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RESEARCH ARTICLE

Bioethanol production from waste potatoes as an environmental waste management and sustainable energy by using cocultures *Aspergillus niger* and *Saccharomyces cerevisiae*

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Abstract

Fermentation of unhydrolyzed potato waste to ethanol by cocultures of *Aspergillus niger* and *Saccharomyces cerevisiae* was investigated at different temperatures (20°C to 50°C) and at different pH (4 to 7). Fermentation was done for 7 days for potato waste and the ethanol content was measured every 24 hours. The optimum pH and temperature for the fermentation of waste potatoes was found 6 and 30°C. With the optimized pH and temperature, fermentation was then carried out at different yeast concentration 3% to 12%. With the change in the concentration of yeast, the time required for the completion of fermentation decreased dramatically. Using a 12%, 9%, 6%, 3% yeast inoculum, maximum ethanol production was completely achieved in 2, 3, 5, 7 days respectively. The maximum ethanol yield from waste potatoes was 12.124%. *Aspergillus niger* strain B gave a higher production of ethanol. The amount of ethanol content increased with the increase in fermentation time.

Introduction

There are two main problems associated with the utilization of fossil fuels. First, the increasing and continuous use of fossil feed stocks as energy source is causing environmental problems and second, the supply of many of these feed stocks is approaching depletion. In recent years, the fermentative production of energy from renewable resources has been considered as an alternative to petrochemical processes. Ethyl alcohol or ethanol probably can be the best known of the alcohols alternative to petroleum. Ethyl alcohol is produced by chemical synthesis and by fermentation or biosynthetic processes.

Apart from the application of ethanol as a solvent, antifreeze as well as a fuel supplement, the major use of it is as an intermediate feedstock in the synthesis of innumerable organic chemicals. Bimolecular dehydration of ethanol gives diethyl ether, which is employed as a solvent and anesthetic. Dehydrogenation of ethanol yields acetaldehyde, which is the raw material for production of a large number of organic chemicals, such as acetic acid, acetic anhydride, chloral, butanol, aldehyde and hexanol. Reaction with carboxylic acids or anhydrides yields esters which are useful in many applications. The hydroxyl group of ethanol may be replaced by halogen to give ethyl halides. Treatment with sulfuric acid gives ethyl hydrogen sulfate and diethyl sulfate, a useful ethylating agent. Reaction of ethanol with aldehydes yields the respective diethyl acetals, and reaction with acetylene produces the acetals, as well as ethyl vinyl ether. These and other ethanol-derived chemicals are used in dyes, drugs, synthetic rubber, solvents,

detergents, plasticizers, surface coatings, adhesives, moldings, cosmetics, explosives, pesticides, and synthetic fiber resins (**Wyman, 1996**).

Currently there is a growing interest for ecological sustainable bio-fuels. In many parts of the world bio-ethanol is already used as additive in some gasoline products instead of toxic MTBE and TAMES (**EC report, 2000**). In India sugarcane molasses is the main raw material for ethanol production but now the short supply and increased cost is the main hindrance for its use. There are about 342 distilleries in the country with an installed capacity of over 3 billion litres of ethanol annually (**Narde, 2009**) which is short of requirement and is met through imports. The efficiency of ethanol production largely depends on the availability of suitable substrate, yeast strain and method employed. The sugary substrates available are comparatively expensive than molasses but can be easily used for ethanol production with some modification in the process. On the other hand cellulosic materials are cheaper and available in plenty but their conversion to ethanol involves many steps and is expensive. The starchy substrates are promising due to their economic viability and availability. Starchy crops like corn, barley, wheat, rice and tuber crops viz. potato, sweet potato are being exploited for the production of bioethanol worldwide (**Shigechi et al., 2004**). The world over production of potatoes in 2007 was 325.3 million tones while in India it was approximately 26 million tons (**Rani et al., 2009**) showing this as a promising crop but is being used for production of ethanol in some countries (**Kobayashi et al., 1998**). Moreover, potatoes are rich in starch, which makes it a cheap substrate for ethanol production. Potatoes can be an appropriate alternate substrate for bioethanol production due to their plenty availability, low cost and easy processing. The problems associated with its processing will also be less than in other grains. It is also semi-perishable food which can be stored for considerable period without spoilage. Good quality alcohol can be produced from potato which can be used for both fuel as well as potable purpose.

