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Research Article

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In-vitro Optimization of Bio-ethanol Production from Agro wastes using *Trichoderma* sps.

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ABSTRACT

This research was aimed at bio-ethanol production by fungi capable of producing cellulases and to convert pre-treated lingo-cellulosic material to fermentable sugars. The lingo-cellulosic material such as sugarcane bagasses, sugarcane leaves, rice husk or wheat bran were used as substrates. Fungi were isolated from soil samples collected from various regions. The pure cultures were screened for the ability to degrade cellulose. The fungi capable of cellulose production were identified as Trichoderma sp based on colony characters, microscopic observation and identification. The substrates were powdered and pretreated with fungal isolates using Mandels' and Reese media. The substrates were used as a carbon source. Then optimization studies were carried out by using five bio-mass substrates at different pH, temperature and incubation period. Analysis was done by using Gas Chromatography. Sugarcane bagasses, Juice waste, Rice husk, Wheat bran, and Dry leaves were treated with Trichoderma isolates. Sugarcane bagasse and juice waste have shown highest concentration of reducing sugars of 45.95 mg/g and 40.56 mg/g respectively and ethanol yield of 51.15 % and 46.5 % respectively. Dry leaves, Wheat bran and Rice husk have shown less reducing sugars of 33.32 mg/g, 30.32 mg/g, and 29.45 mg/g and ethanol yield 11.1 %, 7.15 %, and 6 % respectively as compared with sugarcane bagasse and juice waste.

Key words: *Biodegradation, Bio-ethanol, Trichoderma sp fungi, Ligno-celluloses substrates, Cellulase, Gas Chromatography.*

INTRODUCTION

Long-term economic and environmental concerns have resulted in a great amount of research in the past couple of decades on renewable sources of liquid fuels to replace fossil fuels. Burning fossil fuels such as coal and oil releases CO_2, which is a major cause of global warming[1]. Conversion of abundant lingo-cellulosic biomass to bio-fuel as transportation fuels presents a viable option for improving energy security and reducing greenhouse emissions[2]. Several reviews have been published on the theme of fuel ethanol production especially from lingo-cellulosic biomass[3]. Ligno-cellulosic material from different crop residues have been used for conversion to ethanol[4]. The major lingo-cellulosic material found in great quantities to be considered, especially in tropical countries, is sugarcane bagasse, the fibrous residue obtained after extracting the juice from sugar cane (*Saccharum officinarum*) in the sugar production process[5] and sugarcane trash, the left-over residue of leaves and tops. The presence of lignin in lignocelluloses leads to a protective barrier that prevents plant cell destruction by fungi and bacteria for conversion to bio-fuel. For the conversion of biomass to bio-fuel, the celluloses and hemicelluloses must be broken down into their corresponding monomers (sugars), so that microorganisms can utilize them[6]. But these require pre- treatment for obtaining reducing sugars and conversion of the same to ethanol. The various types of pretreatments and efficient microorganisms have been reviewed here.

MATERIALS AND METHODS

Isolation & Screening of cellulolytic fungi *Trichoderma sp* for conversion of agro biomass into fermentable sugars was isolated from from soil samples by dilution plate methods and plated onto Potato dextrose agar and the isolated colonies were further screened for cellulase production on on Mandels' and Reese agar medium (Selective media).

Substrate Treatment

Five substrates namely Sugarcane bagasses, Juice wastes, Dry leaves, Rice husk and Wheat bran were collected. Each of the substrate wastes were taken and dried in a hot air oven at 100^{o}C for two days and the dried substrate was powdered and sieved into a 1mm sieve. The powder of each substrate was used as carbon source.

Optimization of the substrate, inoculation time, pH, temperature & Production of Bio-Ethanol under both physical and chemical conditions were carried out to estimate the optimum conditions.

