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Effect of Some Environmental Parameters on Biobutanol Production by *Clostridium acetobutylicum* NCIMB 13357 in Date Fruit Medium

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Abstract: Date fruit juice contains high concentration of simple sugars ranging from 65 to 75% (w/w) in dry form. In this study, the potential of date fruit juice as biobutanol fermentation medium by *C. acetobutylicum* was investigated. The fermentation process was carried out at initial pH of 5, 6 and 7, incubation temperature of 30, 35 and 40°C for 72 hours. The date fruit concentrations tested were 10, 20, 30 and 40 g L^{-1}. Medium containing 30 g L^{-1} of date fruit at 35°C incubation temperature with initial medium pH 7.0 gave the highest concentration of solvents of 3.1, 0.1 and 1.1 g L^{-1} butanol, ethanol and acetone respectively. The yield and productivity of biobutanol were 0.32 g g^{-1} and 0.044 g L^{-1}/h respectively, while for total ABE were 0.45 g g^{-1} and 0.06 g L^{-1} h, respectively.

Key words: Biobutanol, *Clostridium acetobutylicum*, dates fruit, solvent

INTRODUCTION

In recent years, the price of crude oil has increased dramatically due to increase of world's oil demand. Excessive use of fossil oil has led to increase of greenhouse gases emission and global warming. This has led to renewed interest in alternative and renewable energy. Biobutanol has higher energy content compared to bioethanol since it has four carbon atom compared to bioethanol which has two carbon only. Biobutanol also has low volatility, less hydroscopic (absorbing or attracting moisture from the air) and less corrosive. Biobutanol can directly used or mixed with gasoline without engine modification (Lee *et al*., 2008).

Date fruit has potential for biobutanol fermentation as it contains high sugars contents and also found abundant as waste in most middle-east countries. There is no studies have been reported specifically to the use of date fruit for biobutanol production by Clostidia.

According to FAO (2005), the world production of dates fruit was 6,924,975 metric ton in 2005. Previous reports showed that dry form of date fruit contains sugar from 62 to 75%, total moisture ranging from 10 to 22%, proteins 2.2 to 2.7%, fibers 5 to 8%, fat 0.4 to 0.7%, ash from 3.5 to 4.2% , total acidity form 0.04 to 0.2% and ascorbic acid from 30 to 50 mg%. (FAO, 1992; Ismail *et al*., 2006; El-Sharnouby *et al*., 2007; El-Sohaimy and Hafez, 2010).

Clostridium acetobutylicum NCIMB 13357 is a gram-positive, rod-shape, anaerobic and spore-forming bacterium, it has ability to produce solvents; acetone, butanol and ethanol (ABE). *C. acetobutylicum* can utilize a large variety of substrates from monosaccharides to polysaccharides in the medium under anaerobic condition and convert it to solvents, with ratio 1:6:3 ABE (Jones and Woods, 1986; Bahl and Durre, 2001).

Butanol has many other applications such as in the plastic industry and as potential fuel extender. Beside that it also can be used as an extractant in the food and flavor industry (Thaddeus *et al*., 2007). Fermentation derived butanol is preferred over petrochemical obtained butanol because of the potential risk of carcinogen carryover (Formanek *et al*., 1997). In this study, the potential of date fruit juice as biobutanol fermentation medium by *C. acetobutylicum* was investigated. Table 1 shows properties of biobutanol and other fuels.

Table 1: Properties of biobutanol and other fuels

	Biobutanol	Gasoline	bioethanol	Methanol
Energy content (MJ L^{-1})	29.2	32	19.6	16
Air-fuel ratio	11.2	14.6	9	6.5
Heat of Vaporization (MJ L^{-1})	0.43	0.36	0.92	1.2
Research octane number	96	91-99	129	136
Motor octane number	78	81-89	102	104

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MATERIALS AND METHODS

Strain and culture media: *C. acetobutylicum* NCIMB 13357 was obtained from biotechnology laboratory, Department of Chemical and Process Engineering, Universiti Kebangsaan Malaysia (UKM), in freeze-dried form and maintained on Reinforced Clostridium Medium (RCM) broth and agar as previously described (Elgadafi and Kalil, 2009). Inoculum was prepared on RCM medium and incubated at 35°C for 24 h under anaerobic condition. Optical density of the 24 h of inoculums (measured using spectrophotometer at 680nm) was approximately 1.1. An inoculum size of 10% (v/v) was used in all fermentation experiments carried out in this study. A good quality inoculum was indicated by the obvious smell of solvent and frothing caused by production of gas by the culture.