The production of industrial and fuel ethanol from starchy biomass commonly involves a three-step process (**Laluce et al., 1984**) : (i) liquefaction of starch by an endoamylase such as α-amylase; (ii) enzymatic saccharification of the low-molecular-weight liquefaction products (dextrins) to produce glucose; and (iii) fermentation of glucose to ethanol. Commercial amylases (frequently those produced by Aspergillus species) are used for liquefaction and saccharification of starch and represent a significant expense in the production of fuel alcohol from starchy materials.

The purpose of this study is the elimination of the enzymatic liquefaction and saccharification step by using symbiotic cocultures of amylolytic and sugar-fermenting organisms and to develop and evaluate a simultaneous single-step system for the enhanced fermentation of waste potatoes to ethanol by using symbiotic cocultures of Aspergillus species, which hydrolyze starch to glucose, and S. cerevisiae, which is non-amylolytic but efficiently ferments glucose to ethanol.

Materials and Methods

Isolation of microorganisms and its maintenance

Soil samples were collected randomly from three different sites from the top 2 cm of the soil profile. Approximately 50 g of soils were collected from each site and put into plastic bags and brought to the laboratory. All the soil samples were air-dried at room temperature (27±1°C) for 24 to 48 h. The dried soil samples were processed to remove stones and plant residues. 100 mg of each soil samples were transferred to labeled test tubes containing five milliliters of sterile saline (0.9% NaCl) (**Knudsen et al., 1995**). In order to suppress bacterial growth, 30 mg/l of streptomycin was added. Each of the test tubes were vortex mixed until all soil was well dispersed throughout the tube. 100 µl of each of the suspension was evenly spreaded on PDA plates with a spreader and incubated at 27±1°C. Mixed colonies on the plates were observed after 5–7 days. Pure culture of *Aspergillus niger* was obtained by streak plate method. It was then maintained on PDA slants at 4^{o}C. Yeast strain *Saccharomyces cerevisiae* (Bakers yeast) (Kwality, India) was obtained from the local market. It was maintained on PDA slants at 4^{o}C.

Starch hydrolysis test of isolated strains of *Aspergillus niger*

A loopful of pure culture was streaked on a sterile plate of starch agar medium. The inoculated plate was incubated at 27°C for 5 to 7 days. After incubation, Iodine reagent was added to flood the growth. Presence of clear zone surrounding colonies confirmed the positive result and accounts for their ability to digest the starch and thus indicates presence of alpha-amylase.

Pretreatment of waste potato substrates

Waste potato tubers were procured from the cold storage situated in Allahabad. Before processing potatoes, it was cleaned and free from sand, stones, soil, and potato foliage. Thoroughly washed unpeeled potatoes were cooked in a

pressure cooker in distilled water containing 0.5% potassium metabisulphite for 30 minutes. Boiled potatoes were mashed, dried at 70°C for about 7 hours in a hot air oven and ground to fine powder.

Simultaneous Saccharification and Fermentation (SSF) of Powdered Potato wastes

Ethanol fermentation was carried out in 250 ml flasks containing 5g powdered potato in 96 ml distilled water. The flasks was sterilized by autoclaving at 121°C for 30 min and inoculated with 4% (v/v) inoculum of *Aspergillus niger* and 3% (w/v) inoculum of Saccharomyces cerevisiae. The flasks were kept for incubation of 7 days for fermentation process and the ethanol content was measured every 24 hours.

Effect of temperature, pH and yeast inoculum on ethanol production

Fermentation of waste potatoes was carried out at different temperatures (20°C to 50°C) at pH 6 and at different pH (4 to 7) at 30°C. The optimum temperature and pH obtained during the course of investigation was further used for fermentation at different yeast concentration 3% to 12%.

Estimation of ethanol content by gas chromatograph

A gas chromatograph equipped with a flame ionization detector (FID) and data acquisition system with computer software (IRIS 32) was used to analyze the ethanol concentration. The installed column was a Capillary column (30 m). Temperature programming was implemented for the liquid sample analysis. During the analysis, the oven temperature was maintained at 80°C. The injector and detector temperatures were 120 and 160°C, respectively. The flow rate for carrier gas (Nitrogen) was set at 30 ml/min. The injection sample volume was 0.2 µl. The volume of standard ethanol used was 0.2 µl. The area of standard ethanol was found to be 1500.

