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Different process designs for bioethanol production

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ABSTRACT

Development of bioethanol production from lignocellulosic residues is one of the greatest challenges nowadays to meet the energy demand sustainably. This study aimed to improve the microbial conversion of bioethanol production using co-culturing of *Streptomyces aegyptia* and *Candida tropicalis* and focused on two trends: process design and substrate adding mode. Two strategies of process design, separated hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) were performed to compare the efficient bioethanol production. Rice straw and manure were pretreated with $Ca(OH)_2$ and further processed for SHF and SSF. Among both strategies, SSF in form of one-pot hydrolysis and fermentation design gave a maximum ethanol production (200 mg/l) and bioconversion efficiency of 1.8% after 5 days of culturing at 30˚C. Substrate adding (batch and fed-batch) in the process design was applied in different modes. In batch mode, all working volume of substrates and medium was applied at the beginning of culture. In the fed-batch, three substrate-feeding modes were carried out. The result showed that the fed-batch system with one-time feeding (75:25) was higher when compared to other modes with ethanol production and bioconversion efficiency of 255 mg/l and 2.3%, respectively.

Keywords:

Batch fermentation, fed-batch fermentation, separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), bioethanol.

1. INTRODUCTION

Continuous efforts are still being made to provide sustainable resources for energy and independence from fossil fuel resources. In this respect, bioethanol can be now favored as the blend or fossil petrol substitute **[1].** The largest potential feedstock for bioethanol is lignocellulosic residues, which include materials generated as wastes from animals and crops **[2]**. There are several properties of bioconversion processes for lignocelluloses based on **[3]:** 1) the mode of

substrate addition (batch and fed-batch), 2) whether hydrolysis and fermentation are performed separately (separate hydrolysis and fermentation -SHF) or together (simultaneous saccharification and fermentation - SSF).

In SHF, hydrolysis and fermentation steps are performed sequentially in separate steps **[4].** The use of two separate reactors increases the total process costs and high sugar concentration after hydrolysis can cause product inhibition to the enzymes and that decrease the rate of hydrolysis **[5].** Simultaneous saccharification and fermentation is a technique for bioethanol production in which the hydrolysis and fermentation processes take place simultaneously **[6].** It would be appropriate because the yeast is immediately converting the hydrolyzed sugars and no inhibiting concentration of sugars can be formed **[7]**. Also, the risk of microbial contamination is decreased, as the overall processes take place in one reactor. However, either hydrolysis or fermentation can be performed under optimal culture conditions **[1].** Several studies investigated the comparison between these modes for ethanol production from lignocellulosic residues**[8–12].**

The fed mode of bioethanol production can be performed in batch, fed-batch, or continuous systems [**13**]. In the batch process mode, substrate and culture medium are provided all at the beginning of the culture process without addition or removal during the process. There are several benefits of the batch process mode including lower risks of contamination and can be controlled easily. Fed-batch fermentation involves the addition of substrate into the reactor at certain intervals without removing the medium. It has been used to overcome the problems of substrate inhibition in batch mode **[14].** Several studies investigated comparisons between these fed modes for ethanol production from lignocellulosic residues **[13, 15, 16].**

Lignocelluloses are rich in cellulose and hemicellulose which need to breakdown for fermentable sugars generation. It is known to use, *Streptomyces aegyptia* in the hydrolysis step (as it contains a battery of lignocellulosic enzymes) and *Candida tropicalis* in the ethanol production (due to its ability for pentose and hexose fermentation) **[26, 27].** This study investigated the bioethanol production using the co-culture of these microorganisms and focused mainly on improving the microbial conversion of bioethanol production by determining the suitable conversion process design and fed mode of rice straw and manure.

2. MATERIAL AND METHODS

2.1. Microorganisms

Strains of actinomycete and yeast used in this study were previously isolated **[17]** and identified by the molecular method as *Streptomyces aegyptia* (Genebank accession number MT534590) and *Candida tropicalis* (Genebank accession number MT874505), respectively. Micromorphology of the strains was examined microscopically and shown in **Photo (1, 2).**

2.2. Lignocellulosic substrates

Rice straw was procured from the animal feed supplier of Suez Canal University (Ismailia, Egypt) and stored in sealed bags until use. Buffalo manure was supplied from the farm of College of Veterinary Medicine of Suez Canal University (Ismailia, Egypt). It was autoclaved immediately after collection at 121ºC for 30 min, dried in an oven at 70 ºC until dryness then stored in a refrigerator until use.