Fresh date medium

Filtrated date fruit: A weight of 100 g of dry date was taken and blended with distilled water to get final volume up to 1000 mL, then autoclaved at 121°C for 15 min, filtered it to remove sold particles and mixed with distilled water to get serial dilution from the stock medium. Different date fruit concentrations 10, 20, 30 and 40 g L^{-1} were used as a fermentation media. A volume of 150 mL of these media was transferred to 250 mL Duran bottle. The pH of the media was adjusted to pH 5, 6 and 7 by using 0.2 M NaOH and 0.1 M HCl and 30, 35, 40°C incubation temperatures, autoclaved again to use in the next experiments. Nitrogen gas was used to generation anaerobic condition inside of fermentation culture.

Experiments were carried out using various concentrations of date fruit ranging from 10 to 40 g L^{-1} *C. acetobutylicum* NCIMB13357, under anaerobic condition, with initial pH 5, 6 and 7, incubation temperatures of 30, 35 and 40°C for 72 hours incubation. Final pH, total sugar consumption g L^{-1}, butanol production g L^{-1}, acetone g L^{-1}, ethanol g L^{-1}, acetic Acid g L^{-1}, butyric acid g L^{-1}, total ABE, total acids, yield of ABE, productivity of ABE g/(L.h), yield of butanol and productivity of butanol were measured during the experiments.

Batch fermentation: The anaerobic batch fermentation was conducted in 250 mL Schott (Duran) bottles with a working volume of 150 mL. These bottles have both inlet and outlet. The initial pH of the medium was adjusted to 7 by 0.2 M NaOH and 0.1 M HCl. The medium was sterilized by autoclaving at 121°C for 15 min. To generate an anaerobic condition, the medium was spared with oxygen-free nitrogen and the vitamin solution was filter-sterilized and added aseptically into the sterilized medium. The batch culture was initiated by inoculation of medium with 10% fresh inoculum that was previously grown on RCM medium for 20 h. The Schott bottles were incubated at 35°C in an incubator under anaerobic conditions. All fermentations were carried out in duplicate and measurements are average values.

An experiments were carried out using various concentrations of Date fruit ranging from 10 to 40 g L^{-1} *C. acetobutylicum* NCIMB13357, under anaerobic condition, with initial pH 5, 6 and 7, under certain incubation temperatures 30, 35 and 40°C and incubation time for 72 hours. Final pH, total sugar consumption g L^{-1}, butanol production g L^{-1}, Acetone g L^{-1}, Ethanol g L^{-1}, Acetic Acid g L^{-1}, Butyric Acid g L^{-1}, total ABE, total acids, yield of ABE, productivity of ABE g/(L.h), yield of butanol and productivity of butanol were measured during the experiments.

Analytical methods: Total carbohydrate was measured by using Anthrone method, previously described (Gerhardt *et al*., 1994; Frolund *et al*., 1996), cell biomass was estimated by optical density at 680nm using UV-visible spectrophotometer. Solvents and Acids concentrations were determined by gas chromatograph with capillary column (Equity™-1 Supelco), previously described (Elgadafi and Kalil, 2009). The pH of all fermentation media was measured with Eutech Instrument pH meter (Model: pH 510;pH/ mV/°C; Cyberscan).

RESULTS AND DISCUSSION

Effect of Initial medium pH on biobutanol Production From Fig. 1 the maximum concentration of biobutanol production was recorded at pH 7.0. The poor biobutanol production was at pH 5.0 and this might be due to the

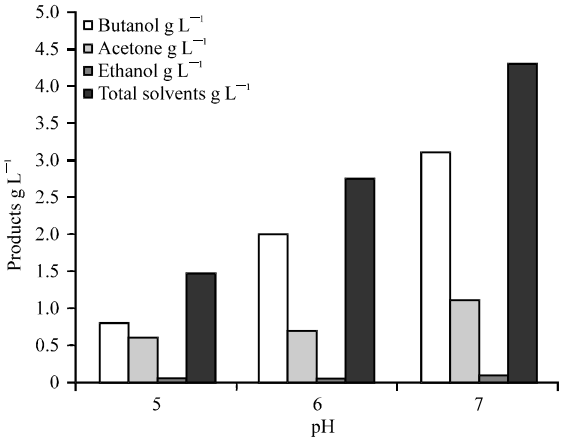

Fig. 1: Effect of pH on Biobutanol, Acetone, BioeEthanol and Total Solvents productions using Date juice as a fermentation media by C. acetobutylicum NCIMB 13357