The formula used for the calculation of percentage of ethanol is given below.

$$\text{Conc. of Ethanol} = \frac{\text{Volume of standard ethanol x Area of unknown sample}}{\text{Area of standard ethanol}}$$

$$\text{\% of Ethanol} = 100 - \left[\frac{\text{Volume of standard ethanol x conc. of ethanol}}{\text{Area of standard ethanol}} \times 100 \right]$$

Results and Discussion

The result of the investigation showed that the fermented potato waste produced a significant amount of ethanol. The volumetric production of ethanol varied according to the variations in temperature, pH and at different yeast concentrations. It also varied according to fermentation time and fungal strains.

Effect of pH on ethanol production

pH value has significant influence on alcoholic fermentation. pH of bioethanol produced from different wastes were determined. Yeast survives in a slightly acidic environment that is with pH between 4 to 6 .The ethanol production of samples was studied for inoculated sample for 7 days regularly and the changes were noted down. The percentage of ethanol production from waste potatoes at 24 hours interval for seven days at different pH by *Aspergillus niger* strain A, strain B and strain C is indicated in table 1-3 respectively. There are variations in ethanol yield from waste potatoes for different fungal strains at pH 6 (Table 4). The changes in ethanol yield from waste potatoes with the variation in pH (4 to 7) for seven days by *Aspergillus niger* strain B is indicated in figure 1. The variation in ethanol yield from waste potatoes for different fungal strains at pH 6 is indicated in figure 2. *Aspergillus niger* strain B was the most efficient strain yielding a higher value of ethanol as compared to other Aspergillus niger strains A and C. The highest bioethanol production was shown by *Aspergillus niger* strain B at pH 6 (11.994%) followed by pH 5 (10.299%), pH 7 (5.759%) and pH 4 (4.261%). **Mohamed and Reddy (1986)** reported optimal ethanol production from potatoes by cocultures of *Aspergillus niger* and *Saccharomyces cerevisiae* in the pH range 5 to 6. **Neelakandan and Usharani (2009)** also reported that the maximum ethanol yield at pH 6 from cashew apple juice using immobilized yeast cells by *Saccharomyces cerevisiae*. Similar finding was also reported by **Sharaghat et al. (2010)** in which the maximum ethanol production from molasses was found at pH 5.6 by using Saccharomyces cerevisiae. **Togarepi et al. (2012)** reported that the maximum rate of ethanol production at pH 6. **Mark et al. (2007)**

also produced the most ethanol by fermentations at initials pH 6.0. **Janani et al. (2013)** also recorded maximum ethanol production from grapes waste at pH 5.4

Table 1: Percentage of ethanol production from waste potatoes at 24 hours interval for seven days at different pH by *Aspergillus niger* strain A at 30ºC.

	Production of Ethanol (in %)						
pH / Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**
4	0.426	0.764	1.819	2.559	2.897	3.706	4.047
5	0.995	1.909	3.988	5.750	6.981	8.933	9.964
6	1.214	2.016	4.755	5.626	8.695	10.904	11.379
7	0.474	0.814	2.486	2.891	4.240	5.305	5.528

Table 2: Percentage of ethanol production from waste potatoes at 24 hours interval for seven days at different pH by *Aspergillus niger* strain B at 30ºC.

	Production of Ethanol (in %)						
pH / Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**
4	0.415	0.826	1.962	2.685	3.104	4.171	4.261
5	1.193	1.866	4.624	5.874	7.617	9.555	10.299
6	1.306	2.244	5.207	6.291	9.259	11.081	11.994
7	0.591	0.913	2.581	3.108	4.347	5.544	5.759

Table 3: Percentage of ethanol production from waste potatoes at 24 hours interval for seven days at different pH by *Aspergillus niger* strain C at 30ºC.

	Production of Ethanol (in %)						
pH / Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**
4	0.303	0.688	1.794	2.366	2.614	3.311	3.436
5	0.915	1.613	3.678	5.433	6.689	7.898	8.704
6	1.110	1.974	4.384	5.514	8.457	9.394	10.151
7	0.451	0.792	2.435	2.653	3.977	4.660	4.777

Table 4: Percentage of ethanol production from waste potatoes at 24 hours interval for seven days by different *Aspergillus niger* strains at pH 6 and at 30ºC.

