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RESEARCH ARTICLE

OPTIMIZATION OF BACTERIAL INOCULUM CONCENTRATION AND pH FOR THE PRODUCTION OF AMYLASE AND PROTEASE USING *Proteus* sp. FROM SAGO EFFLUENT.

S. Kavipriya[1] and P. Kousalya[2].

1. II-M.Sc. Biochemistry, Department of Biochemistry, Bharathidasan College of Arts and Science, Erode, Tamilnadu, India.
2. Assistant Professor, Department of Biochemistry, Bharathidasan College of Arts and Science, Erode, Tamilnadu, India.

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Abstract

Sago is the edible starch in the form of globules extracted from tubers of *Manihotesculenta* commonly known as Tapioca/Cassava plant. Sago effluent contains large amount of organic material which affect the fertility of soil in agricultural lands. The aim of this study was to isolate and identify amylase and protease producing bacterial strain from sago factory effluent and it is identified as *Proteus sp.*Optimization of culture conditions such as various concentration of bacterial inoculum (0.5%, 1.0%, 1.5% and 2.0%) and various range of pH (6, 7, 8, 9) was performed at different time interval (24hrs, 48hrs and 72hrs) for the maximum production of amylase and protease using *Proteus sp.* isolated from sago effluent. Bacterial inoculum concentration of 1.0% and 2.0% shows 100µg/ml of amylase production, 420µg/ml of protease was produced by 0.5%, 1.0% and 1.5% bacterial inoculum concentration and the bacterial inoculum concentration of 0.5% and 1.0% shows 8000µg/ml of protein production at 72 hours incubation period. Optimum pH for the production of amylase and protease was found to be 9. Amylase and protease production at pH 9 was found to be 126µg/ml and 690µg/ml. Protein production at pH 9 was found to be 1200 µg/ml.

Introduction:-

The sago industry is a water-intensive industry, as the processing of sago requires several washing cycles and generates 30,000-35,000 liters of effluent per tonne of sago produce. This effluent is acidic and highly organic in nature with chemical oxygen demand (COD) of 5,000-7,000 mg per liter. Those major pollutants specific for this sago effluent are higher amounts of dissolved organic solids, suspended organic solids, BOD and COD. Thus, these effluents pose a serious threat to the environment and quality of life in the rural area, especially. In the rural areas, it contributes to infertility of the farm land, in case of leaving the untreated effluent in agricultural fields, and to water pollution, when discharged into water bodies. Thus, pollution-control is becoming a major problem for the sago industry. Also, there is no standardized cost effective design developed for the sago manufactures; neither by any institution and nor by the pollution control board itself. Problem posed by pollution and high cost of effluent

Corresponding Author:- P. Kousalya.
Address:- Assistant Professor, Department of Biochemistry, Bharathidasan College of Arts and Science, Erode, Tamilnadu, India.

treatment plants needs to be rectified for the betterment of both economy and environment (Anbukumar *et al*., 2014).
Enzymes are substances present in the cells of living organisms in minute amounts and are capable of speeding up chemical reactions (associated with life processes), without themselves being altered after the reaction. They accelerate the velocity of the reaction without necessarily initiating it (Oyeleke and Oduwole, 2009).
Extracellular proteases are naturally produced by microorganisms mainly to degrade large polypeptides in the medium into peptides and amino acids before cellular uptake. Man has commercially exploited such enzymes to assist in protein breakdown in various industrial processes (Synowiecki, 2010). About 75% of world sales of industrial enzymes applications are hydrolytic enzymes constitutes about 60% (Rai *et al*., 2010). Microorganisms offer an attractive source of protease enzymes because they can be cultured in large quantities in a short period of time using established fermentation techniques, they produce an abundant, regular supply of the desired product and they can be genetically manipulated easily than plants and animals (Dabananda and Kshetri, 2010; Tambekar *et al*., 2009). In addition, the protein products they produce are more stable than those from plants and animals.

Amylases are enzyme which hydrolyze starch molecule to give diverse products including dextrin and progressively smaller polymers composed of glucose units (Windish and Mhatre, 1965).Amylases can be derived from several sources including plants, animals and microorganisms. Microbial enzymes generally meet industrial demands. Today a large number of microbial amylases are available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry (Pandey *et al*., 2000).

This investigation attempts to isolate amylase and protease producing bacterial strain, *Proteus sp.* from sago effluent and optimization of culture conditions such as bacterial inoculum concentration, pH for the maximum production of amylase and protease using isolated *Proteus sp.*

Materials and methods:-

Optimization of enzymes production was carried out by using different bacterial inoculum concentration and pH.Amylase, protease and protein production was analyzed every 24 hours interval upto 72 hours.

Sample collection:-
Fresh sago effluent was collected from a sago effluent treatment plant in and around Salem District. The sample was collected in a 10 liter sterile plastic container. The container used for sample collection was pre-treated by washing with alcohol and later rinsed for three times with distilled water. It was dried for 1 hour at 30±5ºC and allowed to cool at room temperature. At the collection point, container was rinsed with the sample thrice and then filled, corked tightly and taken to the laboratory for further analysis.

Isolation of amylase and protease producing microorganism:-
Sago effluent sample of 100µl was taken for serial dilution technique. The enrichment medium called nutrient agar of 60 ml is prepared and spread plate method is performed for culture of microorganism. The nutrient agar plates were incubated at 37ºC. Isolated colonies were inoculated into the nutrient agar medium and are incubated for 24 hours.