Table 2: Effect of pH on yield and productivity of butanol and ABE

Date fruit concentrations g L^{-1}	Initial pH	Final pH	Temperature°C	Time (h)	ABE yield g g^{-1}	ABE productivity g L^{-1} h^{-1}	Butanol productivity g L^{-1} h^{-1}	Butanol yield g g^{-1}
30	7	3.4	35	72	0.44	0.06	0.044	0.32
30	6	4.2	35	72	0.33	0.039	0.028	0.24
30	5	4.1	35	72	0.14	0.021	0.012	0.075

Table 3: Effect of the substrate concentrations on yield and productivity of butanol and ABE

Date concentration	Initial pH	Temperature°C	Time (h)	ABE Yield g g^{-1}	ABE productivity g L^{-1} h^{-1}	Butanol productivity g L^{-1} h^{-1}	Butanol Yield g g^{-1}
10	7	35	72	0.43	0.027	0.019	0.32
20	7	35	72	0.35	0.039	0.027	0.24
30	7	35	72	0.45	0.06	0.044	0.32
40	7	35	72	0.28	0.044	0.032	0.21

Table 4: Effect of temperature on yield and productivity of butanol and ABE

Date concentration	Initial pH	Temperature°C	Time (h)	ABE Yield g g^{-1}	ABE productivity g L^{-1} h^{-1}	Butanol productivity g L^{-1} h^{-1}	Butanol Yield g g^{-1}
30	7	30	72	0.41	0.046	0.032	0.29
30	7	35	72	0.45	0.06	0.044	0.32
30	7	40	72	0.28	0.031	0.021	0.19

Table 5: Optimum pH and temperature of different strains of solvent producing *Clostridia*

Strain	Substrate	pH[a]	[b]Temperature (°C)	References
C. acetobutylicum DSM 1731	Potato	6.0	37	Grobben *et al.* (1993)
C. acetobutylicum ATCC 824	Glucose	6.5	35	Qureshi and Blaschek (2001)
C. saccharoperbutylacetonicum N 1-4	POME	5.8	30	Ishizaki *et al.* (1999)
C. saccharobutylicum P262	Whey permeate	5.5	34	Maddox *et al.* (1995)
C. Saccharobutylicum P262	DSPS	7.0	37	Badr *et al.* (2001)
C. acetobutylicum NCIMB 13357	RCM	6.0	30	Jaapar *et al.* (2009)
C. saccharoperbutylacetnicum N 1-4	POME	5.8	30	Al-Shorgani *et al.* (2011)
C. acetobutylicum P262	RCM	6.0	35	Madihah *et al.* (2001)
C. saccharoperbutylacetonicum N 1-4	PG	6.5	30	Tashiro *et al.* (2005)
C. acetobutylicum NCIMB 13357	POME	5.8	35	Takriff *et al.* (2009)
C. saccharoperbutylacetonicum N 1-4	TYA	6.5	30	Shamsudin et al. (2006)
C. acetobutylicum NCIMB 13357	RCM-Date fruit	7.0	35	Khamaiseh *et al.* (2012)

[a]The pH was set at the start of fermentation POME is Palm Oil Mill Effluent, [b]The Temperature was set as incubation Temperature RCM is Reinforce clostridium Medium, DSPS is Defibered Sweet Potato Slurry. PG is Potato Glucose, TYA is Tryptone, Glucose and Yeast extract

increased formation of acidic metabolites which destroys the cell's ability to maintain internal pH (Bowles and Ellefson, 1985). Result in lowering the intercellular level of ATP, thereby, inhibiting glucose uptake. However, it is also noted that the final pH of the effluents cultures from all batches having a different initial medium pH was not consistently within the same range. Results in Table 2 shown that at a higher initial pH, there was a greater drop in final pH, with a shorter duration of biobutanol production.