	Production of Ethanol (in %)						
Strain / Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**
A	1.214	2.016	4.755	5.626	8.695	10.904	11.379
B	1.306	2.244	5.207	6.291	9.259	11.081	11.994
C	1.110	1.974	4.384	5.514	8.457	9.394	10.151

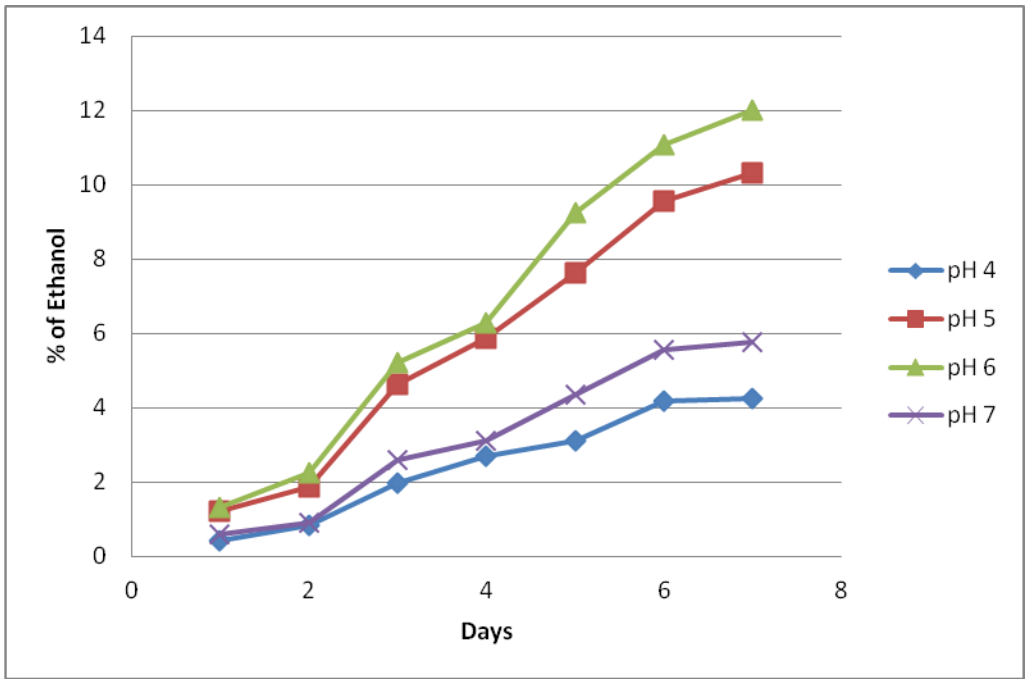

Figure 1: Variation in ethanol yield from waste potatoes with the change in pH (4 to 7) for seven days by *Aspergillus niger* strain B at 30ºC.

