ORIGINAL ARTICLE

Advances in Basic Medical Science

Role of Penicillium Digitatum in Production of Single Cell Proteins from Citrus Reticulablanca Peels

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ABSTRACT

Objective

The potential use of kinnow peels as a substrate for *Penicillium digitatum* to manufacture single cell protein was investigated in this work.

Methodology

Sulfuric acid in concentrations of 0.15, 0.30, and 0.45 N were used to pre-treat the substrate. The kinnow peels hydrolysate (KPH) medium and glucose supplemented kinnow peels hydrolysate medium were employed in this study.

Results

Kinnow peels hydrolysate produced 2.28%, 2.23%, and 2.07% crude protein at 0.15, 0.30, and 0.45 N per 20g of substrate utilized, respectively. Furthermore, on supplemented kinnow peels hydrolysate medium with inorganic nitrogen sources at 0.15, 0.30, and 0.45 N, the percentage of protein in single cell protein was only 2.00%, 2.04%, and 2.15%, respectively. At 0.15, 0.30, and 0.45N, glucose was added to the augmented kinnow peels hydrolysate medium, yielding 2.12%, 2.02%, and 2.12%, respectively.

Conclusion

There was no effect of digestion normalities in case of KPH. The effect of normalities was noted when kinnow peels digest was supplemented with glucose. There was a decreasing trend in SCP production with increase in normalities. No effect was noted when KPH was combined with M.M and glucose.

Keywords

Single Cell Proteins, Hydrolysate, *Penicillium digitatum,* KPH, Amino Acids, kinnow peels

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INTRODUCTION

Malnutrition affects a large proportion of the world's population, particularly those living below the poverty line. There is a large disparity between the demand for high-protein foods and their availability. Because necessary amino acids cannot be substituted by other amino acids, the availability of a specific and unique protein type creates a difficulty. Single cell protein is now the only viable answer to this massive challenge.[1-6]

Furthermore, *Candida arborea* and *Candida utilis* were utilized throughout World War II, and around 60% of the country's pre-war food supply was replaced.[6] The rapidly increasing world population generates the challenge of providing additional necessary food sources. One possible solution to this problem is the production of SCP to meet the need of protein rich food. Bacteria and fungus are top ranked for the synthesis of SCP. Protein contains the elemental nitrogen in addition to carbon, hydrogen and oxygen.[8] Intensive or extensive production of protein from animal sources like meat depends on protein from plants and grass they are grazing on and for the production of animal feeds requires large areas of land. Single cell protein is gaining popularity day by day because they require limited land area for growth and development.[9] Orange, papaya, onion, and sugarcane wastes [10], wheat straw [11], banana wastes [12], pineapple [13], rape straw [14], and watermelon wastes [15], wheat straw,[16] when some of these waste materials are biodegraded by microorganisms, they can generate environmental pollution like

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foul odors. Population growth in combination with rapidly, increasing demand for meat nutrition are creating a protein deficit between the meat available and the expected demand in 2050 and following years. Global demand for meat as a protein source is steadily increasing. Over the past 50 years, meat production was increased more than three times. Each year, 80 billion animals are slaughtered for meat.[17] Throughout the world, the average person consumes nearly 43 kg of meat a year. That is why, using these industrial and agricultural wastes for SCP production helps to regulate and reduce the environmental pollution related with their disposal. Non-pathogenic microbes may use the available nutrients in agricultural wastes for biomass and cellular expansion without harming themselves.[18] *Saccharomyces cerevisiae* is the most widely accepted SCP potential strain, notably in food and feed applications.[19] Single-cell proteins are also high in necessary key amino acids such as lysine and methionine, which are rare and insufficient in most plants and animals for human consumption.[20] The utilization of vegetable and fruit waste in the production of SCP will aid in managing and reducing pollution as well as the waste disposal problem to a certain level, as the waste will be employed in SCP production to meet the world's need for protein-rich food Approximately, 20% of the production of fruits and vegetables in India are going waste every year because in India a surplus amount of apple, cotton, soy bean, and wheat are produced.[20]

Over the last several years, a great deal of research and development has gone into the processing and repurposing of various fruit wastes in order to turn them into useful and nutritious products. Investigations and studies are being carried out in order to examine and learn more about the potential of diverse fruit wastes for producing cost-effective fungal biomass. Kinnow peels were employed as a possible substrate for the manufacture of SCP that might be used in human diet or animal feed in this technique.[21]

METHODOLOGY

For obtaining single cell protein (SCP) from kinnow peels the proposed method by Khan *et al*. [12] and Haddish [7] was followed. Kinnow of same lot were used in this experiment.

