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# PALMYRAH PALM WINE PART II: IMPROVEMENTS IN ALCOHOL PRODUCTION

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*(Date of receipt : 12 October 1986)*
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**Abstract:** The palmyrah palm wine, a traditional mild alcoholic beverage of Northern Sri Lanka, is the spontaneously fermented sap of the young and mature inflorescences of both male and female palmyrah *(Borassus flabellifer)* palms. The palmyrah toddy samples had a mean alcohol content of 5.8%[v/v] and the efficiency of natural fermentation process was found to be 56%. In the present study, it was found that this efficiency of alcohol production can be increased by (i) the addition of inorganic salts such as $NH_4Cl$, $MgSO_4$ and $KH_2PO_4$, (ii) heat sterilization of decalcified palmyrah sweet toddy prior to fermentation by a preselected yeast strain and (iii) the introduction of pure yeast inoculum into collection pots. The percentage increase in alcohol production over the control in each case was found to be 12%, 44% and 25 – 30% respectively.

Previously isolated *Saccharomyces cerevisiae* PY 1 was found to be capable of producing the maximum alcohol within 48 hours of fermentation using a starter inoculum potential of $10^7$ cells/ml, thus suggesting that the rate of fermentation can be significantly increased by increasing the inoculum potential.

## 1. Introduction

Palmyrah palm *Borassus flabellifer* grows naturally in the drier regions of Sri Lanka. A mild alcoholic beverage, popularly known as 'palmyrah toddy' or 'palmyrah wine' is obtained from this palm by 'tapping' the inflorescences. This palmyrah wine is the spontaneously fermented sap of the young and mature inflorescences of both male and female palms.

The unfermented sap, commonly referred to as 'sweet toddy' or 'neera', contains 10 – 16.5 % w/v sugar, mainly in the form of sucrose. This sugar is converted into ethyl alcohol during fermentation by wild yeasts and bacteria usually found in 'toddy' collecting pots.[10] From preliminary studies it was found that the observed levels of alcohol in palmyrah 'toddy' was in the range of 5 – 6 % v/v whereas the theoretical yield lies in the range of 9 – 10 % v/v.[11]

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It appears that a considerable part of sugars in the sap is utilized by microorganisms resulting in products other than alcohol during the early stages of natural fermentation.[8] The usual methods of tapping and collecting fermented coconut toddy account for a loss of about 1 – 1.5 % alcohol by injudicious handling;[9] about the same percentage of alcohol can be lost from palmyrah toddy for similar reasons.

There will be a greater demand for ethanol in the near future to meet the energy crisis. Also, improvements in the efficiency of alcohol production will lead to increased production of palmyrah arrack – a product obtained by distilling palmyrah toddy. It is therefore important to formulate methods of controlled fermentation to obtain the maximum yield of alcohol. As reported earlier, the yeasts belonging to the genus *Saccharomyces* are the best fermentors isolated from naturally fermented palmyrah toddy.[5] Therefore *Saccharomyces* yeasts were used in these studies to improve alcohol production.

## 2. Materials and Methods

### 2.1 Collection of Materials and preparation of Experimental Media

a) Fresh unfermented sap:

This was collected in sterile MacCartney bottles by holding the bottle at the tip of the inflorescence for about 3 minutes. The sample was immediately stored at 5°C or was heated in a boiling water bath for 15 minutes to inactivate the microorganisms which may have contaminated the sap. This sample was mainly used to determine the sugar content of unfermented sap.

b) Sweet toddy:

Palmyrah sweet toddy was collected for 14 hours in earthenware pots; the inner surface of these pots were coated with slaked lime.

The lime used in the collection of sweet toddy was removed initially by sedimentation and later by precipitation as calcium phosphate by adding superphosphate. Precipitation was enhanced by heating to about 40 – 50°C and by centrifugation. This centrifuged, decalcified sweet toddy was a clear, colourless liquid with a pH around 6.5 – 7. For experiments where sterile sap medium was required, this decalcified sweet toddy was sterilized by autoclaving at 15 lb/in$^2$ pressure (121°C) for 15 minutes.

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c) Partly fermented toddy:

This was obtained by collecting the palmyrah sap in earthenware pots by adopting the traditional process of toddy collection. Usually the samples were obtained in mornings after a collection period of about 14 hours.

2.2 Methods

A) Routine analytical methods:

Amount of sugar in a sample was estimated according to the Somogyi's semimicro method.[2] Alcohol content was determined using an ebulliometer.[5]

B) Experimental procedures:

*2.2.1 Heat sterilization of palmyrah sweet toddy and alcohol production:*

Decalcified palmyrah sweet toddy medium was prepared as described in 2.1.(b); 500 ml aliquots of this medium was fermented under both sterile and non–sterile conditions using an overnight culture of *Saccharomyces cerevisiae* PY 1. The inoculum potential was $10^5$ cells/ml; the alcohol content of the experimental media was measured after 48 hours.

*2.2.2 Effect of inorganic salts on alcohol production:*

Partly fermented palmyrah toddy samples were supplemented with (i) $NH_4Cl$ – 0.8 g/l; (ii) $MgSO_4$ – 0.2 g/l; $NH_4Cl$ – 1.0 g/l and $KH_2PO_4$ – 1.0 g/l. The alcohol contents of these media were determined after 48 hours of total fermentation.

Sterile, decalcified palmyrah sweet toddy was supplemented with (i) $MgSO_4$ –0.2 g/l; $NH_4Cl$ – 1.0 g/l and $KH_2PO_4$ – 1.0 g/l (i) $KNO_3$ – 0.5 g/l and $NH_4NO_3$ – 0.5 g/l. These media, supplemented with salts, were fermented using the yeast *S. cerevisiae* PY 1 for 48 hours and the alcohol content determined.

*2.2.3 Effect of inoculum potential on fermentation:*

500 ml portions of the sterile, decalcified palmyrah sweet toddy were inoculated with different inoculum potentials of an overnight culture of *S. cerevisiae* PY 1. The initial cell density ranged from $10^4$ to $10^8$ cell/ml. The sugar and alcohol contents of these experimental media were measured periodically by the routine methods.

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*2.2.4 Improvements in alcohol production by introducing pure yeast inoculum into the collection pots:*

In this experiment pure cultures of *S. cerevisiae* PY 1 and *Saccharomyces chevalieri* PY 10 were introduced separately into clean collection pots and their yield of alcohol was compared with that obtained by the usual practice.

Inoculum for each collection pot was prepared by growing the particular yeast strain for 24 hours in 500 ml of yeast extract peptone–glucose (2%) broth to obtain a final cell density of the order $10^8$ cells/ml. The yeast cells were separated by centrifugation and washed well with sterile water. This yeast residue was then transferred to clean earthenware pots normaly used for the collection of toddy.

The toddy samples were collected from these pots after about 15 hours and allowed to ferment for a further period of 25 hours, after which their alcohol content was determined. Control samples were obtained by collecting the toddy from the usual pots having a sediment of wild yeasts and bacteria, including the test strains *S. cerevisiae* PY 1 and *S. chevalieri* PY 10.

Toddy samples were collected from the same pots and analysed again on the 7th and 15th day after the introduction of pure yeast inocula. This experiment was carried out from March 1983 to June 1983 with samples of toddy from two male palmyrah palms, and the procedure was repeated 5 times.

## 3. Results And Discussion

### 3.1 Heat Sterlization of Palmyrah Sweet Toddy and Alcohol Production:

The results of experiment 2.1 were statistically analysed according to Bailey[3] and are presented in Table 1.

