation and bioethanol production. The optimum temperature for bioethanol production was 37 °C, and pH is 5 from rice bran substrate. Bioethanol is the best alternative source of fuel and considered as fuel of future. Exploration of low-cost substrate and use of an efficient microorganism will open new doors for the bioethanol-production technology.

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