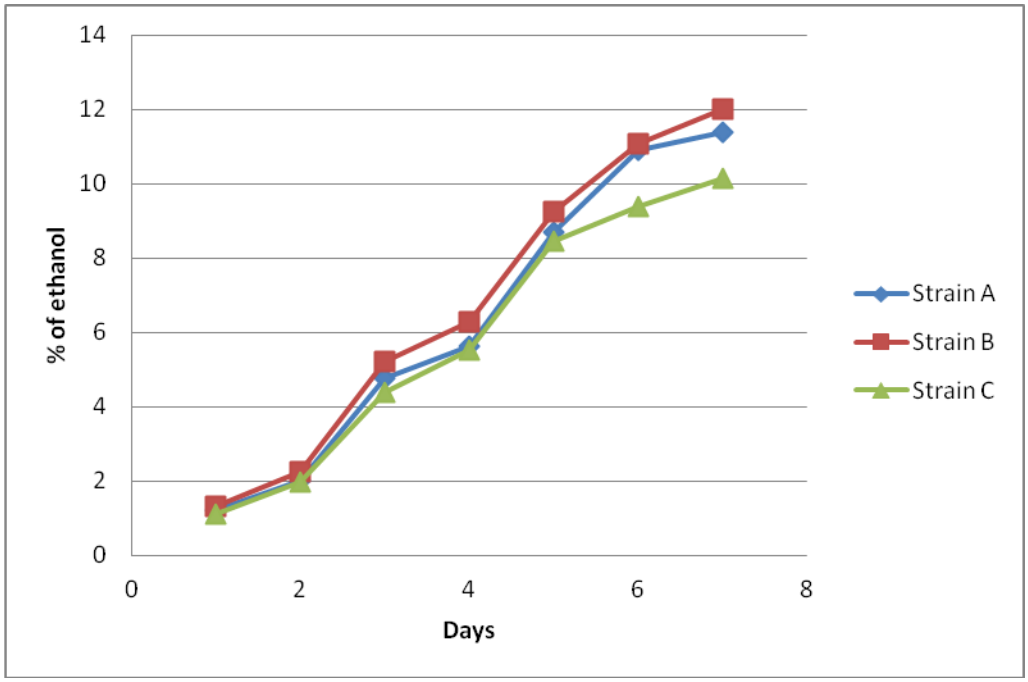

Figure 2: Variation in ethanol yield from waste potatoes by different *Aspergillus niger* strains at pH 6 and at 30ºC.

Effect of temperature on ethanol production

Temperature plays a major role in the production of ethanol, since the rate of alcoholic fermentation increases with the increase in temperature.The ethanol production was studied for inoculated sample for 7 days regularly and the changes were noted down. Ethanol production from waste potatoes at 24 hours interval for seven days at different temperatures by *Aspergillus niger* strain A, strain B and strain C was measured (Table 5 - 7). The variation in ethanol yield from waste potatoes for different fungal strains at 30ºC is indicated in Table 8. The variation in ethanol

yield from waste potatoes with the change in temperature (20ºC to 50ºC) for seven days by *Aspergillus niger* strain B is indicated in figure 3. The variation in ethanol yield from waste potatoes for different fungal strains at 30ºC is indicated in figure 4. *Aspergillus niger* strain B was the most efficient strain yielding a higher value of ethanol as compared to other Aspergillus niger strains A and C. The maximum ethanol production was observed at temperature 30ºC with 12.124%, followed by 40ºC (10.598%),, 20ºC (4.518%) and 50ºC (3.177%) respectively. **Janani et al. (2013)** also reported maximum ethanol yield at temperature 30°C. **Hadeel et al. (2011)** reported that the maximum ethanol production from rambutan fruit biomass using yeast Saccharomyces cerevisiae at temperature 30ºC. **Neelakandan and Usharani (2009)** also reported that the maximum ethanol yield from cashew apple juice using immobilized yeast cells by Saccharomyces cerevisiae that was obtained at 32 ºC. **Manikandan and Viruthagiri (2010)** found the optimum temperature to be 30ºC for the ethanol production from corn flour using *Aspergillus niger* and non starch-digesting and sugar-fermenting *Saccharomyces cerevisiae*. **Togarepi et al. (2012)** also reported maximum rate of ethanol production at a temperature of 30ºC. **Magdy et al. (2011)** reported that temperature in the range of 25-30°C is commonly found optimum for thermophilic *S. cerevisiae* strain for production of ethanol in SSF of various substrates, i.e. apple pomace (**Hang et al., 1986**), carob pod (**Roukas, 1994**), sweet sorghum (**Kargi and Curme, 2004**), etc. Rani et al. (2010) recorded maximum ethanol content of 56.8 g/l after 48 h of fermentation at 30°C. **Asli (2010)** observed best ethanol production rate at 32°C. On the other hand, when the temperature increases, enzymes begin to denature or unfold and thus become inactive. Each enzyme will have a different temperature range where it becomes inactive. Even if one essential enzyme stops working, the organism fails to grow. Hence, the first essential enzyme that gets deactivated defines the maximal temperature at which that organism can grow. At the lower end, it gets more complicated. Usually, the enzymes are not inactivated but rather just slow down (**Sanchez, 2007)**

Table 5: Percentage of ethanol production from waste potatoes at 24 hours interval for seven days at different temperatures by *Aspergillus niger* strain A at pH 6.

	Production of Ethanol (in %)						
Temp / Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**
20ºC	0.406	0.823	1.775	2.584	3.172	4.019	4.269
30ºC	1.187	1.955	4.969	5.907	8.703	10.711	11.559
40ºC	1.128	1.937	4.362	5.728	7.813	9.884	10.340
50ºC	0.426	0.563	1.308	1.817	2.378	2.521	2.860

Table 6: Percentage of ethanol production from waste potatoes at 24 hours interval for seven days at different temperatures by *Aspergillus niger* strain B at pH 6.