The results show that the alcohol produced from heat–sterilized sweet toddy is greater than that from non–sterilized sweet toddy. Although fermentation of sweet toddy is arrested during collection by the addition of calcium, there is still a large number of bacterial and yeast cells. These grow when the pH of the decalcified sweet toddy is adjusted to 6.5–7.0 due to decalcification and compete with the inoculated yeast strains, reducing the level of alcohol production. For maximum alcohol production, palmyrah sweet toddy, therefore should be sterilized prior to inoculation with the desired yeast strains. The only disadvantage in heat sterilization is that the toddy may have a slightly altered, bitter flavour. This may be due to caramelisation of sugars in sweet toddy during heat sterilization.

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Table 1 Effect of heat sterilization of palmyrah sweet toddy on alcohol production

| Condition of fermentation | w/v % mean sugar content | v/v % mean alcohol content |
|---|---|---|
| Control (unsterilized) | 15 | 4.7 a |
| Heat sterilized palmyrah sweet toddy medium | 15 | 6.79 b |

The values denoted by the different letters a and b are statistically different at 5 % level (p=0.05)

Number of Experiments: 10

## 3.2 Effect of Inorganic Salts on Alcohol Production:

Results of the Experiment 2.2 were statistically analysed according to Bailey[3] and are presented in Table 2.

Table 2 Effect of inorganic salts on alcohol production

| Sample | Condition of fermentation | Control mean alcohol | Experimental mean alcohol |
|---|---|---|---|
| Partly fermented natural toddy | Addition of $NH_4Cl$ | 4.874 a | 5.452 b |
| Partly fermented natural toddy | Addition of $Mg^{++}$, $NH_4^+$ and $PO_4^{---}$ | 4.406 c | 4.771 d |
| Autoclaved palmyrah sweet toddy + PY 1 | Addition of $Mg^{++}$, $NH_4^+$ and $PO_4^{---}$ | 4.824 e | 5.388 f |
| Autoclaved palmyrah sweet toddy + PY 1 | Addition of nitrates | 4.888 g | 4.100 h |

The values denoted by the different letters a & b, c & d, e & f and g & h are statistically different at 5 % level (p=0.05)

* The mean alcohol contents are expressed in v/v %.

Number of experiments: 7

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These studies reveal that the addition of $NH_4Cl$ into partly fermented palmyrah toddy significantly increased alcohol production in toddy. However, the % increase over the control in this case is approximately 12, which is much lower than that obtained for heat sterilized sweet toddy fermented with the yeast *S. cerevisiae* PY 1 (44 %), without the addition of $NH_4^+$. This may by due to the mixed microflora present in the partially fermented toddy. Also, as reported by Nathanael[9], 1 – 1.5 % of the alcohol would have been lost during the collection of the partially fermented toddy.

The addition of $Mg^{++}$, $NH_4^+$ and $PO_4^{---}$ increases the yield of alcohol significantly over the control. In the case of natural toddy, addition of these salts has led to an 8.26 % increase in alcohol production over the control whereas in the case of sweet toddy fermentation, this increase was approximately 12 %. However, this 12 % increase over the control can be achieved by the addition of $NH_4Cl$ alone into the fermenting medium.

Jansz[6] reports that the addition of $NH_4Cl$ at a prefermentation stage improves the flavour of toddy, but it does not affect significantly the yield of alcohol. Kalyananda[7] states that the effect of $NH_4Cl$ on fermention depends on when it is added. It has been reported that the $NH_4Cl$ supplies the yeasts with an easily digestible source of nitrogen, resulting in increased sugar utilization and higher yields of alcohol.[1]

From the results presented in Table 2, it is obvious that the addition of nitrates suppresses the production of alcohol. It may be that palmyrah sap has sufficient levels of nitrates and any addition would lead to inhibitory levels of nitrate. It may be also due to the fact the *S. cerevisiae* strains cannot utilize nitrate since they lack the ability to reduce it to $NH_4^+$ ions.[4]

### 3.3 Effect of Inoculum Potential on Fermentation:

The resúlts of the Experiment 2.3 are presented in Figures 1 and 2.

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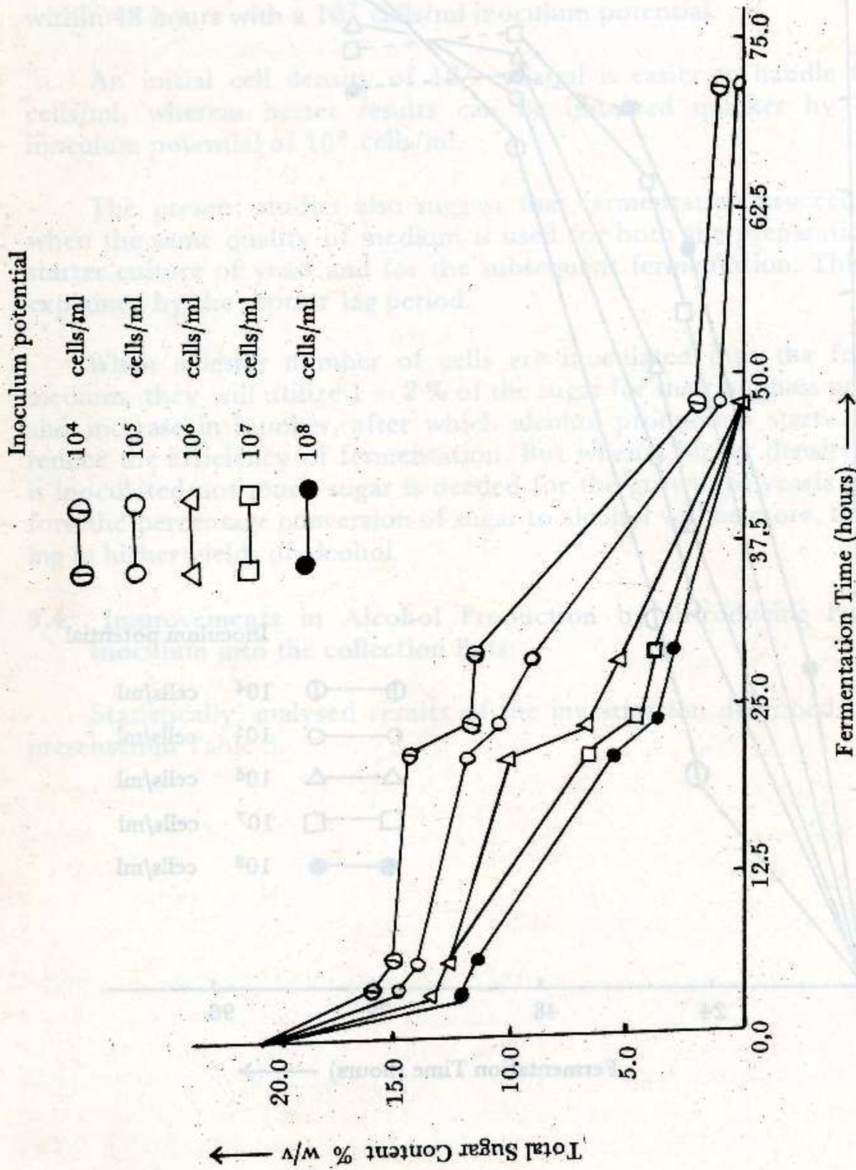


Figure 1: Effect of inoculum potential on total sugar content of palmyrah sweet toddy medium fermented by the yeast *Saccharomyces cerevisiae* PY 1