2.3. Media

- *Streptomyces aegyptia* was maintained on starch casein agar medium **[18]** containing (g L^{-1}): soluble starch, 10.0; casein (vitamin-free), 0.3; KNO_3, 2.0; NaCl, 2.0; K_2HPO_4, 2.0; $MgSO_4.7H_2O$, 0.05; $CaCO_3$, 0.02; $FeSO_4.7H_2O$, 0.01.

- *Candida tropicalis* was maintained on yeast-peptone glucose (YPG) agar medium **[19]** containing (g L^{-1}): yeast extract, 10; peptone, 20; glucose, 20.
- Medium of salt mineral **[20]** was applied in hydrolysis and fermentation processes, containing (g L^{-1}): Yeast extract, 1.0; KH_2PO_4, 0.3; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01; $ZnSO_4$, 0.001.

2.4. Pretreatment of substrates

Pretreatment of substrates was conducted for all designs used in this study in a 250-ml capacity bottle containing 4.0 g of the rice straw and manure mixture (1:1). Pretreatment with $Ca(OH)_2$ was done at 100°C, with a water loading of 6 ml H_2O/g air-dried substrates, and an alkaline loading of 2 wt% of oven-dried substrates.

2.5. Separate Hydrolysis and Fermentation (SHF)

In SHF, hydrolysis and fermentation steps were performed sequentially in separate processes. The mineral salt medium contained the pretreated substrates (4g/100 ml) was autoclaved for 20 minutes. Afterwards, $1x10^7$ equivalent CFU/ml *Streptomyces aegyptia* spores were inoculated. Cultures were grown at 30 ºC in an orbital incubated shaker at 120 rpm for 72 hours. Next, the medium after hydrolysis was centrifuged and the hydrolysate was inoculation with 5ml of *Candida tropicalis* culture ($1\chi10^8$). Cultures were statically incubated at 30 ºC for 72 hours. Ethanol contents were monitored every 24 hours.

2.6. Simultaneous Saccharification and Fermentation (SSF)

In SSF, hydrolysis and fermentation took place simultaneously. Pretreated substrates (4g) supplied with the mineral salt medium (100 ml) were autoclaved for 20 minutes. Afterwards, $1x10^7$ equivalent CFU/ml *Streptomyces aegyptia* spores were added together with 5ml of *Candida tropicalis*. The process was conducted in the shaking incubator under 30°C with velocity agitation of 120 rpm for 120 hours. Ethanol contents were monitored every 24 hours.

One–pot hydrolysis and fermentation as a type of simultaneous saccharification and fermentation (SSF) was performed as above except that culture of *Streptomyces aegyptia* was grown at 30 ºC in an orbital incubation shaker at 120 rpm for 72 hours. Subsequently, the fermentation process was conducted in the same flask by adding 5ml of *Candida tropicalis* culture and static incubating for 72 hours.

2.7. Batch submerged processes

In this mode, all working volume of substrates and the medium was applied at the beginning of culturing and autoclaved then inoculated by $1x10^7$ equivalent CFU/ml *Streptomyces aegyptia* spores. Cultures were grown at 30 ºC in an orbital incubation shaker at 120 rpm for 72 hours. Next, fermentation was conducted by adding 5ml of *Candida tropicalis* and static incubated for 72 hours at 30^0C. Samples were taken every 24 hours for reducing sugar and ethanol analysis.

2.8. Fed-batch submerged processes

Three substrate-feeding modes were carried out in this process:

- The first mode was one-time substrate feeding with the initial working volume of 50 and 75% of the total used volume marked as 50:50 and 75:25, respectively.
- The second mode was two-time substrate feeding with the initial working volume of 50% followed by feeding twice with 25% of the total working volume marked as (50:25:25).