Analytical methods:

After spore inoculation, the media was incubated at room temperature for a duration of 7 days. After the incubation period the media was filtered and the amount of reducing sugars were estimated by DNS method. The filtered supernatant was autoclaved and inoculated with 3% v/v of *Saccharomyces cerivaceae.* The media was incubated for 15 days and the samples were collected to check ethanol production at regular alternative days like 4, 7, 10 & 13 days. The supernatants were collected and the Bio-ethanol assay was carried out using Gas Chromatography method.

Assay Method:

The sample / Tube showing the highest production value, was considered as the best solid substrate. The best solid substrate was selected and used in subsequent experiments for optimization.

Calculation:

$$\text{Ethanol concentration (µL/ 0.2 µL)} = \frac{\text{Area of Sample} \times \text{Vol. of Std Ethanol}}{\text{Area of Std Ethanol}}$$

$$\% \text{ of Ethanol} = 100 - \left\{ \frac{\text{Vol. of Control} - \text{Vol. of Sample}}{\text{Vol. of control}} \times 100 \right\}$$

Distillation & Ethanol estimation:

The ethanol, produced from the fermentation process was purified by fractional distillation & The ethanol was estimated by Gas chromatography analysis.

Estimation of total carbohydrate, Reducing and Non reducing sugar.

Determination of Total Carbohydrate

The carbohydrate content of untreated and pretreated raw material in the culture broth was measured by phenol sulphuric acid method (Krishnaveni *et al.,* 1984) using glucose as standard. The amount of total sugars present in the sample is calculated using the standard curve.

Determination of Reducing Sugars:

Reducing sugars in untreated and pretreated raw material in the culture broth were determined by DNS method (Miller, 1972) with glucose as standard. The amount of reducing sugars present in the sample is calculated using the standard curve.

Determination of Non-reducing Sugars

The concentration of non reducing sugars was determined by taking the difference in concentrations of Total sugars and reducing sugars.

Non-reducing sugar = (Total sugar – Reducing sugar)

RESULT AND DISCUSSION

Among the five substrates, sugarcane bagasses pre-treated with *Trichoderma sp* isolate has given maximum ethanol yield (51.15 %) followed by juice waste pretreated with the same culture 46.5 %. The

other substrates (Wheat bran, Rice husk, Dry leaves) pretreated with *Trichoderma sp* isolate have moderately increased the ethanol content. At optimum condition, the bio-ethanol concentration of sugarcane bagasse distilled sample was 87 % at pH 6 and temperature 30 ^{0} C after 13 days incubation. Similar to our result, (*Frain et al., 1982)* also obtained the same result with his study on solid state fermentation of *Trichoderma reesei* for cellulase production on agro residues around ~13 days incubation. Earlier studies have revealed that fungi required slightly acidic pH for optimum growth. pH is known to affect the synthesis and secretion of cellulase for degradation of cellulose (Ting *et al. 2005*). The obtained results of similar research were agreed that reported by *Rashmi Kataria, and Sanjay Ghosh.(2011)*. However, there are reports in which higher ethanol obtain at higher temperature.

3.4 Sugar estimation:

Total sugar, reducing sugar, non-reducing sugar content of each substrate was determined using Phenol sulphuric acid method and DNS method respectively. Estimation of sugars was done for untreated and pretreated samples and the concentrations of sugars were compared. Concentration of reducing sugar, non reducing sugar and total sugar of treated samples as compared with the untreated (control) samples is shown in Table.

Table 1: Sugar content of Substrates before and after inoculation of Fungi

Sr. No.	Substrate name	Before Fungal Inoculation			After Fungal Inoculation		
		Reducing sugar (mg/ml)	Non-reducing sugar (mg/ml)	Total sugar (mg/ml)	Reducing sugar (mg/ml)	Non-reducing sugar (mg/ml)	Total sugar (mg/ml)
1	Dry Leave	0.62	1.07	1.69	33.32	21.02	54.34
2	Juice waste	0.88	1.15	2.03	40.56	31.34	71.90
3	Rice husk	0.56	0.92	1.48	29.45	18.86	48.31
4	Sugarcane bagasse	0.98	1.27	2.25	45.95	30.05	76.0
5	Wheat bran	0.51	0.90	1.41	30.32	19.09	49.41