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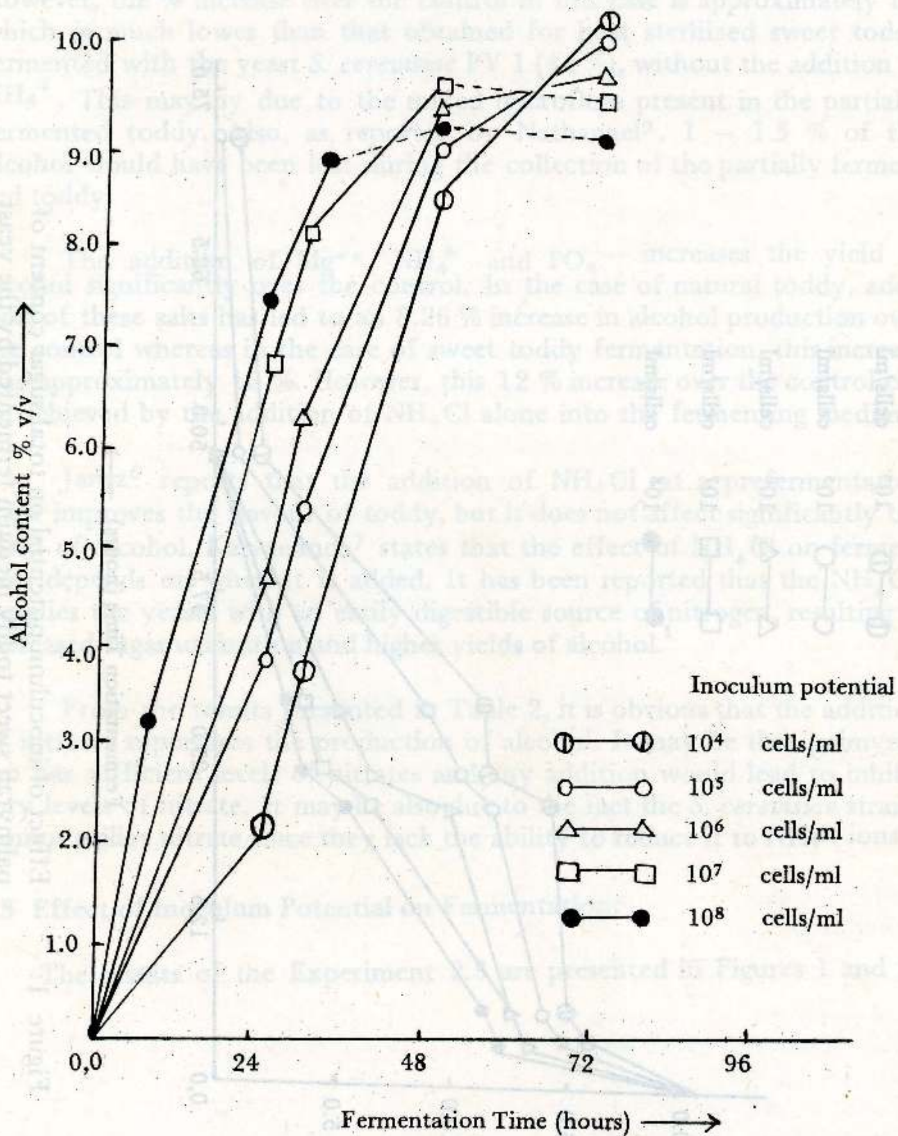


Figure 2: Effect of inoculum potential on alcohol production by *Saccharomyces cerevisiae* PY 1 in palmyrah sweet toddy medium.

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These studies reveal that the rate of fermentation can be significantly increased by increasing the inoculum potential. It was found that an inoculum potential of $10^7$ cells/ml used up all the sugar within 48 hours of fermentation thus suggesting that the maximum alcohol can be obtained within 48 hours with a $10^7$ cells/ml inoculum potential.

An initial cell density of $10^7$ cells/ml is easier to handle than $10^8$ cells/ml, whereas better results can be obtained quicker by using an inoculum potential of $10^8$ cells/ml.

The present studies also suggest that fermentation proceeds rapidly when the same quality of medium is used for both the preparation of the starter culture of yeast and for the subsequent fermentation. This may be explained by the shorter lag period.

When a lesser number of cells are inoculated into the fermenting medium, they will utilize 1 – 2 % of the sugar for their biomass production and increase in number, after which alcohol production starts. This will reduce the efficiency of fermentation. But when a higher density of yeast is inoculated not much sugar is needed for the growth of yeasts and therefore the percentage conversion of sugar to alcohol will be more, thus resulting in higher yields of alcohol.

### 3.4 Improvements in Alcohol Production by Introducing Pure Yeast Inoculum into the collection Pots:

Statistically analysed results of the investigation described in 2.4 are presented in Table 3.

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Table 3 Effect of introducing a pure yeast inoculum into the collection pots

| Palm | Day | Treatment | Average alcohol content v/v % | | % increase over the control |
|---|---|---|---|---|---|
| 1 | 1 | Control | 4.278 | a | – |
| | | *Saccharomyces cerevisiae* | 5.558 | b | 29.92 |
| | | *Saccharomyces chevalieri* | 5.345 | b | 24.94 |
| | 7 | Control | 3.800 | a | – |
| | | *S. cerevisiae* | 4.868 | b | 28.11 |
| | | *S. chevalieri* | 4.600 | b | 21.05 |
| | 15 | Control | 5.200 | a | – |
| | | *S. cerevisiae* | 5.930 | b | 14.04 |
| | | *S. chevalieri* | 5.770 | b | 10.96 |
| 2 | 1 | Control | 4.900 | a | – |
| | | *S. cerevisiae* | 5.810 | b | 18.57 |
| | | *S. chevalieri* | 5.490 | b | 12.04 |
| | 7 | Control | 4.990 | a | – |
| | | *S. cerevisiae* | 5.770 | b | 15.63 |
| | | *S. chevalieri* | 5.620 | b | 12.62 |
| | 15 | Control | 5.507 | a | – |
| | | *S. cerevisiae* | 6.212 | b | 12.80 |
| | | *S. chevalieri* | 6.108 | b | 10.91 |

The values denoted by the different letters a and b are statistically different at 5 % level ($p=0.05$)

The whole experiment was repeated 5 times.

The results clearly indicate that a significant increase in alcohol production can be achieved by introducing pure yeast inoculum into the collection pots. It was also noted that, with time, there was a drop in the quantity of alcohol produced. However, once inoculated, the pots could be used for two weeks with an appreciable increase in the yield of alcohol over the control. Kalyananda[7] suggests that instead of introducing yeast inoculum into collection pots, the addition of $NH_4^+$ into the collection pots would significantly increase the alcohol content of coconut toddy.

Though these experiments indicate that the production of alcohol in toddy can be increased by (i) the fermentation of heat–sterilized sweet toddy with pre–selected, efficient yeast strains in the presence of $NH_4^+$ salts and (ii) the addition of pure yeast inoculum into the collection pots, there are limitations in applying these two methods. The limitations in the

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fermentation of heat–sterilized sweet toddy are (i) the risk of altered flavour and aroma from the caramelization of sugars in sweet toddy (ii) the need to have the sweet toddy samples collected and to have the lime present in those samples removed by the addition of superphosphate and (iii) the difficulties arising from large quantities of palmyrah sweet toddy having to be sterilized without delay.

Introduction of pure yeast inoculum into collection pots also involves the preparation of a high density of yeast inoculum in a convenient form so that it can be easily handled by the tapper.

## Acknowledgement

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fermentation of heat-sterilized sweet toddy are (i) the risk of altered flavour and aroma from the caramelization of sugars in sweet toddy (ii) the need to have the sweet toddy samples collected and to have the lime present in those samples removed by the addition of superphosphate and (iii) the difficulties arising from large quantities of palmyrah sweet toddy having to be sterilized without delay.

Introduction of pure yeast inoculum into collection pots also involves the preparation of a high density of yeast inoculum in a convenient form so that it can be easily handled by the tapper.