- The third mode was three-time substrate feeding with the initial working volume of 25% followed by substrate feeding three times with 25% of the total working volume marked as (25:25:25:25).

The total time processes for hydrolysis and fermentation were 144 hours. $1x10^7$ equivalent CFU/ml *Streptomyces aegyptia* spores were inoculated at the beginning of the process followed by adding *Candida tropicalis* culture after 72 hours. In the first strategy, the feeding was applied after 72 hours; while in the second strategy the feeding was every 48 hours. In the third strategy, the feeding was every 24 hours. Samples were taken every 24 hours for reducing sugar and ethanol analysis.

2.9. Reducing sugar and ethanol determination

Reducing sugar ware determined by the 3,5-dinitrosalicylic acid DNS method **[21]**. One ml of broth cultures were withdrawn and centrifuged at 10,000 rpm for 3 min. Clear supernatant (0.5 ml) was mixed with 1.0 ml of 3, 5-dinitro salicylic acid (DNS) regent. The mixture was boiled for 5 min in vigorously boiling water, then cooled on ice and its optical density was determined at 540nm. The amount of reducing sugar was determined using a standard curve for pure glucose as a standard control. Ethanol was measured using the tri-n-butyl phosphate (TBP) solvent extraction method **[22],** and its content was estimated by the spectrophotometric method **[23].**

3. RESULTS

3.1. Process design

The production of bioethanol was performed in two process configuration modes, separate enzymatic hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) processes. In simultaneous saccharification and fermentation (SSF) mode the yeast, *Candida tropicalis* was added at beginning of culturing. However, in the other two modes (Separate Hydrolysis and Fermentation (SHF) & one–pot hydrolysis and fermentation) yeast, *Candida tropicalis* was added after three days of hydrolysis. In this study, the SSF model was considered a better process than SHF due to its rapid and highest ethanol production. Ethanol concentration was 100 mg/l (1%) in 120 hours of fermentation by SHF process and 173 mg/l (1.5%) of ethanol in 72 hours by SSF process. The highest ethanol concentration (200 mg/l) was observed in one pot hydrolysis and fermentation mode **as shown in figure (1).** The figure performed that time producing ethanol was shorter in simultaneous saccharification and fermentation (SSF) mode.

3.2. Substrate addition

In batch and fed-batch processes, **Fig (2, 3)** depicted the reducing sugar hydrolysis profiles of *Streptomyces aegyptia* and ethanol fermentation profiles of *Candida tropicalis*, respectively. Results showed that the one-time feeding (75:50) system performed better at sugar and ethanol concentrations of 255 mg/l and 260 mg/l, respectively. Ethanol concentration reached the highest level in the batch process mode at 96 h of culture. However, in one-feeding time the highest level of ethanol reached 120 h, and in two and three-feeding reached 144h.

4. DISCUSSION

Conversion of rice straw and manure residues into bioethanol is performed in sequential steps, including pretreatment, hydrolysis, and fermentation. The recalcitrant structure of rice straw and manure makes the pretreatment step essential for improving sugar and ethanol yield. The condition

of the pretreatment process applied in this study was chosen based on previous studies **[24, 25]**. Hydrolysis of (hemi) cellulose to sugar can be done using different lignocellulose-degrading microorganisms such as Streptomycetes **[26]**. The produced hexoses (six-carbon sugars) and pentoses (five-carbon sugars) are fermented by only a few microorganisms such as Candida **[27].** Several factors could improve the bioconversion of lignocellulose into bioethanol. This study aimed to compare the efficiency of ethanol production from rice straw and manure using different configuration modes. The effects of different substrate-feeding strategies on the efficiency of sugar and ethanol production were also studied.