Acid production during the course of fermentation provides some buffering effect which results in the subsequent attainment of an equilibrium pH level for the media having high initial pH. This implies that the fermentative microorganisms could not adapt to the rapid change in environment and thus might have been inhibited. Conversely, at a lower initial pH level, the starting environment might not be suitable for butanol producers. However, with their adaptation and limited self-adjustment of environmental conditions, such as pH, they started to produce hydrogen gradually at a moderate rate. These observations agree well with the pH studies conducted by Alshiyab *et al.* (2008). The optimum pH range of 7.0±0.2 for the maximum rate of hydrogen production observed in this study was also in strong agreement with the reported values of similar studies of pH 6.5, using glucose (Qureshi and Blaschek, 2001); pH 6.0, using RCM (Jaapar *et al.*, 2009); and pH 7.0, using DSPS (Badr *et al.*, 2001). The results underline that the optimum pH for maximizing of butanol production is dependent both on the type of microorganisms and the substrates used, Table 5. Even so, it is difficult to draw a satisfactory correlation between the initial medium pH and the butanol production, since the experiments were carried out in uncontrolled pH conditions.

The cumulative butanol production reached maximum at pH 7.0 with 3.1 g L^{-1} of butanol, 4.3 g L^{-1} total solvents and 1.1 g L^{-1} total acids. Yield of butanol and ABE and productivity of butanol and ABE are shown in Table 2.

Effects of initial date fruit concentrations on biobutanol production: Production of biobutanol in anaerobic fermentation is accompanied by the breakdown of an organic substrate like glucose, sucrose, fructose in the present studies. So, the initial carbohydrates concentration plays an important role in the amount of

butanol production during the course of fermentation. At a relatively low initial carbohydrate concentration, the amount and rate of fermentation was also low, according to the law of mass action (Fabiano and Perego, 2002). Table 3 reveals that the maximum butanol production (3.1 g L^{-1}) was accomplished with 30 g L^{-1} of the initial date fruit concentration corresponds to product yield, $Y_{p/s}$ of 0.45 g butanol (g date fruit)$^{-1}$. At 40 g L^{-1} of date fruit the final maximum biobutanol concentration was 2.3 g L^{-1} with 0.032 g L^{-1}/h and 0.21 g g^{-1} of the biobutanol productivity and Yield, respectively. It had already been reported that substrate inhibition gets predominant at higher glucose concentration because this modifies the metabolic pathways (Oh *et al*., 2002) Fig. 2, 3. On the other hand, at 10 g L^{-1} of date fruit concentration, the maximum biobutanol concentration, Yield and productivity of biobutanol were 1.3 g L^{-1}, 0.32 g g^{-1} and 0.019 g L^{-1}h, respectively. As well, the maximum biobutanol produced at 20 g L^{-1} of date fruit concentration medium was 1.9 g L^{-1} with 0.027 g g^{-1} biobutanol Yield and 0.24 g L^{-1} h of biobutanol productivity. The lower butanol production indicates that the carbon flux at high carbohydrate concentrations is more directed to the production of reduced by-products such as ethanol and (or) organic acids (Nath *et al*., 2006) (Fig. 2, Table 3).

At date fruit concentration of 10 g L^{-1}, the total acids was 2.6 g L^{-1}, whereas the acetic acids was 1.5 g L^{-1} and butyric acids was 1.1 g L^{-1}. The total concentration of the acids was 2.1 g L^{-1} at 20 g L^{-1} of date fruit concentration. At 30 g L^{-1} of date fruit, the concentrations of acetic acid and butyric acid were 0.5 g L^{-1} and 0.6, respectively. At high concentration of 40 g L^{-1} date fruit, the acetic acid and butyric acid concentrations were 1.5 g L^{-1} and 1.1 g L^{-1} respectively with total acids was 2.6 g L^{-1}. This probably indicates that the chances of forming some acids (as metabolites) increase with high initial glucose concentration, whereby, pH drops appreciably and results in a lower butanol production albeit at the cost of higher percent consumption of glucose (Mizuno *et al*., 2000; Oh *et al*., 2002, Nath *et al* 2006).

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This suggests that at higher substrate concentration may quickly become inhibitory through pH depletion, acid production, or increased hydrogen partial pressure. Therefore, addressing these inhibitory mechanisms is necessary to achieve a high butanol production rate at a higher substrate concentration (Van Ginkel *et al*., 2001).