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# STUDIES ON SOME NOVEL SILICA MOLECULAR SIEVES

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*(Date of acceptance : 09 February 1988)*

**Abstract:** Four different silica end members of zeolites (zeosils) namely silica-ZSM–48, silica ZSM–22, silica–ferrierite and silica–ZSM–5 have been synthesized and their structural features and properties determined. Long chain polyamines act as templates for the formation of silica–ZSM–22 and silica–ZSM–48. The use of amines with side $NH_2$ groups or straight chain amines at elevated temperatures, favours the formation of silica–ZSM–48. Ethylenediamine–boric acid pair acts as a template for silica–ferrerite formation while the use of highly branched tertiary amines leads to the formation of silica–ZSM–5. Available data indicate that these four zeosils are isostructural with the corresponding zeolites. These molecular sieves contain 3–dimensional 4–connected network of corner linked$[SiO_4]$ tetrahedra, possessing channel like voids. Silica–ZSM–22 and silica–ZSM–48 contain 1D channel systems but the latter has wider channels. Silica–ferrierite possesses 2D channels while silica–ZSM–5 possesses a 3D channel system. All these frameworks are thermally stable upto $1000^0C$, even after the removal of templates. They are hydrophobic, organophyllic and possess neither catalytic nor ion exchange properties.

## 1. Introduction

One of the major scientific and technological developments since the World War II has been the development of synthetic zeolites as molecular sieve adsorbents, ion exchangers and catalysts. More than sixty different zeolite framework topologies[1] are known today and it is expected that many more will be synthesized in the near future.

A series of high silica zeolites has been synthesized in the presence of very small amounts of aluminium and they include ZSM–5,[5] ZSM–11,[6] ZSM–22,[7] ZSM–23,[12] ZSM–48,[13] and ZSM–12.[8] High–silica zeolites are thermally very stable and are of considerable importance as industrial catalysts. ZSM–5 group of zeolites in particular is used as a catalyst[10] in the conversion of methanol to gasoline, ethylbenzene synthesis , xylene isomerization and toluene disproportionation. They also possess unusual hydrophobicity[11] leading to potential applications in the separation of hydrocarbons from polar compounds such as water and alcohols.

Synthesis of pure silica (Al free) end–member of ZSM–5 and ZSM – 11 referred to as silicalite 1[3] and silicalite 2[2] respectively have been reported recently. Silica end members of zeolites have been given the name zeosils.[9] This series of silica polymorphs constitute a hitherto unknown and a novel group of porous compounds, which are of considerable importance as organophillic, hydrophobic and thermally stable molecular sieves. This paper reports the synthesis, properties and structural features of three new

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zeosils namely silica–ZSM–48, silica–ZSM–22 and silica–ferrierite which are apparently isostructural with the corresponding zeolites ZSM–48, ZSM–22 and silica–ferrierite respectively.

## 2. Methods and Materials

### 2.1 Synthesis

Silica molecular sieves have been crystallized hydrothermally from aqueous homogeneous 0.5M silicic acid solutions prepared by hydrolysing tetramethoxysilane, $Si(OCH_3)_4$, in water or in 2M aqueous ethylenediamine solution. The hydrolysis has been carried out under vigorous and continuous agitation. Special care has been taken to avoid any contamination by aluminium or any other cations.

The resulting silicic acid solutions were filled into silica glass tubes. After addition of organic amines (20% by volume) or organic amine (20% by volume) and 0.1M boric acid solution(10% by volume), the tubes were sealed in air. The silica tubes were then subjected to thermal treatment in an oven at $160^0 - 200^0C$ for a period ranging from 7 days to 3 months. The total pressure generated in the silica tubes is about 150 bar. The same synthesis procedure was adopted to synthesize all the zeosils described in this paper, except for different guest species employed(Table 2).

### 2.2 Identification and analysis

The crystallisation products were identified by optical microscopy and powder X–ray diffraction with Cu $K_\alpha$ radiation. A Gandolfi camera had been used for the purpose of identification while a Phillips diffractometer had been used for the accurate measurement of *d* values. Some crystals were subjected to electron diffraction by Phillips 802 electron microscope. Chemical analysis was carried out using a CAMECA Microprobe Analyzer. Boron contents were determined by using JY 38 Plasma spectrograph. Thermogravimetric analysis (TGA) was performed using a Dupont 1090 Thermal Analyzer.

## 3. Results and Discussion

### 3.1 Zeosil formation

Different polymorphs of silica such as quartz, cristobalite, keatite, etc., with high framework densities (Table 1) crystallize from aqueous silicic acid solutions under hydrothermal conditions in the absence of guest species. The presence of small gaseous molecules, short chain amines, cyclic amines, etc., results in the formation of clathrasils having lower framework densities and possessing polyhedral cavities or cages.[4] The "windows" of these cages are too small for the free movement of guest molecules. On the other hand,

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long chain amines act as templates for the formation of zeolite–type aluminium free frameworks (zeosils) possessing channel like voids.

Table 1. Framework densities ($d_f$)* of different silica polymorphs, clathrasils and zeosils

| Phase | df |
|---|---|
| (i) Silica polymorphs[9] | |
| Coesite | 29.3 |
| Quartz | 26.6 |
| Keatite | 25.1 |
| Cristobalite | 23.2 |
| Tridymite | 22.9 |
| (ii) Clathrasils[4] | |
| Melanophlogite | 19.0 |
| Dodecasil 1H | 18.5 |
| Dodecasil 3C | 18.6 |
| Deca–dodecasil 3R | 17.6 |
| Nonasil | 19.3 |
| Silica–sodalite | 17.4 |
| (iii) Zeosils | |
| Silica–ferrierite[14] | 19.3 |
| Silica–ZSM–22[7] | 19.7 |
| Silica–ZSM–48[13] | 19.9 |
| Silica–ZSM–5[3] | 17.8 |
| Silica–ZSM–11[2] | 17.9 |

Note: * df may be defined as number of $SiO_2$ units per 1000 A $^3$ in the structure.

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Four zeosils namely silica—ZSM—48, silica—ZSM—22, silica—ferrierite and silica—ZSM—5 (silicalite 1) have been crystallized in the present study. Successful guest species and the temperatures of synthesis are given in Table 2. Unbranched long chain amines such as diethylamines, 1—amino-butane at 160—180$^0$C yield silica—ZSM—22. 1,4,8,11- tetraazaundecane, 1,5,8,12—tetraazadodecaneand 1,5,9,13—tetraazatridecane guest molecules also give the same zeosil at 160$^0$C (Table 2). On the other hand, diethylene-triamine, triethylenetetramine, tetraethylenepentamine and 1,2—diamino-propane which contains a side—$NH_2$ group, act as guests for the formation of silica —ZSM—48. Furthermore, 1,4,8,11—tetraazaundecane, 1,5,8,12—tetraazadodecane and 1,5,9,13—tetraazatridecane too form silica—ZSM—48 at relatively higher temperatures (180—200$^0$C).

It was not possible to synthesize silica—ferrierite using only the poly-amines as the guest species. Presence of boric acid was found to be essential for the crystallization of silica—ferrierite. Of the guest species attempted ethylenediamine—boric acid pair was found to be the most efficient guest system for the formation of silica—ferrierite. Highly branched amines such as triethylamine, tripropylamine, tributylamine as well as long chain polyamines—boric acid pairs act as templates for the formation of silica—ZSM—5.