In separate hydrolysis and fermentation (SHF) design, the hydrolysis and fermentation steps are performed sequentially in a separate reactor. However, the hydrolysis and fermentation processes in the simultaneous saccharification and fermentation process (SSF) design take place simultaneously in a single reactor with the same culture conditions **[6, 28]**. In this study, *Candida tropicalis* was added at the beginning of the culture in the SSF system resulting in faster ethanol detection (2 days of culturing) than in the SHF system (4 days of culturing). Consequently, volumetric ethanol productivity in the SSF system was 58% more than produced by the SHF system. It proved the fact that the continued process of hydrolysis with fermentation increased the amount of potential fermentable sugars for ethanol production. It would be also appropriate because the yeast is immediately converting the hydrolyzed sugars and no inhibiting concentration of sugars can be formed. Sugar accumulation is considered one of the most important factors that inhibit a battery of enzyme activity used in the bioconversion process **[7]**. These results also demonstrated the positive relationship between *Streptomyces aegyptia* and *Candida tropicalis* for ethanol production.

That pattern was similar to the results in one study **[8]** that compared SHF and SSF systems for bioethanol production from empty fruit bunch and found that ethanol yield of 4.7% was produced by SHF mode in 72 hours of fermentation and 6.05% of ethanol in 24 hours by SSF mode. Increasing in ethanol concentration between 72 and 120 h with a longer operational time of SHF in comparison with the SSF system was observed in this study and was similar to the behavior results of another study **[1].**

The problem with SSF in this study was the different time taking for hydrolysis by *Streptomyces aegyptia* and fermentation by *Candida tropicalis*. The growth rate and nature of actinomycete, *Streptomyces aegyptia* and yeast, *Candida tropicalis* are different. Thus, the time taken by *Streptomyces aegyptia* for lignocellulosic enzyme secretion was longer than sugar to be available at a sufficient level once the yeast, *Candida tropicalis* was grown in the medium **[26, 29]**. This point was overcome in one–pot hydrolysis and fermentation. In one-pot hydrolysis and fermentation, the hydrolysis and fermentation processes could perform sequentially in the same pot. Sugar produced from hydrolysis is sequentially metabolized by *Candida tropicalis*, thereby alleviating problems caused by product inhibition in the SHF process. Although one-pot hydrolysis and fermentation and SSF systems were performed in the same pot, the one-pot hydrolysis and fermentation design mode was preferred over SSF because *Streptomyces aegyptia* allowed degrading of the lignocellulose for some time before adding *Candida tropicalis* and that increased the ethanol concentration. This mode was also reported by other studies **[30],** in which maximizing bioethanol concentration was obtained from cellulose in a single reactor by two different microorganisms, a cellulase producer *Acremonium cellulolyticus* and an ethanol producer *Saccharomyces cerevisiae*. Integrated pretreatment, hydrolysis, and fermentation steps in a one-pot SSF process were carried out in one study, and optimized conditions for ethanol yielded 67.56% of the theoretical maximum**[11].**

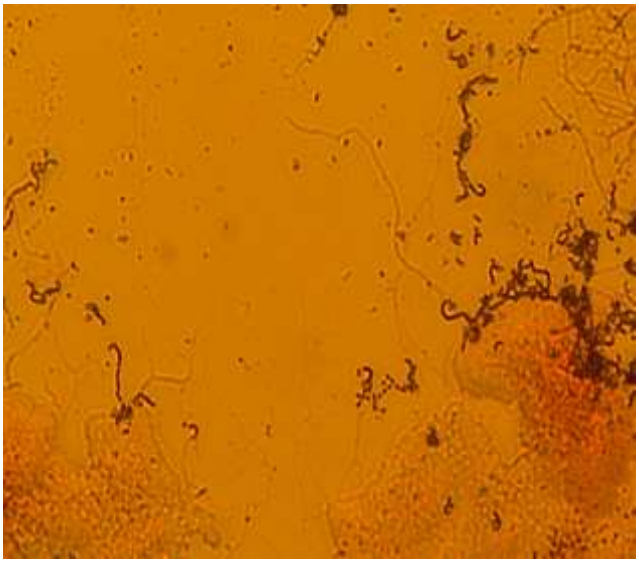

Photo (1): Light microscope photomicrograph of *Streptomyces aegyptia* (200 x magnification), showing substrate mycelium and hock ends of aerial mycelium.

Photo (2): Microscopic examination of *Candida tropicalis* (200x magnification of light microscope), showing pseudohyphae and yeast cells.