	Production of Ethanol (in %)						
Temp / Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**
20ºC	0.422	0.770	1.827	2.715	3.307	4.314	4.518
30ºC	1.194	2.311	5.240	6.415	8.906	10.888	12.124
40ºC	1.093	1.961	4.646	6.015	7.947	9.839	10.598
50ºC	0.542	0.682	1.441	1.929	2.659	3.007	3.177

Table 7: Percentage of ethanol production from waste potatoes at 24 hours interval for seven days at different temperatures by *Aspergillus niger* strain C at pH 6.

	Production of Ethanol (in %)						
Temp / Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**
20ºC	0.416	0.699	1.489	2.023	2.365	3.019	3.293
30ºC	1.132	2.072	4.475	5.798	8.296	9.332	10.063
40ºC	0.941	1.753	4.464	5.435	7.315	8.127	9.074

50ºC	0.274	0.608	1.143	1.566	1.857	1.989	2.063

Table 8: Percentage of ethanol production from waste potatoes at 24 hours interval for seven days by different *Aspergillus niger* strains at pH 6 and at 30ºC.

	Production of Ethanol (in %)						
Strain / Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**
A	1.187	1.955	4.969	5.907	8.703	10.711	11.559
B	1.194	2.311	5.240	6.415	8.906	10.888	12.124
C	1.132	2.072	4.475	5.798	8.296	9.332	10.063

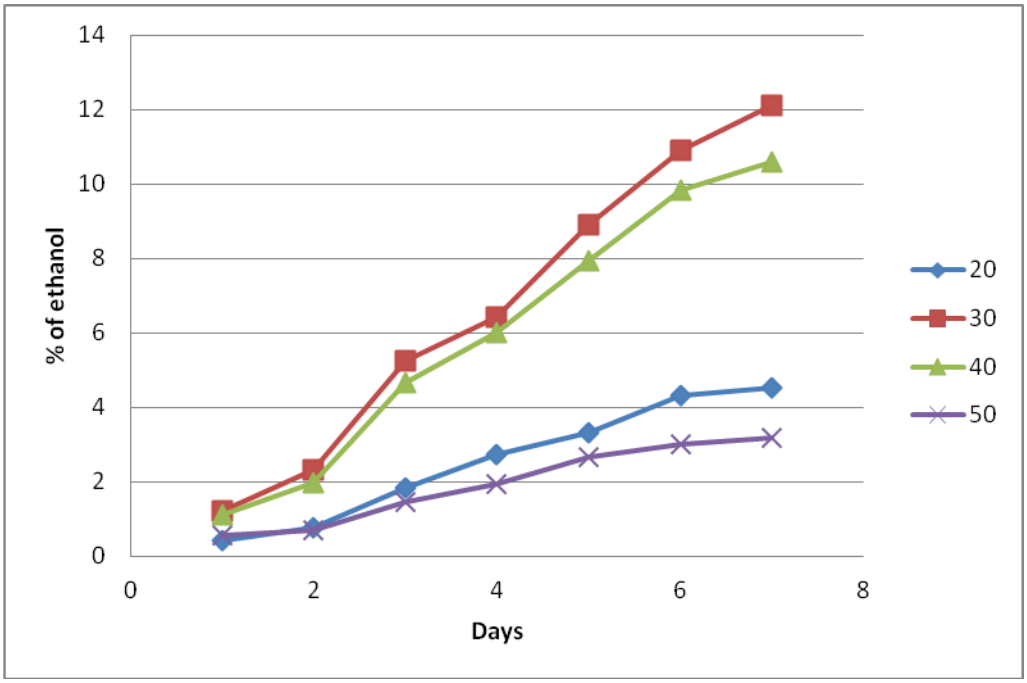

Figure 3: Variation in ethanol yield from waste potatoes with the change in temperature (20ºC to 50ºC) for seven days by *Aspergillus niger* strain B at pH 6.