It is evident that amines having relatively short chains, particularly at lower temperatures, favour the formation of silica—ZSM—22. Elevation of temperature or the increase of width of amine chains by the introduction of a side—$NH_2$ group lead to the formation of silica—ZSM—48 possessing relatively wide 1D channel systems (Table 4). Thus, it is apparent that increase in width of the guest molecule and the high mobility of atoms and molecules at elevated temperatures favour the formation of wide channel 1D zeosils. Highly branched tertiary amines form silica—ZSM—5 because these amines could readily be accomodated in a 3D channel system with a possible location of their tertiary N atoms at the intersection points with the chains protruding into the channel voids.

It has been observed that in the presence of polyamines and boric acid, zeosils with 2D and 3D channel systems are formed. Analytical data of these as—synthesized as well as ignited products suggest that boron is not present in the framework but it is almost exclusively associated with the guest species. Thus, ethylenediamine—boric acid pair can be considered as the effective template for silica—ferrierite synthesis. It is likely that in zeosils synthesized in the presence of amine—boric acid pairs, the B—N groups are located at the channel intersections with side chains stretching into the cavities.

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Table 2. Successful guest species and the temperatures for zeosil synthesis

| Zeosil | Guest species 160°C | 180°C | 200°C |
|---|---|---|---|
| Silica–ZSM–48 | Diethylenetriamine | Diethylenetriamine | Diethylenetriamine |
| | Triethylenetetramine | Triethylenetetramine | Triethylenetetramine |
| | Tetraethylenepentamine | Tetraethylenepentamine | Tetraethylenepentamine |
| | 1,2–diaminopropane | 1,2–diaminopropane | 1,4,8,11–tetraazaundecane |
| | | 1,3–diaminopropane | 1,5,8,12–tetraazadodecane |
| | | 1,4,8,11–tetraazaundecane | 1,5,9,13–tetraazatridecane |
| | | 1,5,8,12–tetraazadodecane | |
| | | 1,5,9,13–tetraazatridecane | |
| Silica–ZSM–22 | Diethylamine | Diethylamine | |
| | 1–aminobutane | 1–aminobutane | |
| | 1,3–diaminopropane | | |
| | 1,4,8,11–tetraazaundecane | | |
| | 1,5,8,12–tetraazadodecane | | |
| | 1,5,9,13–tetraazatridecane | | |
| Silica–ferrierite | Ethylenediamine + Boric acid | Ethylenediamine + Boric acid | |
| Silica–ZSM–5 | Triethylamine | Triethylamine | Diethylenetriamine + Boric acid |
| | Tripropylamine | Tripropylamine | Triethylenetetramine + Boric acid |
| | Tributylamine | Tributylamine | Tetraethylenepentamine + Boric acid |
| | Triethylenetetramine + Boric acid | Triethylenetetramine + Boric acid | |
| | Tetraethylenepenamine + Boric acid | Tetraethylenepentamine + Boric acid | |

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### 3.2 Structural features

X–ray powder patterns of silica–ZSM–48, silica–ZSM–22 and silica–ferrierite were indexed on the basis of orthorhombic cells and the least square refinement gave accurate unit cell parameters. These unit cell parameters are compared with the corresponding zeolites in Table 3. For completeness ZSM–5 and silica–ZSM–5 are also included in the table. In general there is a slight decrease in all unit cell parameters with respect to their values in the corresponding zeolites. This is expected due to the complete replacement of larger Al by smaller Si in zeosils.

X–ray powder patterns of these products were very similar to those of the corresponding zeolites indicating that they are isostructural, while microprobe analysis of the products confirmed the presence of only silicon and absence of aluminium in the frameworks. Thus, it is apparent that all these zeosils contain 3–dimensional 4–connected networks of $SiO_4$ tetrahedra possessing channel like voids.

### 3.3 Silica–ZSM–48

The cell parameters of silica–ZSM–48 were fixed from both X–ray powder diffraction and electron diffraction data. Approximate values for $a^*$, $b^*$ and $c^*$ from electron diffraction patterns of the (hol)–and(hKO)– reciprocal lattices of silica–ZSM–48 were used to compute approximate direct cell parameters. The electron diffraction patterns are shown in Figure 1. The cell parameters were refined to $a$ = 1421(4), $b$ = 20.09(5) and $c$ = 8.40(3) Å , using the best–resolved peaks in the powder X–ray pattern. Powder reflections indicate $C$ or $I$ centering suggesting Immm, Imma, Cmcm and Cmmm symmetries. All specimens investigated by TEM showed weak reflections indicating the space groups Pmnm or $P2_1$ nm. Disregarding the weak reflections the space group Immm was found to be the most plausible. Twinning of silica–ZSM–48 is a common feature and the twin plane is perpendicular to [001].

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Table 3. Comparison of unit cell data

| Compound | a °A | b °A | c °A |
|---|---|---|---|
| ZSM–5[5] | 20.1 | 19.9 | 13.4 |
| Silicate 1[3] (silica–ZSM–5) | 20.07(1) | 19.86(1) | 13.36(1) |
| Ferrierite[14] | 14.16(1) | 19.16(1) | 7.50(1) |
| Silica–ferrierite | 13.89(1) | 18.56(1) | 7.25(1) |
| ZSM–22[7] | 13.86(3) | 17.41(4) | 5.04(2) |
| Silica–ZSM–22 | 13.85(1) | 17.42(1) | 5.03(1) |
| ZSM–48[13] | 14.24(3) | 20.14(4) | 8.40(2) |
| Silica–ZSM–48 | 14.21(1) | 20.09(1) | 8.39(1) |

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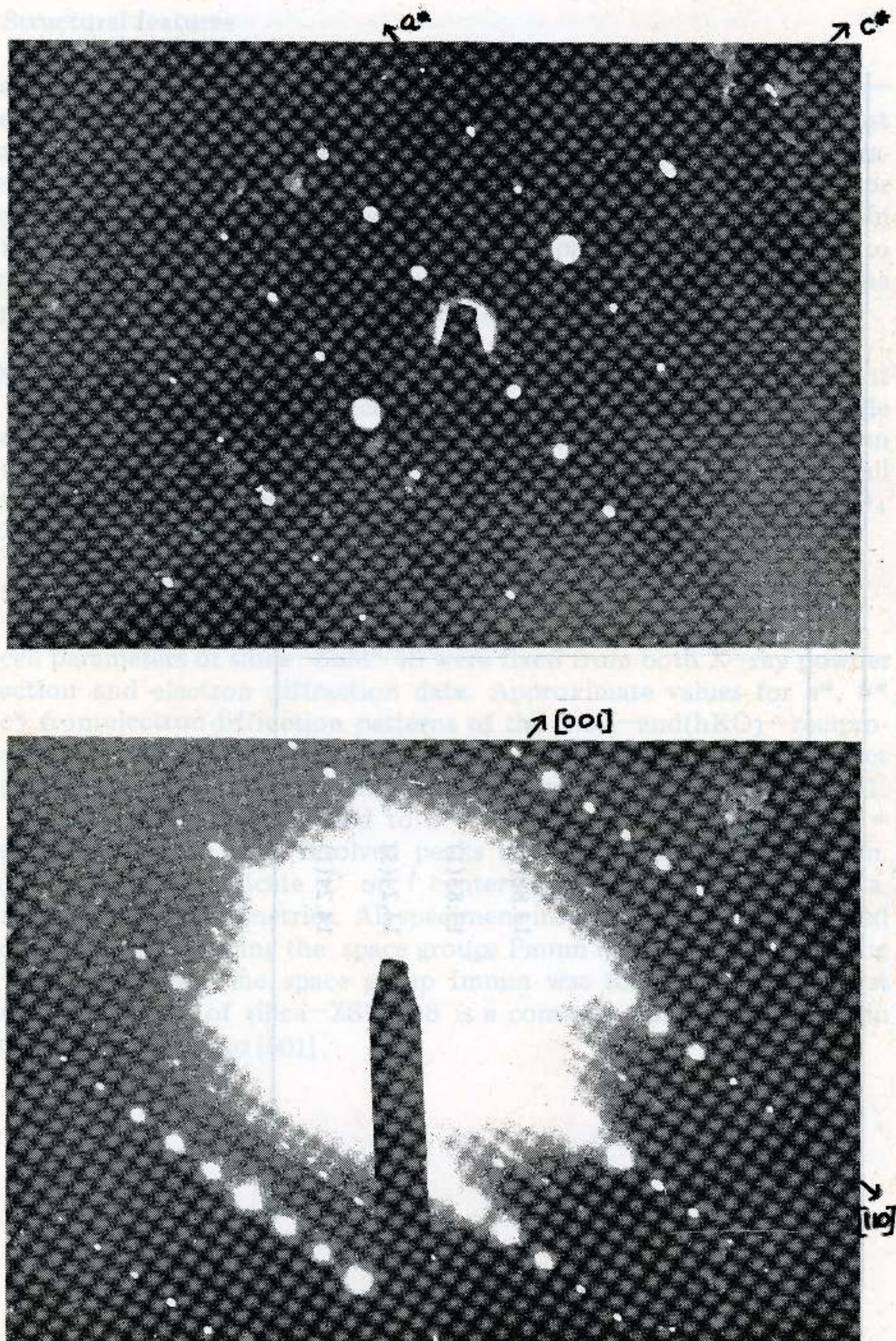