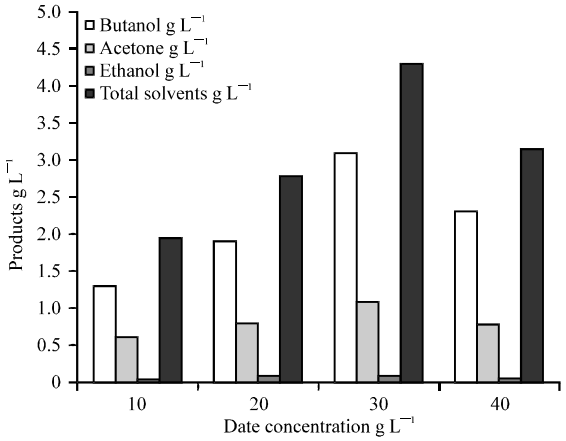

Fig. 2: Effect of the Substrate concentrations on Butanol, Acetone, Ethanol and Total Solvents productions using Date juice as a fermentation media by *C. acetobutylicum* NCIMB 13357

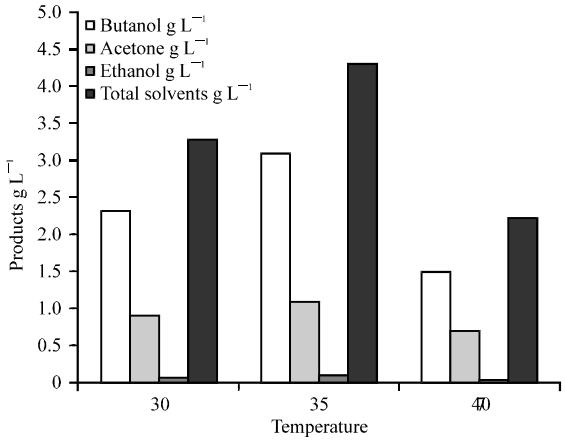

Fig. 3: Effect of the Temperature on Acetic Acid, Butyric Acid and Total acids productions using Date juice as a fermentation media by *C. acetobutylicum* NCIMB 13357

Effect of temperature on the production of biobutanol: The effect of temperature on butanol production was investigated at temperatures of 30, 35 and 40°C (Fig. 3). During those experiments, initial pH and initial carbohydrate concentration of the fermentation medium were kept at 7.0±0.2 and 30 g L^{-1}, respectively. Maximum concentration of butanol was at 35°C with 3.1 g L^{-1} of biobutanol and total solvents were 4.3 g L^{-1} Fig. 3. In addition, the yield of biobutanol and productivity were 0.32 g g^{-1} and 0.044 g L^{-1}/h, respectively. In more detail, the productivity and yield of ABE were 0.0.06 g L^{-1} h and 0.45 g g^{-1}, respectively

(Table 4). The temperature reported in this study was the same temperature reported by Qureshi and Blaschek, 2001 for biobutanol production by *C. acetobutylicum* ATCC 824 and 34°C for biobutanol production by *C. saccharobutylicum* P262 was reported by Maddox *et al.* (1995). Although, the strains are different, the best temperature for biobutanol production was 35°C. This may be the temperature that a further increase of it resulted in a subsequent reduction of biobutanol production. Comparison of the result with other reported works in literature reveals that *C. saccharobutylicum* P262 gives the maximum butanol production at 34°C (Maddox *et al.*, 1995), while *C. acetobutylicum* DSM 1731 produces maximum butanol at 37.0°C (Grobben *et al.*, 1993).

Elgadafi and Kalil (2009) reported that at the incubation temperature of 30°C was the most suitable temperature for a maximum biobutanol production by *C. acetobutylicum* NCIMB 13357. The decrease in the butanol production at higher temperature can be attributed to an increase in denaturation rate of the enzymes (Fabiano and Perego, 2002). In fermentation or enzymatic processes, it is known that the positive kinetic effect of an increase of temperature prevails over the negative effect on the biocatalyst activity, up to the threshold temperature beyond which thermal deactivation of biocatalyst takes place (Slininger *et al.*, 1990). Thermal deactivation at higher temperatures leads to inactivation of the enzymes responsible for controlling metabolic pathways in the fermentative hydrogen production process.