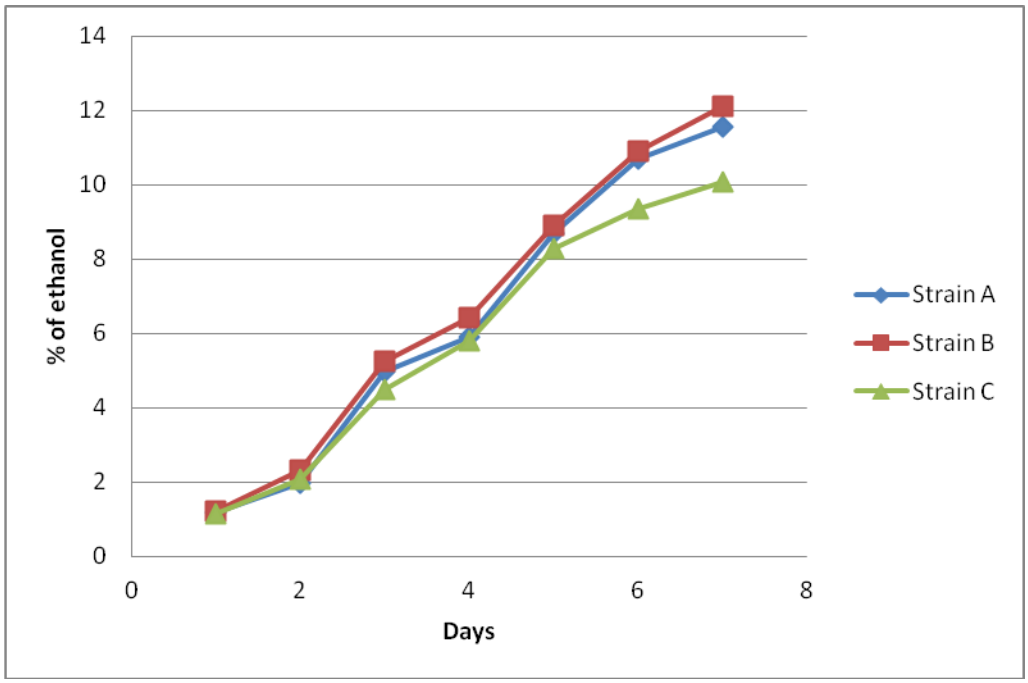

Figure 4: Variation in ethanol yield from waste potatoes by different *Aspergillus niger* strains at pH 6 and at 30ºC.

Variation of ethanol production due to yeast concentration

The ethanol yield of inoculated samples was measured at 24 hours interval and was recorded upto 7 days of incubation. Different concentrations of yeast were used as inoculums for fermentation. The fermentation time decreased dramatically with increase in yeast (*Saccharomyces cerevisiae*) concentration. Increasing *Saccharomyces cerevisiae* inoculum in the cocultures from 3% to 12% gave a dramatic increase in the rate of ethanol production. Using a 12%, 9%, 6%, 3% yeast inoculum, maximum ethanol production was completely achieved in 2, 3, 5, 7 days respectively. The percentage of ethanol production from waste potatoes at 24 hours interval for seven days at different yeast concentrations by *Aspergillus niger* strain A, strain B and strain C is indicated in table 9 - 11 respectively. The variation in ethanol yield from waste potatoes with the change in yeast concentration (3% to 12%) for seven days by *Aspergillus niger* strain B at the optimum temperature (30ºC) and at the optimum pH (pH 6) is indicated in Figure 5. **Mohamed and Reddy (1986)** reported that the increasing *Saccharomyces cerevisiae* inoculum in the cocultures Aspergillus niger and *Saccharomyces cerevisiae* from 4% to 12% gave a dramatic increase in the rate of ethanol production from potato starch. **Ocloo and Ayernor (2010)** also reported that the time taken for the fermentation to be completed was affected significantly by the yeast concentration. The results obtained supported the fact that the speed of fermentation depends on the yeast concentration, the higher the concentration, the shorter the fermentation period required to achieve maximum alcohol yield **(Kordylas, 1990)**. **Ueda et al. (1981)** reported 5 days fermentation period for raw cassava root starch using 15% yeast suspension. **Togarepi et al. (2012)** reported increased production rate rapidly with the increase in the amount of yeast up to the yeast concentration of 8 g/20 g fruit pulp. Beyond that point the rates no longer significantly increased. At this point the substrate becomes limiting and increasing the yeast amount does not increase the rate of reaction.

Table 9: Percentage of ethanol production from waste potatoes at 24 hours interval for seven days at different yeast concentrations by *Aspergillus niger* strain A at the optimum temperature (30ºC) and at the optimum pH (pH 6).

	Production of Ethanol (in %)						
Yeast Conc / Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**
3%	1.240	2.045	4.685	5.610	8.353	10.306	11.514
6%	1.641	4.058	7.109	10.302	11.501	11.510	11.529
9%	3.384	7.910	11.533	11.538	11.546	11.600	11.634
12%	5.443	11.54	11.548	11.549	11.555	11.562	11.568

Table 10: Percentage of ethanol production from waste potatoes at 24 hours interval for seven days at different yeast concentrations by *Aspergillus niger* strain B at the optimum temperature (30ºC) and at the optimum pH (pH 6).