Figure 1 Electron diffraction pattern of silica—ZSM—48 (a) hol reciprocal lattice, (b) hko reciprocal lattice.

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Considering the structures of the zeolites, ZSM−48[13] and ferrierite[14] it may be possible to propose a structure for silica−ZSM−48. A structure based on ferrierite sheets linking via oxygen atoms located on mirror planes and consisting of 1D channel system may be visualized for this zeosil. Figure 2 shows a projection of the structure along[001].

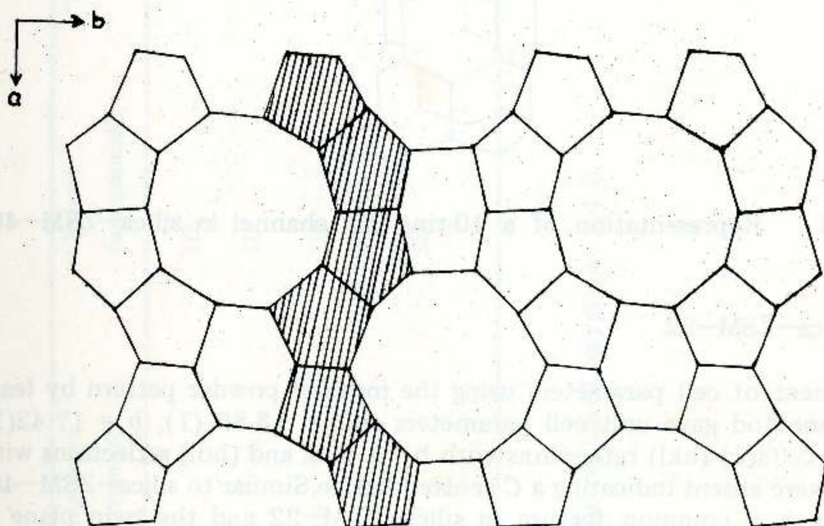


**Figure 2** **Projection of the structure of silica−ZSM−48 along (001). One chain of zig-zag edge-sharing 5−membered rings is shaded.**

The framework is composed of 5−, 6− and 10− membered rings of $[SiO_4]$ No 4−rings are present. Edge−sharing 5−membered rings of $SiO_4$ tetrahedra form sinusoidal chains which traverse along a direction producing elliptical (d ~ 5.3 x 5.6 Å) 10−membered ring openings. The 10−T−ring channels (Figure 3) are not interconnected and therefore, represent a 1D channel system parallel to[001]. This channel system is similar in size to the straight channels[5] of the ZSM−5 framework.

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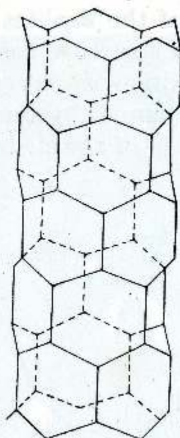

Figure 3 Representation of a 10-ring 1D channel in silica–ZSM–48.

3.4 Silica–ZSM–22

Refinement of cell parameters using the indexed powder pattern by least square method gave unit cell parameters as $a$ = 13.85 (1), $b$ = 17.42(1) and $c$ = 5.03(1) (hkl) reflections with h + k ≠ 2n and (hol) reflections with 1 ≠ 2n were absent indicating a $C$ centred lattice. Similar to silica–ZSM–48, twinning is a common feature in silica–ZSM–22 and the twin plane is often [110].

As in the case of silica–ZSM–48, the structure consists of 5–, 6– and 10– membered rings of $SiO_4$ tetrahedra. Figure 4 gives a projection of the structure along [001]. Zig–zag chains of 5–T–rings are running along the $a$ direction forming 1D, 10–T–ring channels parallel to $c$ axis. The channels are considerably smaller (d ~ 4.7 x 5.5 Å ) than those in silica–ZSM–48 (Table 4).

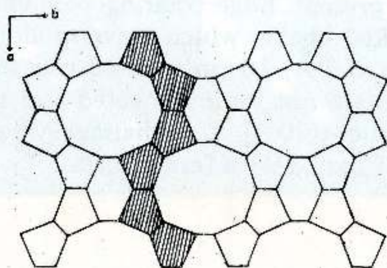

Figure 4 Projection of the structure of silica–ZSM–22 along (001). One chain of zig-zag 5–membered rings is shaded.

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Table 4. Some important structural features

| Zeosil | Most probable space group | $SiO_4$ ring types in the structure | Channel dimensionality | *Channel characteristics(A) |
|---|---|---|---|---|
| Silica–ZSM–48[13] | Immm | 5,6,10 | 1D | <u>10</u> 5.3 x 5.6 |
| Silica–ZSM–22[7] | $Cmc2_1$ | 5,6,10 | 1D | <u>10</u> 4.7 x 5.5 |
| Silica–ferrierite[14] | Immm | 5,6,8,10 | 2D | <u>10</u> 5.4 x 5.8 ⟷<br><u>8</u> 3.5 x 4.7 |
| Silica–ZSM–5[3] | $Pn2_1a$ | 4,5,6,10 | 3D | <u>10</u> 5.4 x 5.6 ⟷<br><u>10</u> 5.1 x 5.4 |

N.B.* These values have been calculated assuming the radius of oxygen as 1.4 A.

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Comparison of dimensions of different guest molecules with the channel dimensions of silica–ZSM–22 indicates that only unbranched aliphatic chains can occupy the channels without appreciable distortion of the framework or the guest chains. It can be shown that the long chain amines almost completely fill the available space within the channels (Figure 6).