Figure 1:Bioethanol Production From Different wastes by trichorderma isolates

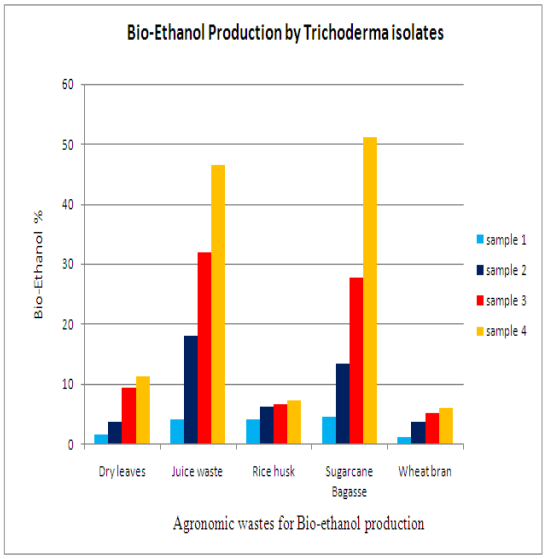


Figure 2: Bio-ethanol Production at different incubations intervals(days)

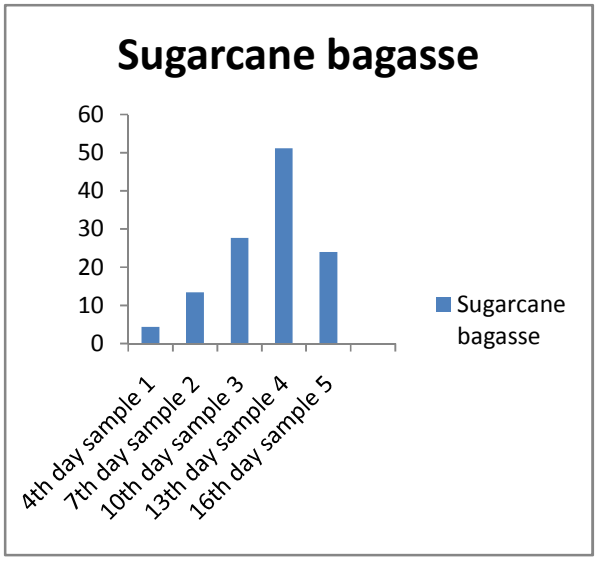


Figure 3: Optimization of pH conditions for Bio-ethanol production

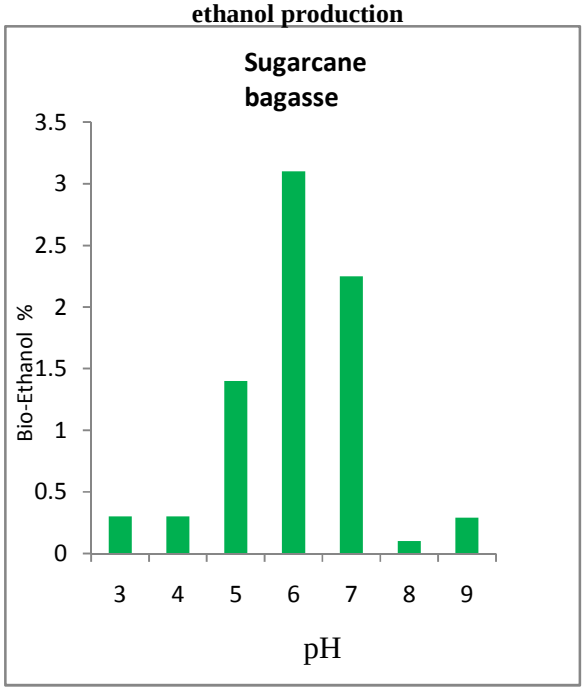
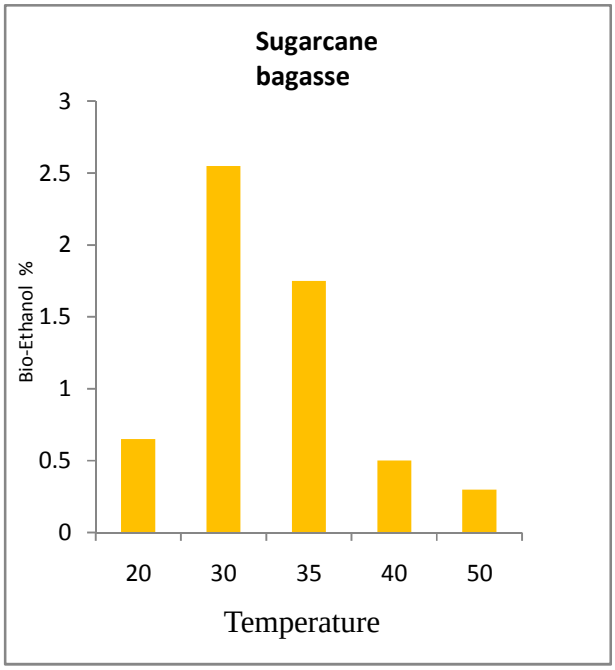