To remove dust particles, the collected kinnow were washed with distilled water and dried in the open air. After that, the kinnows were peeled. To remove any contamination that stuck to the kinnow peels while peeling them off, the peels were washed with distilled water. The peels were then dried for three days in a hot air oven at 100°C for two hours at a time, until all of the moisture in the kinnow peels was vaporized and only the dried peels remained (Fig.1). Using an electric grinder, the dried kinnow peels were crushed to a fine powder.

The peels of the collected kinnow were kept in a beaker dipped partially with 20mL of water for a week. A standard laboratory method was used to isolate the fungus species from kinnow peels.

The fungal hyphae were collected and inoculated in SDA. The purified fungal specie was obtained using serial dilution (Fig. 2). The dried and powdered peels prepared, were then used as a substrate for the growth of isolated fungus and production of SCP. Kinnow peels were degraded to simple components by chemical treatment. Twenty grams of crushed kinnow peels were treated with different concentrations of 0.15, 0.30 and 0.45 N of sulfuric acid. These mixtures were frequently agitated on flame for one hour or until converted into a slurry, maintaining the level of solution constant. After cooling to room temperature, the slurry was autoclaved. Insoluble peel debris were removed by filtration. The autoclaved slurry was cooled to room temperature. The solubilized kinnow peels were incorporated into the culture medium as a carbon and energy source for fungus.

Figure 1 (a) Kinnow peels were dried for three days in a hot air oven at 100°C for two hours at a time, until all of the moisture in the kinnow peels was vaporized and only the dried peels remained (b) A standard laboratory method was used to isolate the fungus species from kinnow peels. The peels of the collected kinnow were kept in a beaker dipped partially with 20mL of water for a week

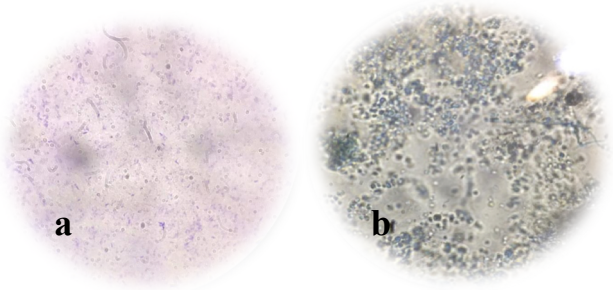

Figure 2. The purified fungal specie was obtained using serial dilution of sulphuric acid and microscopically observed at (a) 40x and (b) 100x

The culture medium prepared for fungal growth had the following composition per liter solution, fumaric acid 2.0 g, $(NH_4)_2SO_4$ 2.5 g, KH_2PO_4. $2H_2$ 1.0 g, $MgSO_4$ 0.5 g, (NH_4) Fe $(SO_4)_2.12H_2O$ 0.2 mg, $ZnSO_4.7H_20$ 0.2 mg; $MnSO_4.H_2O$, 0.1 mg; thiamine 0.1 mg and soluble extract of 20 kinnow peels. The final volume of the medium was made to 1 liter by adding $dH_2O$and pH was adjusted to 5.5 with pH meter.

In conical flasks, submerged fermentation was carried out in triplicate. The first experiment used a medium containing supplemented kinnow peels hydrolysate (SKPHM). Fumaric acid

2.0 g, (NH4)2SO4 2.5 g, KH2PO4.2H2 1.0 g, MgSO4 0.5 g; (NH4) Fe (SO4)2. 12H2O 0.2 mg, ZnSO4.7H20 0.2 mg, MnSO4.H2O 0.1 mg,thiamine 0.1 mg; (NH4) Fe (SO4)2. 12H2O 0.2 mg, ZnSO4.7H20 0.2 mg, MnSO4. The second medium, glucose supplemented kinnow peels hydrolysate medium (GSKPHM), had all of the same components as SKPHM but added 2 mg/L glucose. Only kinnow peels hydrolysate medium (KPHM) was used in the third experiment. In a 250 mL conical flask plugged with cotton wool and autoclaved, 100 mL of culture media was supplemented with solubilized filtrate of 20g kinnow peels. The sterilized media was cooled to room temperature before 1.0 mL of fungal spores were added.