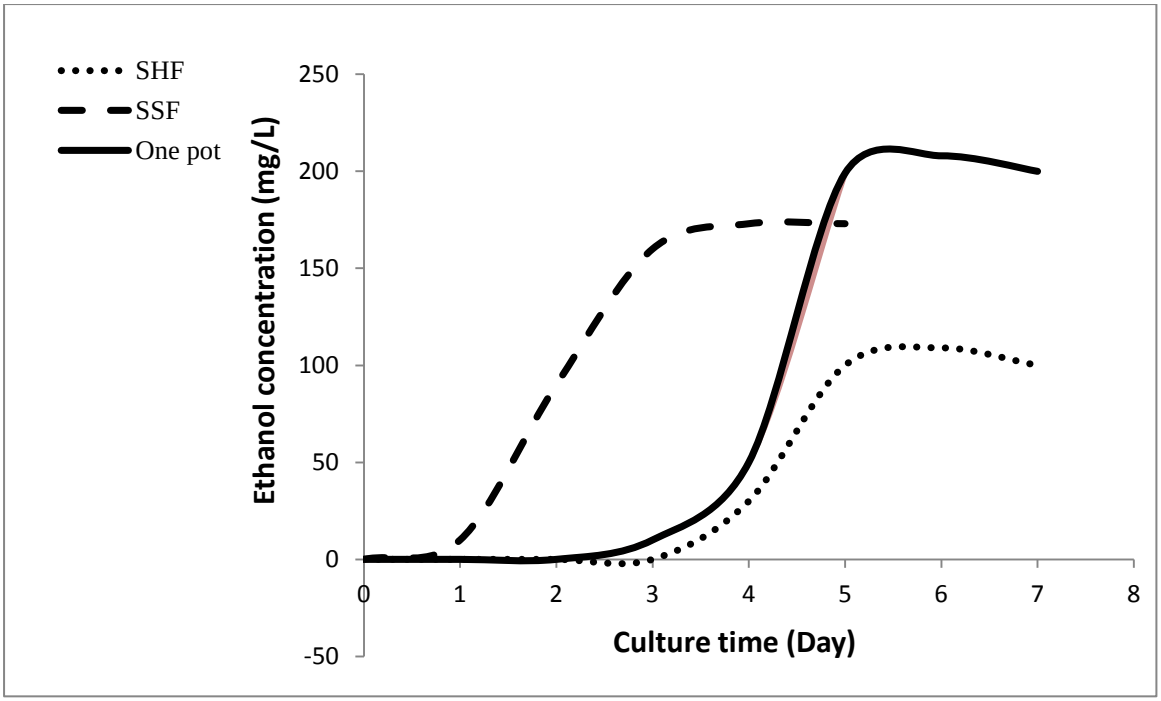

Figure (1): Ethanol Concentrations during separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), and one-pot hydrolysis and fermentation.