Identification of Bacteria:-
The isolated pathogens were identified on the basis of Gram's reaction and biochemical characteristics (MacFaddin, 1980) and results were identified with the help of Bergey's Manual of systematic Bacteriology.

Estimation of Amylase:-
Total reducing sugar was estimated by dinitrosalicyclic (DNS) acid reagent method (Miller, 1959). DNSA reagent was prepared for 50 ml. Addition of DNSA reagent to the standard solution. The whole reaction mixture was incubated in boiling water bath for 15 minutes. Subsequently absorbance was measured at 540 nm. Following the similar procedure, the filtrate obtained at different acid and alkali concentrations was estimated for reducing sugar by adding DNSA reagent.

Estimation of Protease:-
Tyrosine standard was prepared by taking 50 mg of tyrosine in 100 ml of distilled water and 500 µg/ml was taken. Then 1.0 ml of sample was taken at different concentration and 5.0 ml of sodium bicarbonate was added in each test

tube and after 30 minutes incubation 1.0 ml of folin reagent was added in each test tube and the optical density value was recorded at 660 nm in spectrophotometer (Singh *et al.*, 1999).

Estimation of Protein:-

Bovine serum albumin standard was prepared and then 0.3 µl of standard solution was taken and add 0.3 µl of NaOH and kept for 10 minutes in boiling water. And then after few minutes add 3.0 ml of complex reagent to each test tube and incubated for 10 minutes at room temperature. And then add 0.3 µl of folin phenol reagent and kept for 30 minutes incubation at room temperature. Blue colour development was measured at 660 nm in spectrophotometer (Lowry *et al.*, 1951).

Results and discussion:-

Various biochemical tests were done to identify the pathogen from sago effluent. The result obtained is given in the Table 1. The organism is identified as *Proteus* species. This *Proteus* species was used for production of amylase and protease.

Table 1:- Biochemical characterization of selected pathogen:-

S.No.	Biochemical tests	Results
1	Gram staining	Gram negative rod
2	EMB agar	Colorless
3	Macconkey agar	Pale colonies
4	Mannitol agar	Negative
5	Indole production test	Positive
6	Voges-proskauer test	Negative
7	Citrate utilization test	Negative
8	Catalase test	Positive
9	Oxidase test	Positive
9	Nitrate reduction test	Positive
10	Triple sugar iron agar test	Acid butt/Alkaline slant
11	Methyl Red Test	Positive

Effect of various concentration of Bacterial Inoculum on amylase and protease production:-

Optimization of various concentrations (0.5%, 1.0%, 1.5% and 2.0%) of bacterial inoculum for amylase, protease and protein production was done (Table 2).

Bacterial inoculum concentration for maximum amylase production (100µg/ml) was recorded at 1.0% and 2.0%.Bacterial inoculum concentration for maximum protease production (420µg/ml) was recorded at 0.5%, 1.0%, 1.5%. Bacterial inoculum concentration for maximum protein production (8000µg/ml) was recorded at 0.5%, 1.0% respectively.

Table 2:- Effect of various concentration of Bacterial Inoculum on Amylase and protease production:-

S.No	Bacterial Inoculum (%)	Amylase (µg/ml)			Protease (µg/ml)			Protein (µg/ml)		
		Incubation period in hours			Incubation period in hours			Incubation period in hours		
		24	48	72	24	48	72	24	48	72
1.	0.5%	80	80	92	260	310	420	3200	7500	8000
2.	1.0%	92	92	100	290	310	420	2600	7500	8000
3.	1.5%	80	86	92	280	310	420	2000	7500	7500
4.	2.0%	72	86	100	280	290	360	1900	2600	4400

Effect of various concentration of pH on amylase and protease production:

Optimization of various concentrations (6, 7, 8, 9) of pH was done. The results are shown in the Table 3. The optimum pH for this study was found to be pH 9.Maximum production of amylase at pH 9 was found to be 126 µg/ml. Maximum production of protease at pH 9 was found to be 690 µg/ml. Maximum production of protein at pH 9 was found to be 1200 µg/ml.

Table 3:- Effect of various concentration of pH on Amylase and protease production.

S.No.	pH	Amylase (µg/ml)			Protease (µg/ml)			Protein (µg/ml)		
		Incubation period In hours			Incubation period In hours			Incubation period In hours		
		24	48	72	24	48	72	24	48	72
1	6	28	40	86	160	240	440	200	800	1100
2	7	22	34	86	130	210	490	200	800	1100
3	8	20	40	86	120	220	480	900	1000	1000
4	9	22	22	126	130	220	690	100	800	1200

Summary and Conclusion:-

The isolated bacteria were identified as *Proteus sp.* based on the biochemical confirmation. The isolated bacteria were tested for suitability of different inoculums such as 0.5%, 1.0%, 1.5% and 2.0%. It was found that 1.0% bacterial inoculum concentration shows maximum production of amylase ,protease and protein.The isolated bacteria were tested for maximum production of amylase,protease and protein at various pH ranges from 6-9. Optimum pH for amylase,protease and protein production was recorded at pH 9.The present study reports the production of Protease and Amylase by Sago effluent as a sole source of enzyme production using *proteus sp.* This proteolytic bacterium could be used effectively for industrial purpose.

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