The total nitrogen content of the dried biomass was calculated using the traditional Kjeldhal's Method. Each sample received 10 mL of 98 percent sulfuric acid poured into 5.0 mL. To increase the speed and efficiency of the digestion process, 0.19 mg of potassium sulphate was added to raise the boiling point of sulfuric acid, and 0.16 mg of copper sulphate was used as a catalyst. The samples were boiled for 30 minutes to 3 hours at 420°C, or until they became transparent. After the samples have been digested, they are allowed to cool to room temperature before being diluted with 50mL distil water. Then they were moved to the distillation unit. The conversion of organically bonded nitrogen into ammonium ions is aided by digestion.

The ammonium ions are transformed to ammonia during the distillation process by adding 50mL of NaOH (35%) to the sample and heating until 100mL of distillate is collected in the receiving vessel. To catch the dissolved ammonia gas, the receiving vessel was filled with a 25 mL absorbing solution of boric acid (4%). Steam distillation is used to transport the ammonia into the reception vessel. The ammonia is caught quantitatively by the boric acid solution, which results in solvated ammonium ions. The nitrogen content is then determined by titrating the ammonium borate produced with standard sulfuric acid with 0.1N sodium hydroxide and an appropriate indicator (phenol red) to identify the reaction's end point (Fig.3).

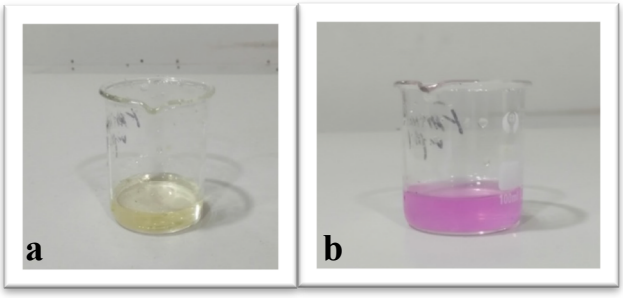

Figure.3. The titration of the ammonium borate produced with standard sulfuric acid with 0.1N sodium hydroxide and an appropriate indicator (phenol red) to identify the reaction's end point. (A) Sample color before and (B) after titration.

RESULTS

The kinnow peels were treated with 0.15, 0.30 and 0.45 normal of sulfuric acid to make it as carbon and energy source for the fungi. The fungus was then inoculated and incubated for 14 days at 37°C. The protein prepared were then quantified using Kjeldahl Method. The results are based on 20g of the substrate used. With 0.15, 0.30 and 0.45 normal sulfuric acid and prepared kinnow peels hydrolysate medium (KPH) the percent protein were calculated. The percent protein obtained at different normalities is given in Table 1. At 0.15 N the protein calculated were 2.278%, at 0.30 N they were 2.233% while at 0.45 N the protein detected were 2.278%. KPH was combined with Mendel's media and treated with 0.15, 0.30 and 0.45 normal solution of sulfuric acid. The percent protein obtained are shown in table 1. At 0.15N the protein yields were 2.004%, at 0.30N the amount was 2.039% while at 0.45 normality 2.152% protein was obtained. When KPH and Mendel's media was combined with glucose and treated with 0.15, 0.30 and 0.45 normal sulfuric acid. At 0.15N the protein obtained was 2.118%, at 0.30N it was 2.022% and at 0.45N the protein obtained was 2.118%.

The protein percent obtained at 0.15 N sulfuric acid treated different carbon source was calculated. The protein obtained with only KPH were 2.278%, with that of KPH and M.M were 2.004% while with combination of KPH + M.M + Glucose yield was 2.118% of protein. The analysis was also made for 0.45N sulfuric acid treated with carbon source. The protein% from only KPH were 2.074%, from KPH + M.M were 2.152% while from KPH combined M.M and G was 2.118%. Similar analysis when made for 0.30N sulfuric acid treated with different carbon source. At 0.30N the protein analyzed in KPH only were 2.233%, with KPH + M.M were 2.039% while KPH + M.M + Glucose produced 2.022% of protein as shown in (Table 1. a to f)

DISCUSSION

Single cell proteins are the biomass produced by many microbial species such as bacteria, fungi and algae. This biomass contains proteins, amino acids, vitamins and lipid content.[13] The microorganisms utilize waste materials, agricultural waste and garden waste. Single cell proteins can be used as a vital supplement of proteins and regarded as a quantitative approach against malnutrition.[14] SCP have a wide field of applications in animal nutrition, in food stuffs.[15] The production process and research and development of SCP are encompassing various fields of science including genetics, microbiology, biotechnology, economics, agriculture, food technology and veterinary sciences.[16]