	Production of Ethanol (in %)						
Yeast Conc / Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**
3%	1.533	2.087	5.174	6.420	8.717	10.821	12.106
6%	1.946	4.184	7.512	10.755	11.989	12.003	12.011
9%	3.459	8.290	11.969	11.993	12.066	12.087	12.108
12%	5.684	11.973	11.96	12.006	12.058	12.127	12.040

Table 11: Percentage of ethanol production from waste potatoes at 24 hours interval for seven days at different yeast concentrations by *Aspergillus niger* strain C at the optimum temperature (30ºC) and at the optimum pH (pH 6).

	Production of Ethanol (in %)						
Yeast Conc / Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**
3%	1.078	1.997	4.215	5.246	7.651	9.304	9.932
6%	1.395	3.634	6.634	8.757	9.866	9.901	9.904
9%	3.014	7.022	9.927	9.927	9.929	9.933	9.934
12%	4.546	9.897	9.920	9.923	9.926	9.954	9.956

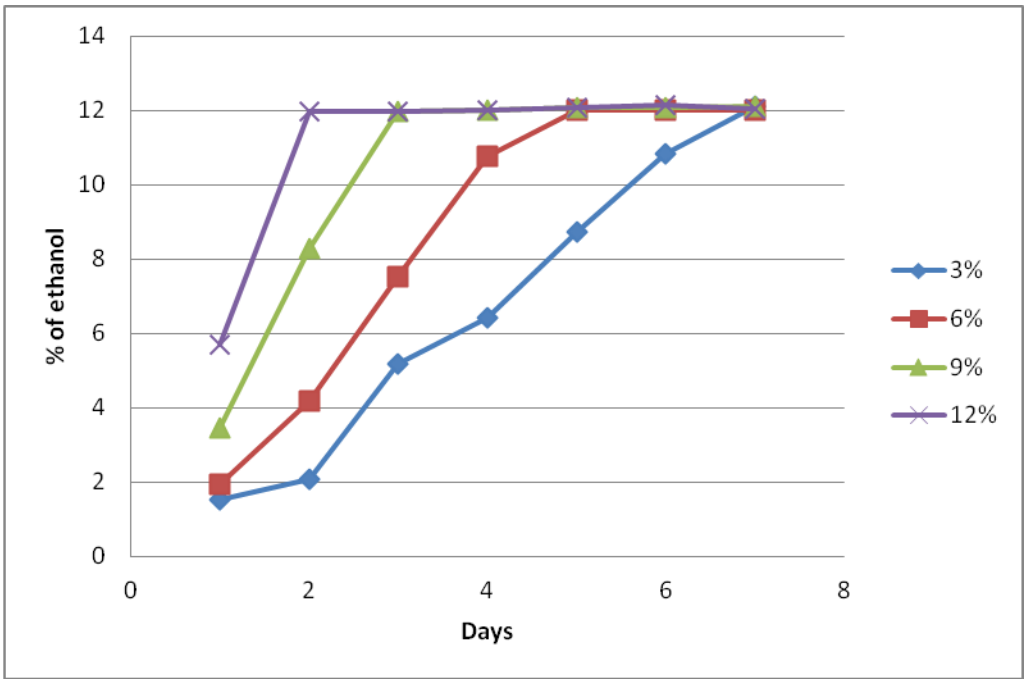

Figure 5: Variation in ethanol yield from waste potatoes with the change in yeast concentration (3% to 12%) for seven days by *Aspergillus niger* strain B at the optimum temperature (30ºC) and at the optimum pH (pH 6).

Conclusion

The optimum pH for the fermentation of waste potatoes was found to be 6 while the optimum temperature was found to be 30°C. Fermentation was then carried out at different yeast concentration 3% to 12%. With the change in the concentration of yeast, the time required for the completion of fermentation decreased dramatically.The amount of ethanol content increased with the increase in fermentation time. Simultaneous fermentation of starch to ethanol can be conducted efficiently by using cocultures of the amylolytic fungus *Aspergillus niger* and a non-amylolytic sugar fermenter, *Saccharomyces cerevisiae*.

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