Figure 4: Optimization of Temperature for Bio-ethanol



Result Table (Uncal - C:\Documents and Settings\Administrator\Desktop\milan pH ethanol\milan\Ethanol-std)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	0.823	1457.289	684.712	100.0
	Total	1457.289	684.712	100.0

Result Table (Uncal - C:\Documents and Settings\Administrator\Desktop\milan pH ethanol\milan\control ethanol)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	0.913	10.013	2.796	100.0
	Total	10.013	2.796	100.0

Result Table (Uncal - C:\Documents and Settings\Administrator\Desktop\milan pH ethanol\milan\dRy leave\dry leave sample 4)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	1.023	161.543	22.605	54.6
2	1.257	73.374	6.245	24.8
3	2.243	3.288	0.515	1.1
4	2.760	2.760	0.437	0.9
5	3.257	3.452	0.475	1.2
6	3.443	10.011	1.367	3.4
7	3.600	21.592	2.440	7.3
8	3.783	19.824	2.263	6.7
	Total	295.844	36.348	100.0

Result Table (Uncal - C:\Documents and Settings\Administrator\Desktop\milan pH ethanol\milan\juice waste\juice waste sample 4)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	0.840	679.779	281.193	96.3
2	1.093	6.039	2.106	0.9
3	1.233	20.166	2.557	2.9
	Total	705.983	285.855	100.0

Result Table (Uncal - C:\Documents and Settings\Administrator\Desktop\milan pH ethanol\milan\rice husk\rice husk 4)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	1.040	103.927	21.500	58.9
2	3.550	31.817	2.908	18.0
3	3.760	40.693	3.408	23.1
	Total	176.437	27.816	100.0