When SCP is created from waste material, it provides a cost-effective protein source that may be utilized animal feed or further processed for human use. Bacteria and fungus are two microorganisms that might produce SCP.[17,18] They were an

S. No.	Factors	Normality			Negative Control
a) Effect of different normality on protein yield using KPH only.					
1.	Normality	0.15N	0.30N	0.45N	-
2.	Carbon source	KPH	KPH	KPH	-
3.	Volume of sample used	20ml	20 ml	20 ml	20ml
4.	Concentration of KP used	20gm/100ml	20gm/100ml	20gm/100ml	0
6.	Fermentation pH	5.5	5.5	5.5	7.0
7.	Fermentation temperature	370C	370C	370C	370C
8.	% Protein yield	2.278	2.233	2.074	0
b) Effect of different normality on protein yield using KPH with M.M.					
1.	Normality	0.15N	0.30N	0.45N	-
2.	Carbon source	KPH + M.M	KPH + M.M	KPH + M.M	-
3.	Volume of sample used	20ml	20 ml	20 ml	20ml
4.	Concentration of KP used	20gm/100ml	20gm/100ml	20gm/100ml	0
6.	Fermentation pH	5.5	5.5	5.5	7.0
7.	Fermentation temperature	370C	370C	370C	370C
8.	% Protein yield	2.004	2.039	2.152	0
c) Effect of different normality on protein yield using KPH combined with M.M and glucose.					
1.	Normality	0.15N	0.30N	0.45N	-
2.	Carbon source	KPH+M.M+ Glucose	KPH+M.M+ Glucose	KPH+M.M+ Glucose	-
3.	Volume of sample used	20ml	20 ml	20 ml	20ml
4.	Concentration of KP used	20gm/100ml	20gm/100ml	20gm/100ml	0
6.	Fermentation pH	5.5	5.5	5.5	7.0
7.	Fermentation temperature	370C	370C	370C	370C
8.	% Protein yield	2.118	2.022	2.118	0
d) Effect of different carbon source of protein yield.					
1.	Normality	0.30N	0.30N	0.30N	-
2.	Carbon source	KPH	KPH+M.M	KPH+M.M+Glucose	-
3.	Volume of sample used	20ml	20 ml	20 ml	20ml
4.	Concentration of KP used	20gm/100ml	20gm/100ml	20gm/100ml	0
6.	Fermentation pH	5.5	5.5	5.5	7.0
7.	Fermentation temperature	370C	370C	370C	370C
8.	% Protein yield	2.233	2.039	2.022	0
e) Effect of different carbon source of protein yield.					
1.	Carbon source	KPH	KPH+M.M	KPH+M.M+ Glucose	-
2.	Normality	0.45N	0.45N	0.45N	-
3.	Volume of sample used	20ml	20 ml	20 ml	20ml
4.	Concentration of KP used	20gm/100ml	20gm/100ml	20gm/100ml	0
5.	Time of Fermentation	15 days	15 days	15 days	15 days
6.	Fermentation pH	5.5	5.5	5.5	7.0
7.	Fermentation temperature	370C	370C	370C	370C
8.	% Protein yield	2.074	2.152	2.118	0
f) Effect of different carbon source of protein yield.					
1.	Carbon source	KPH	KPH+M.M	KPH+M.M+ Glucose	-
2.	Normality	0.30N	0.30N	0.30N	-
3.	Volume of sample used	20ml	20 ml	20 ml	20ml
4.	Concentration of KP used	20gm/100ml	20gm/100ml	20gm/100ml	0
6.	Fermentation pH	5.5	5.5	5.5	7.0
7.	Fermentation temperature	370C	370C	370C	370C
8.	% Protein yield	2.233	2.039	2.022	0

Table 1 showing the effect of different normality on protein yield using only KPH (section a), KPH with M.M (section b), KPH combined with M.M and Glucose (section c). Sections d-f are showing effect of different carbon source of protein yield. Time of fermentation was 15 days for all.

degraded to fermentable sugars in the first example by treating them with a strong acid (H2SO4) of varying normality. Second, protein yield was tested by varying the medium composition with different supplements, such as glucose and Mendel's media.[4,16]