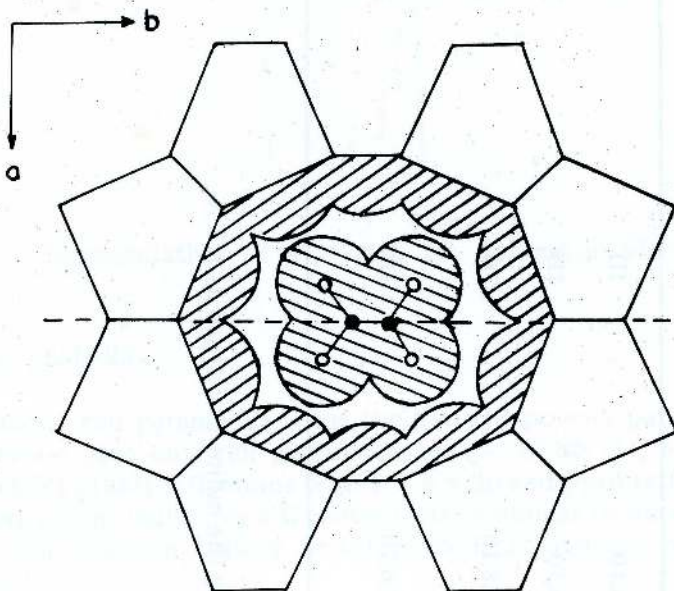


Figure 6 Cross section of a 10–T–ring channel of silica–ZSM–22 along (001) showing the position of a guest molecule.

### 3.5 Silica–ferrierite

Least square refinement of the powder data gave unit cell parameters as $a$ = 13.89(1), $b$ = 18.56(1) and $c$ = 7.25(1). The powder pattern was very similar to naturally occurring ferrierite. [14] The most probable space group was found to be Immm.

The framework consists of 5–,6–,8– and 10–membered rings of [$SiO_4$] tetrahedra. The structure is characterized by the presence of zig–zag chains of 5–membered rings parallel to [001] forming a 2D channel system. The main channels are running along [001] with 10–T–ring openings (d ~ 5.4 x 5.8 Å) while side channels with 8–T–ring openings (d ~ 3.5 x 4.7 Å) are parallel to [010]. Figure 5 shows the projection of the structure along [001].

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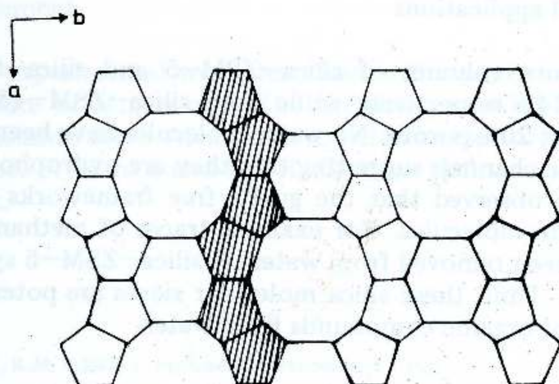


**Figure 5 Projection of the structure of silica-ferrierite along (001). One chain of zig-zag 5-membered rings is shaded.**

Main structural features of silica–ZSM–48, silica–ZSM–22 and silica–ferrierite are compared with those of silica–ZSM–5 (silicalite 1)[3] in Table 4. Silica–ZSM–5 framework contains 4–membered rings in addition to 5–,6–and 10–membered rings of [$SiO_4$] The framework is traversed by a 3D channel system containing straight and sinusoidal channels with 10–T–ring openings. The channel openings are elliptical with free diameter intermediate between those of large pore (faujasite type) and small pore (Linde type A) zeolites.

### 3.6 Thermal behaviour

Silica–ZSM–5, silica–ZSM–48, silica–ZSM–22 and silica–ferrierite frameworks were found to be thermally stable up to about 1000°C. A continuous weight loss has been observed in TGA up to about 900–1000°C at which temperature a constant weight has been obtained. During thermal treatment the organic guest species are decomposed, oxidized and removed from the channels resulting in guest-free frameworks. The framework is unaffected even after prolonged heat treatment at 900°C, as shown by powder X–ray patterns. However, in the temperature range 1000–1100°C the silica frameworks transform slowly to the thermodynamically stable phase, cristobalite.

TGA of silica–ZSM–5 shows that, the weight loss occurred in two stages in the temperature range 300–800°C indicating possible self–blocking at the channel intersections. Similar but relatively less self–blocking is observed in silica–ferrierite. For instance weight loss of silica–ferrierite in TGA occurs in 2 stages although the two stages are not clearly visible unlike in the case of silica–ZSM–5. Fragments of the guest species located at the channel intersections may be responsible for this blocking. However, in silica–ZSM–48 and silica–ZSM– 22 with 1D channel systems the self–blocking is not evident and their weight loss is gradual and continuous in the temperature range 350–800°C.

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### 3.7 Properties and applications

Intracrystalline pore volumes of silica—ZSM—5 and silica—ferrierite are about 33% and 24% respectively while both silica—ZSM—48 and silica—ZSM—22 are about 20% porous. No water molecules have been observed to be located in these channels suggesting that they are hydrophobic. Furthermore, it has been observed that the guest—free frameworks have greater affinity for organic molecules. For example traces of methanol, propanol and hexane have been removed from water by silica—ZSM—5 synthesized in the present study. Thus, these silica molecular sieves are potentially useful in the separation of organic compounds from water.

Furthermore, it has been observed that the guest-free frameworks have a greater affinity for organic molecules. A series of aqueous solutions have been prepared using 5—10 ppm of (i) methanol, (ii) propanol and (iii) hexane, with deionized water. To these solutions guest-free silica—ZSM—5 has been added and equilibrated for 6 hours. The contents of methanol, propanol and hexane have been estimated using High Performance Liquid Chromatography (HPLC) before and after this treatment. The solutions obtained after equilibration with silica—ZSM—5 followed by filtration, were completely free of any organic compounds showing that these molecular sieves are capable of separating organic compounds from water.

Table 5. Framework decomposition temperatures of some zeolites and zeosils

| Zeolite/Zeosil | Framework decomposition Temperature (approximately) |
|---|---|
| Ferrierite[14] | 960$^0$C |
| ZSM—22[7] | 940$^0$C |
| Silica—ferrierite | 1100$^0$C |
| Silica—ZSM—22 | 1060$^0$C |

As mentioned earlier they are thermally very stable in comparison (Table 5) with the corresponding zeolites. The frequent occurrence of 5—membered rings of [$SiO_4$] tetrahedra throughout these structures may be responsible for the high thermal stability of these zeosils. In addition, they are stable to most mineral acids except HF. However, since these molecular sieves have electrically neutral frameworks, neither catalytic activity nor cation exchange properties may be expected. Nevertheless, they may perhaps be employed as catalyst carriers in industrial catalysis.

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# OBSERVATIONS ON THE FORAGE POTENTIAL OF VELVET BEAN

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**Abstract:** A field trial was conducted with velvet bean (*Mucuna* spp.) planted during the Maha season of 1983/84. Whole crop samples of the above ground dry matter were taken at four different stages of growth (60, 90, 120 and 150 days) to assess the interaction of dry matter yield and forage quality during maturity. Dry matter yield increased with maturity, but quality parameters steadily declined. The results suggest that the velvet bean forage should be harvested around 90 days. Harvested at this maturity, a dry matter yield of 3.1 tonnes per hectare, with a crude protein content of 20.6% and 55.4% *in vitro* digestibility, could be obtained.

## 1. Introduction

The usefulness of legumes as components in forage mixtures is well recognised. The beneficial effects attributed to the legume derive partly from its ability to fix atmopheric nitrogen and partly from the high protein contents of its dry matter.[14,20] In Sri Lanka, at least on account of the latter reason, establishment of legumes is justified.

Velvet bean (*Mucuna* spp; *Syn Stizolobium* spp),[20] which was originally introduced as a cover crop in coconut plantations, has the potential as a forage crop. The major characteristics of this forage legume has been recently reviewed[11] and, in fact, its use is recommended for the hill country region.[12] The study reported herein is a preliminary attempt to assess the productivity and nutritive value of velvet bean forage at four different growth stages. Such assessment has not been reported previously for velvet bean grown in Sri Lanka.