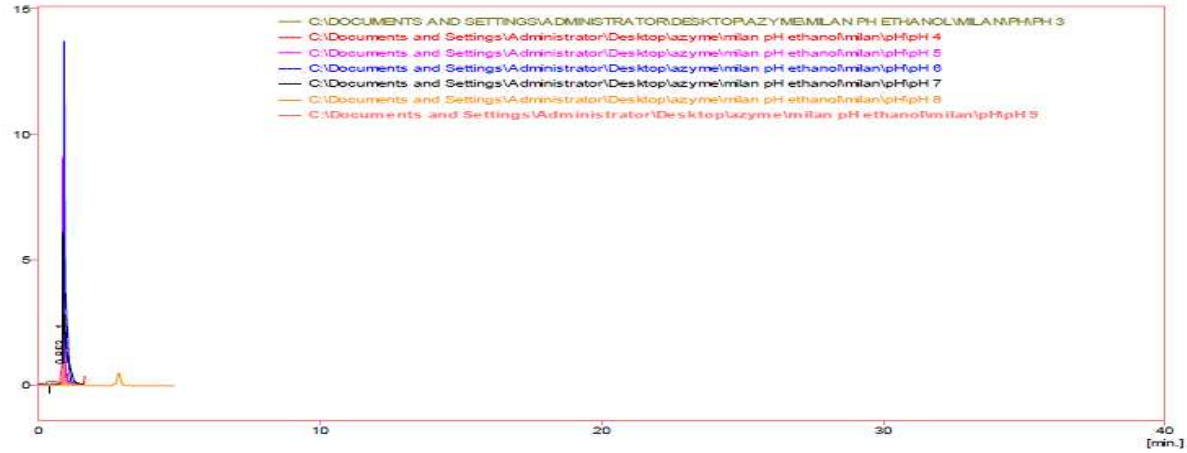
Result Table (Uncal - C:\Documents and Settings\Administrator\Desktop\milan pH ethanol\milan\wheat bran\whea bran sample 4)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	0.987	86.813	17.450	58.1
2	1.543	2.576	0.316	1.7
3	3.267	2.152	0.362	1.4
4	3.460	16.953	1.959	11.3
5	3.787	40.882	2.821	27.4
	Total	149.375	22.909	100.0

Result Table (Uncal - C:\Documents and Settings\Administrator\Desktop\milan pH ethanol\milan\sugarcane\sugarcane bagasses sample 4)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	0.967	745.292	123.770	97.5
2	1.473	10.824	1.160	1.4
3	2.497	3.838	0.362	0.5
4	3.413	1.331	0.211	0.2
5	3.810	3.222	0.452	0.4
	Total	764.507	125.956	100.0

Column	: Capillary	Detection	: FID
Mobile Phase	: Carrier gas	Temperature	: Oven temp 80 C
Flow Rate	: 2 bar	Pressure	: 5 lb/in2
Note	: pH optimization		
Autostop	: None	External Start	: Start - Restart, Down
Detector 1	: Signal 1	Range 1	: Bipolar, 1250 mV, 10 Samp. per Sec.



Result Table (Uncal - C:\DOCUMENTS AND SETTINGS\ADMINISTRATOR\DESKTOP\AZYME\MILAN PH ETHANOL\MILAN\PH\PH 3)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	0.890	4.748	1.032	100.0
	Total	4.748	1.032	100.0

Result Table (Uncal - C:\Documents and Settings\Administrator\Desktop\azyme\milan pH ethanol\milan\pH\pH 4)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	0.897	4.174	1.073	100.0
	Total	4.174	1.073	100.0

Result Table (Uncal - C:\Documents and Settings\Administrator\Desktop\azyme\milan pH ethanol\milan\pH\pH 5)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	0.793	0.868	0.552	4.0
2	0.870	20.720	9.059	96.0
	Total	21.588	9.611	100.0

Result Table (Uncal - C:\Documents and Settings\Administrator\Desktop\azyme\milan pH ethanol\milan\pH\pH 6)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	0.907	45.585	13.615	100.0
	Total	45.585	13.615	100.0

Result Table (Uncal - C:\Documents and Settings\Administrator\Desktop\azyme\milan pH ethanol\milan\pH\pH 7)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	0.867	32.440	6.011	100.0
	Total	32.440	6.011	100.0