Kinnow peels are a viable contender for the creation of SCP, according to the results of this experiment. Kinnow peels in various medium at varied concentrations of sulfuric acid (0.15, 0.30, and 0.45N) give us a protein percentage of over 2% on average per 20mg of sample utilized. Minor variances were detected at various normalities; however, these changes were not significant.[21,22]

At 0.15N treatment, the peak yield for KPH was 2.278 percent. At 0.45 normality, the lowest yield was 2.074 percent. In the instance of KPH mixed with Mendel's medium, the maximum yield was 2.152 percent at 0.15N compared to 2.004 percent at 0.15 normality, indicating a reversal of the trend. Furthermore, when KPH was coupled with Mendel's medium and glucose, the yield was 2.118 percent at 0.15 and 0.45 normalities, but 2.022 percent at 0.30 normalcy. As a result, there was no discernible and significant influence of normalities on total protein output. Hamdy (2013) conducted a study in which orange peels were utilized to synthesize single cell protein.[1-5] For per 100g of material utilized, the protein produced was 20.59%. Changes in yield might be attributed to differences in carbon source percent composition (1 percent w/w of sucrose, glucose, and fructose as carbon and nitrogen sources).[6-9] Furthermore, at 0.1 N of NaOH, *Saccharomyces cerevisiae* was employed. SCP's high output might be attributed to several carbon sources and low NaOH normalcy.

Mondal et al. (2012) employed orange peels as a carbon source and substrate for the development of single cell protein synthesis in another investigation.[10-12] Per 100g of substrate, 30.5 percent protein was generated.[13-16] It's possible that the increased protein production is due to the usage of *Saccharomyces cerevisiae*, which has distinct pH, temperature, and fermentation times. They found that *Saccharomyces cerevisiae* generates the best protein at pH 5.5 and temperatures ranging from 28 to 32 degrees Celsius.[17-19] When the carbon source was changed and the normality for each source was maintained constant in our experiment, a distinct picture emerged.

KPH projected 2.278 percent of protein at 0.15 normality, compared to 2.004 percent from KPH + M.M. KPH + M.M. with glucose yielded a 2.118 percent intermediate result. At 0.30 normality, a similar pattern was seen, with a good quantity of protein from KPH and the least amount 2.022 percent from KPH coupled with Mendel's medium and glucose at normality.

Khan et al. (2010) grew *Saccharomyces cerevisiae* for 24 hours at 24 ^{0}C on various fruit wastes such as sweet orange peel.[20] The crude protein content per 100g substrate was calculated to be 26.26%. S. cerevisiae was grown on fruit wastes without the need of any supplements like as inorganic nitrogen sources, carbon sources, or glucose sources. Our and this study's findings reveal variance owing to the use of various organisms and varied fermentation times. The high yield is attributable to the organism being kept at an optimal temperature.

Citrus aurantium, Citrus sinensis, and Citrus paradise were employed as substrates in a study by Azam *et al.* (2014), to generate SCP utilizing the fungus *Aspergillus niger and Saccharomyces cerevisiae. C. aurantium* had the greatest protein level of 13.37 percent per 100g substrate, whereas the other orange peels had protein values of 11.9 percent and 11.53 percent, respectively, for *C. paradise and C. sinensis.*[21-22] The results reveal some differences from our research due to the use of a large amount of monosaccharide as a carbon source. The inclusion of a large amount of carbon source optimizes single-cell protein production.

Every year a high volume of kinnow peels are wasted. To make a better use the result obtained from our experiment suggests that's different bacterial and fungal species i.e., *Saccharomyces cerevisiae* can be used for the production of protein from KP. Different digestion acids and bases are also recommended. Kinnow peels at different level of their maturity may also be used to maximize the protein yield. Fermentation time may also be used to optimize the yield. Furthermore, composition of nitrogen source may also affect the overall production of SCP and hence the use of different nitrogen sources is recommended.

CONCLUSION

There was no effect of digestion normalities in case of KPH. The effect of normalities was noted when kinnow peels digest was supplemented with glucose. There was a decreasing trend in SCP production with increase in normalities. No effect was noted when KPH was combined with M.M and glucose. At 0.15 N H_2SO_4 digestion the maximum yield of protein was obtained from KPH. At 0.30 N H_2SO_4 digestion there was a decreasing trend from KPH to KPH+M.M to KPH+M.M+glucose. At 0.45 N H_2SO_4 digestion no effect was noted on the overall protein yield.

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