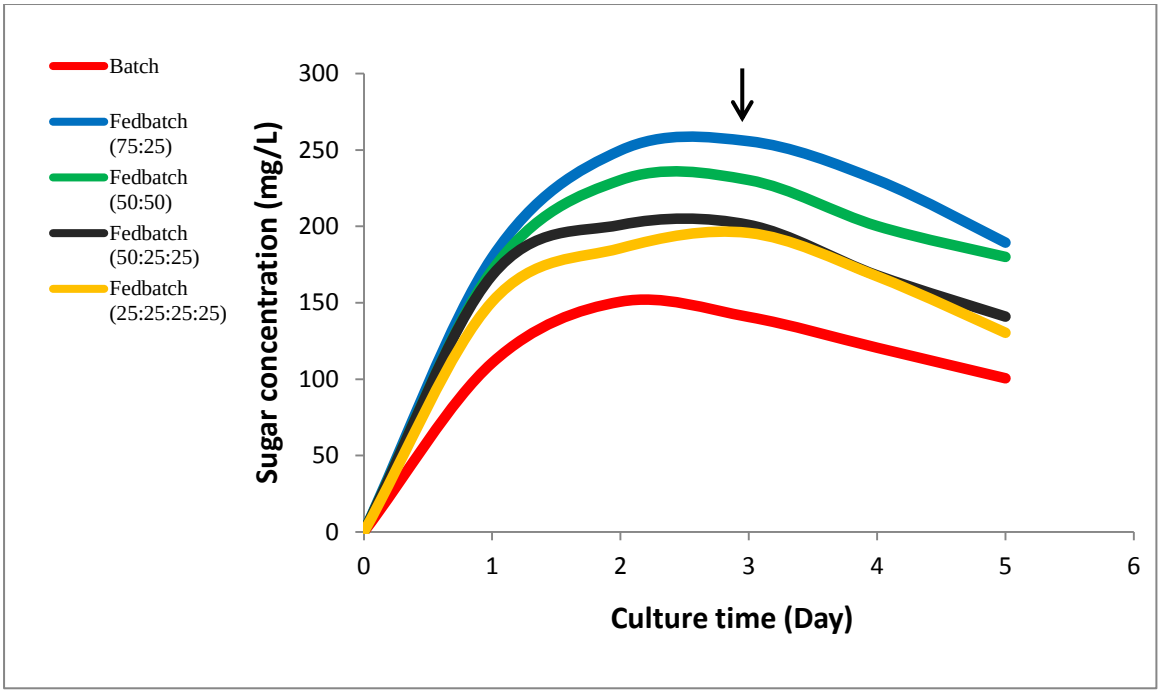

Figure (2): Sugar hydrolysis profiles of *Streptomyces aegyptia* in batch and fed-batch processes. Fed-batch feeding include: one-time feeding **(75:25 and 50:50) and** two-time feeding **(50:25:25) and** three-time feeding **(25:25:25:25)** strategies**.** The arrow indicated *Candida tropicalis* adding time.

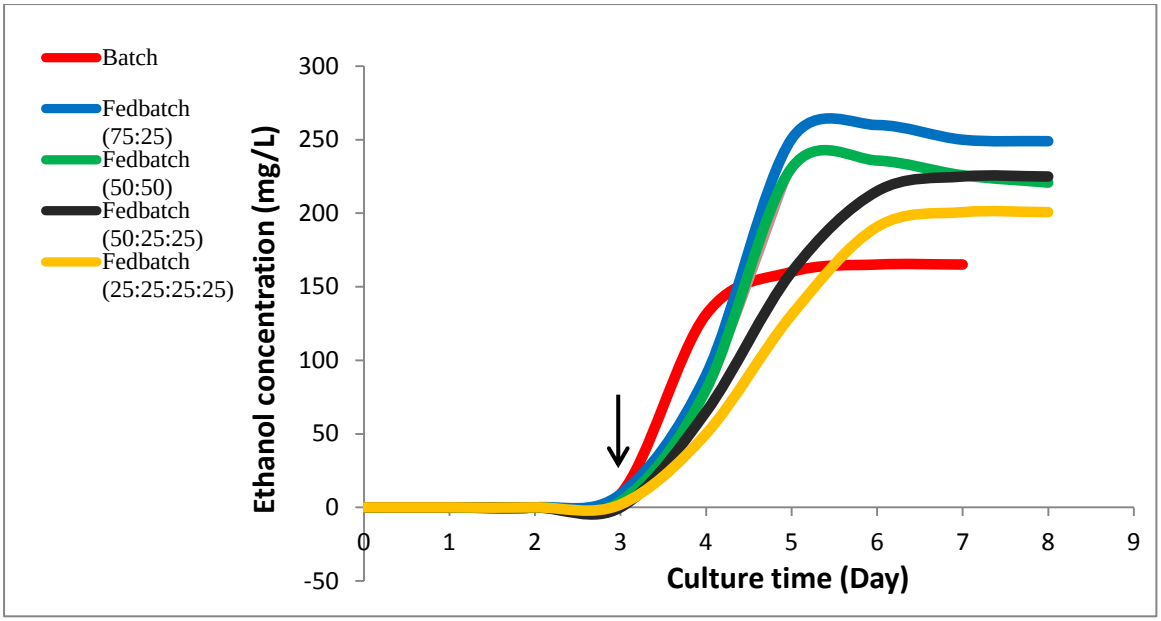

Figure (3): Ethanol fermentation profiles of *Candida tropicalis* in batch and fed-batch processes. Fed-batch feeding include: one-time feeding **(75:25 and 50:50) and** two-time feeding **(50:25:25) and** three-time feeding **(25:25:25:25)** strategies**.** The arrow indicated *Candida tropicalis* adding time.