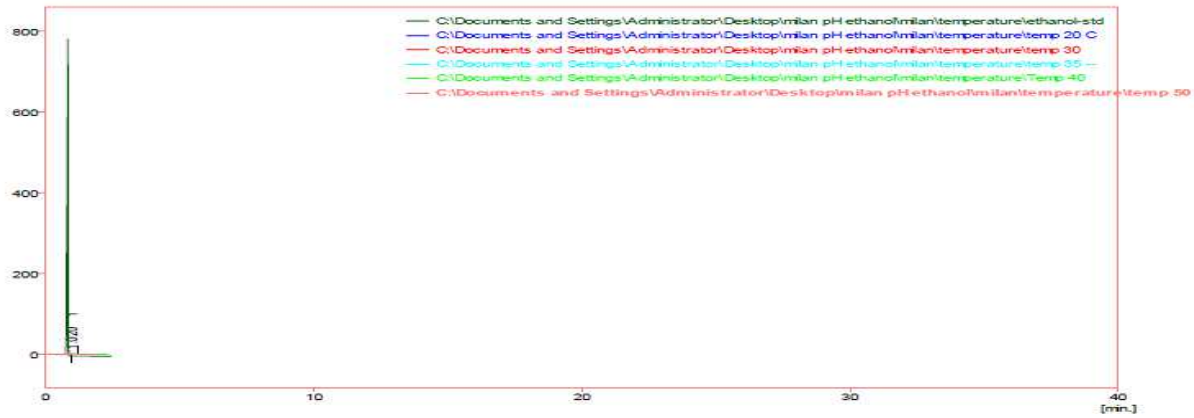
Result Table (Uncal - C:\Documents and Settings\Administrator\Desktop\azyme\milan pH ethanol\milan\pH\pH 8)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	0.890	1.442	0.843	27.6
2	2.850	3.792	0.468	72.4
	Total	5.235	1.311	100.0

Result Table (Uncal - C:\Documents and Settings\Administrator\Desktop\azyme\milan pH ethanol\milan\pH\pH 9)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	0.853	4.255	0.569	100.0
	Total	4.255	0.569	100.0

Column	: Capillary	Detection	: FID
Mobile Phase	: Carrier gas	Temperature	: Oven temp 80 C
Flow Rate	: 2 bar	Pressure	: 5 lb/in2
Note	: Temperatuer optimizaion		
Autostop	: None	External Start	: Start - Restart, Down
Detector 1	: Signal 1	Range 1	: Bipolar, 1250 mV, 10 Samp. per Sec.



Result Table (Uncal - C:\Documents and Settings\Administrator\Desktop\milan pH ethanol\milan\temperature\ethanol-std)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	0.823	1457.289	684.712	100.0
	Total	1457.289	684.712	100.0

Result Table (Uncal - C:\Documents and Settings\Administrator\Desktop\milan pH ethanol\milan\temperature\temp 20 C)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	0.900	9.314	3.996	100.0
	Total	9.314	3.996	100.0

Result Table (Uncal - C:\Documents and Settings\Administrator\Desktop\milan pH ethanol\milan\temperature\temp 30)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	0.873	37.140	9.622	100.0
	Total	37.140	9.622	100.0

Result Table (Uncal - C:\Documents and Settings\Administrator\Desktop\milan pH ethanol\milan\temperature\temp 35 –)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	0.907	25.323	5.590	100.0
	Total	25.323	5.590	100.0

Result Table (Uncal - C:\Documents and Settings\Administrator\Desktop\milan pH ethanol\milan\temperature\Temp 40)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	1.007	7.177	1.180	100.0
	Total	7.177	1.180	100.0

Result Table (Uncal - C:\Documents and Settings\Administrator\Desktop\milan pH ethanol\milan\temperature\temp 50)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	1.020	4.089	1.202	100.0
	Total	4.089	1.202	100.0

CONCLUSION

This research has been carried out in order to produce Bio-ethanol from ligno-cellulolytic wastes by using cellulolytic fungi like *Trichoderma sp.* ***Trichoderma sp*** fungi obtained from the soil sample were able to successfully degrade the lingo-cellulolytic wastes to produce bio-ethanol. Upon performing optimization studies, the production of bio-ethanol is maximum at **pH 6** and a **temperature 30°C.** Higher yield bio-ethanol was obtained from the **sugarcane bagasse 51.15 %** compare to rest of the waste used in this study.

Fungal cultures having the potential to degrade cellulosic materials were identified in this study. These cultures can be used to hydrolyze the pretreated ligno-cellulytic waste material for the production of Bio-ethanol which can be used as an alternative to the depleting fossil fuels.

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