This suggests the occurrence of a similar deactivation mechanism in connection with the progressive denaturation of the enzyme which kinetically determines fundamental pathways in some other butanol producing enteric bacteria such as *E. aerogenes* (Fabiano and Perego, 2002).

For most of Clostridia, growth is optimal at pH 5.5-7.0 and temperature 30 to 37°C. For a given organism the optimal pH for growth and temperature may vary depend on strain used and the formulation of the medium, Table 5 summarizes the pH and temperature used in previous studies. Many of the early reports relating to the industrial production of solvents and acids noted that the initiation of solvent production occurred only after the pH had decreased to around 4.5 -5.0 (Jones and Wood, 1986). These observations have been confirmed in a number of studies (Monot *et al.*, 1984; Soni *et al.*, 1992) which have been reported that culture maintained at high pH produced mainly acids, whereas in fermentation maintained at low pH, solvents production usually predominates. The *C. acetobutylicum* ATCC 824 has been reported to produce good level of solvent between pH 5.5 and 4.3 (Monot *et al.*, 1984).

Previous report has shown that the optimum pH range for solvent production is much higher for *C. acetobutylicum* NCIMB 13357 which was used for the industrial production of solvent (Jaapar *et al.*, 2009). Whereas the optimum pH and temperature were set at about pH 6 and temperature 30°C with butanol production of 0.8 g L^{-1} with RCM medium.

Effect of agitation on the production of biobutanol: A set of tests was performed at varying agitation speed from 0.0 to 200 rpm at intervals of 50 rpm, keeping the other operative conditions (temperature at 35°C, pH 7.0 and substrate concentration at 30 g L^{-1} of date fruit) constant. Result shows in Fig. 4, maximum rate of butanol production was recorded at agitating speed 50 rpm and it was 3.0 g L^{-1}. At the same time, the concentration of biobutanol produced by this bacterium without shaking was 3.1 g L^{-1}. We suggested that when the speed of agitation increased, the ability of *C. acetobutylicum* to produce butanol decreased. At 50 rpm was the maximum butanol concentration production was 3.0 g L^{-1}. Jaapar *et al.* (2009) reported that more hydrogen is produced in the static culture which is 5 times higher than the amount produced in the shake culture. The maximum amount of hydrogen produced in shake culture was only 11.57 mL while in the static culture was 54.37 mL. When the agitation increased its led to destroy the cells or don't allow to bacteria to breakdown and uptake of glucose or

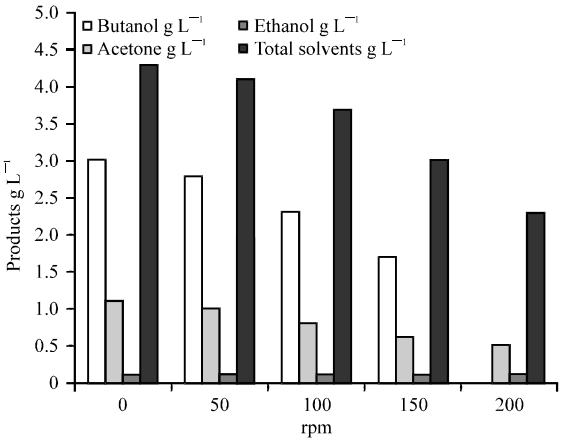

Fig. 4: Effect of agitation speed on butanol, acetone, ethanol and total solvent productions by *C. acetobutylicum* NCIMB 13357

carbohydrate inside the cell as well outside. Results show that this operational parameter such as agitation will affect the biobutanol production by *C. acetobutylicum* NCIMB 13357. Therefore, biobutanol production using this bacterium should be carried out in static condition rather than shake condition especially for small scale culture.

CONCLUSION

This study shows that date fruit has the potential to be used as fermentation suitable medium for biobutanol production by *C. acetobutylicum* NCIMB 13357. The maximum yield of biobutanol was 0.32 g (g carbohydrate)$^{-1}$ when using 30 g of carbohydrate date fruit was used as the main substrate in the fermentation media. An initial medium pH of 7.0±0.2 and a process temperature of 35°C were found to be the most favorable suitable condition for optimum maximum biobutanol production. The agitation speed of 100 rpm gave the maximum biobutanol production by *C. acetobutylicum* NCIMB 13357.

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