Ethanol fermentation profiles in the batch and fed-batch processes were studied. In the batch system, all substrate concentration (4g/l) was added at the beginning of the process and that batch mode formed the less sugar concentration (150 mg/l) by *Streptomyces aegyptia*. It proved the fact that adding all insoluble substrates concentration in batch mode was found to affect the viscosity of the medium and the ratio of substrate to microbial enzymes in the culture, resulting in inhibiting the microbial activity and decreasing the sugar production **[31]**. Periodic substrate addition in fed-batch systems showed an increase in sugar production. The highest sugar concentration (255 mg/l) was in the one-time feeding system (75:25) followed by the one-time feeding system (50:50) than the two-time feeding system (50:25:25) and the three-time feeding system (25:25:25:25). Sugar concentration in the fed-batch (75:25) system was more 59% than in the batch system. It has been reported that fed-batching can prevent substrate inhibition during culturing**;** however, the nutrient addition should be controlled to be always sufficient to provide the food needed for microbial growth and more sugar production [**14**]. Extensive feeding (such as two and three-time feeding systems) couldn't be sufficient for higher microbial activity and sugar production. The trend of the ethanol concentrations was the same as the sugar concentrations, in which the one-time feeding system (75:25) produced the highest ethanol concentration (260 mg/l) followed by (50:50) than (50:25:25) and (25:25:25:25). Ethanol concentration in the fed-batch (75:25) system was more 63% than the batch system. These results elucidated that the best mode for the fed-batch cultures of *Streptomyces aegyptia* and *Candida tropicalis* was substrate concentrations of 3 g/l at the beginning of culture and feeding substrate at concentrations of 1 g/l after three days of incubation. Adding substrate concentrations of 4 g/l could inhibit ethanol production and also extensive feeding (such as two and three-time feeding systems) couldn't be sufficient for higher microbial activity and ethanol production. That behavior was similar to the results of several other studies **[13, 15, 16].** The effects of glucose feeding modes with different concentrations were performed in a study performed on *Saccharomyces cerevisiae* to evaluate ethanol production by batch and fed-batch and found that ethanol concentrations and yield were significantly higher in the fed-batch culture system than those of the batch cultures **[13]**. Another study **[15]** compared fed and fed-batch fermentation of sweet sorghum by *Saccharomyces cerevisiae* and found that fed-

batch fermentation improves the ethanol production efficiency in terms of ethanol concentration (120 g l^{-1}) and product yield (0.48 g g^{-1}) in compare with ethanol concentration (100 g l^{-1}) and product yield (0.42 g g^{-1}) in batch mode. The fermentation process for ethanol production by the fed and the fed-batch system was also carried out in a study **[16]** and found that ethanol yield (0.21 g/g) was higher in fed-batch mode compared with batch mode (0.12 g/g).

The time profiles of ethanol production during batch and fed-batch systems were different in this study. The maximum ethanol concentration level was reached in the batch process mode at 4 days of culture. However, in one-feeding time reached 5 days, and in two and three-feeding reached 6 days. The results indicated that the higher the substrate concentration, the faster the maximum ethanol rate will be reached **[15]**.

5. CONCLUSION

Bioconversion of rice straw and manure was applied by co-culture of *Streptomyces aegyptia* and *Candida tropicalis*. One-pot hydrolysis and fermentation mode design gave maximum ethanol production (200 mg/L) and bioconversion efficiency of 1.8% after 5 days of culturing at 30˚C. Fed-batch mode system with one-time feeding (75:25) was higher compared to other modes with ethanol production and bioconversion efficiency of 255 mg/L and 2.3%, respectively.

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