## 2. Materials and Methods

### 2.1 Description

Several selections of velvet bean are known to exist in Sri Lanka,[16] but the exact classification is difficult as the taxonomy of the species is confusing.[6] They are all annual-perennial legumes with vigorously growing vines which may extend up to 6 m in length. The leaves are trifoliate with large, ovate leaflets. The white to dark purple flowers appear in long pendant clusters. Pods, 10 to 14 in a cluster, are borne singly and have a greyish-white pubescence of short, silky hairs. Three seedcoat colour types, namely black, white and mottled, are common. [15,20,23]

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Originating in India, it is now naturalized in all tropical countries. Their distribution has been extended into the temperate countries by breeding.[23] The legume is tolerant to drought due to its deep roots.[6,15]

The velvet bean variety used in the present evaluation was black-seeded. The seeds were obtained from the Coconut Research Institute, Lunuwila.

**2.2 Growing conditions**

Velvet beans were planted (three seeds per hill) at a spacing of 60 x 60 cm during the 1983/84 Maha season in an experimental plot (6.0 x 12.0 m) at the Department of Animal Science, University of Peradeniya. The soil at the site was reddish brown latasolic soil with a pH of 6.1. A well distributed rainfall was received during the experimental period.

The plot received a basal application of one tonne of poultry litter/ha (equivalent to approximately 52 kg N, 36 kg $P_2O_5$ and 35 kg $K_2O$ per hectare) at planting. The stand was thinned to two plants per hill after one month and bamboo trellises were provided for support. The supports were given mainly to prevent matting of plants from adjacent hills and to facilitate the yield estimations.

**2.3 Sampling procedures**

The flowering in velvet beans commences around 90 days and continues for another 50 – 70 days. The sampling of the forage was done at 60 days (pre-bloom), 90 days (start of bloom), 120 days (mid-bloom) and 150 days (post-bloom). At each age of harvesting, twenty plants (from ten hills) were randomly selected and cut 10 cm above ground. Leaves and stems (and pods at 120 and 150 days of age) from each plant were hand-separated, and weights were recorded. Sub-samples (ca 500 g) of the different anatomical parts were removed to the laboratory for dry matter determination. Further sub-samples (ca 2 kg) were taken and dried at 60$^0$C in an unitherm oven for 36 – 48 hours. The dried samples were then ground and stored in air tight plastic jars for subsequent laboratory evaluation.

Velvet bean pods and seeds are also reported to have been used for livestock feeding.[6,15] For this reason, samples of green pods (immature and mature), seeds and pod husks were also collected for analysis.

**2.4 Analytical procedures**

The dry matter, crude protein, ether extract and ash contents were determined according to standard procedures.[2] The detergent fibre composition was determined using the method of Goering and Van Soest.[9] *In vitro* organic matter digestibility (IVOMD) was determined according to the method of Tilley and Terry[22] as modified by Barnes.[3] Rumen liquor was

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collected from two fistulated cattle which have been previously fed a mixture of straw and legumes. Five standard samples of known *in vivo* digestibility were used in each *in vitro* run to correct for run-to-run variation. The standard samples consisted of legume and straw samples.

## 3. Results and Discussion

The forage dry matter yields of velvet bean increased with maturity (Table 1). The observed yields are in the same range as those reported by King *et al.*[13] in Australia and by Takahashi and Ripperton[21] in Hawaii.

Table 1. Some productive parameters of velvet bean forage.

| Age (days) | Dry matter (%) | Total dry matter production[a] | | Major anatomical fractions (%) | | |
|---|---|---|---|---|---|---|
| | | g/plant/cut[b] | kg/ha/cut[c] | Leaf | Stem | Reproductive parts |
| 60 | 18.2 | 61 | 1660 | 54.6 | 45.4 | – |
| 90 | 21.1 | 114 | 3103 | 47.9 | 52.1 | – |
| 120 | 23.7 | 161 | 4383 | 41.1 | 55.1 | 3.8 |
| 150 | 20.4 | 194 | 5282 | 30.3 | 60.4 | 9.3 |

a Includes reproductive parts in plants harvested at 120 and 150 days of age.
b Mean of twenty plants.
c Extrapolated yield based on a plant density of 27,225/ha.

The relative dry weight distribution of different anatomical parts (Table 1) show that at 60 days the leaves constituted 55% whereas only 45% was stems. This changed during growth to 30% leaves and 61% stems by 150 days. The reproductive parts, mainly pods, constituted the balance 9%. The general tendency of a decreasing leaf:stem ratio during growth is in agreement with previous studies reported for other tropical and temperate legumes.[1,20]

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Table 2. Chemical composition and IVOMD of velvet bean forage at four different stages of growth (percentage DM basis).

| Parameter | Plant age (days) | | | |
|---|---|---|---|---|
| | 60 | 90 | 120 | 150 |
| Crude protein | 24.80 | 20.61 | 14.75 | 12.84 |
| Ether extract | 2.78 | 2.72 | 2.51 | 2.39 |
| Ash | 5.52 | 5.79 | 4.55 | 6.43 |
| Acid detergent fibre | 33.15 | 40.12 | 45.83 | 47.91 |
| Cell wall | 40.26 | 51.35 | 56.78 | 59.15 |
| Cell contents | 59.74 | 48.65 | 43.22 | 40.85 |
| Hemicellulose | 7.11 | 11.23 | 10.95 | 11.24 |
| Cellulose | 27.85 | 31.12 | 36.24 | 37.50 |
| Permanganate lignin | 3.87 | 5.92 | 7.75 | 8.96 |
| Silica | 0.65 | 1.07 | 1.63 | 1.85 |
| IVOMD | 66.41 | 55.35 | 50.60 | 42.23 |

As shown in Table 2, the velvet bean forage contained 20.6 – 24.8% crude protein during the pre-bloom stage. The crude protein content declined rapidly with flowering, due to a redistribution of nitrogen to the seeds.[5] Despite this decline, the crude protein content of the legume herbage (even at 150 days) remained at a higher level than of common grasses available in Sri Lanka.[10]

Cell walls are a major part of the plant. The amount and composition of the cell walls is probably the most important factor influencing the nutritive value of forages.[4] But studies of the composition of the cell walls in tropical forages are scanty. As such, comparison of the present data with other work is not possible. The data (Table 2) shows that all cell wall components steadily increased with maturity.

The *in vitro* digestibility of the velvet bean forage decreased from 66.4% to 42.2% as the plant matured. This is to be expected, since the general trend of decline in the digestibility of forage crops with plant maturity is well documented.[8,19] This decrease can be attributed to a combination of factors *inter alia* decreasing leaf to stem ratio, decreasing amount of crude protein, increasing amount of cell walls and increasing lignification. Although not reported separately in the present data, the fact that the chemical composition of leaves and stems during crop maturity followed distinctly different patterns needs a special mention. The contents

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of crude protein, cell walls and lignin and *in vitro* digestibility of the leaf fraction changed only a small extent during maturity, while in the stems all these parameters decreased rapidly.

Table 3. Chemical composition and IVOMD of some reproductive parts of velvet bean.

| Parameter | Immature green pod | Mature green pod | Mature seed | Pod husk |
|---|---|---|---|---|
| DM (%) | 30.66 | 41.56 | 88.50 | 91.60 |
| % DM basis | | | | |
| Crude protein | 14.68 | 19.29 | 26.45 | 3.13 |
| Ether extract | 4.21 | 4.80 | 4.32 | 3.88 |
| Ash | 3.94 | 3.85 | 3.46 | 4.87 |
| ADF | 27.33 | 31.89 | 10.91 | 40.70 |
| Cell wall | 53.96 | 59.11 | 21.90 | 64.81 |
| Cell contents | 46.04 | 40.89 | 78.10 | 35.19 |
| Hemicellulose | 22.63 | 27.22 | 10.99 | 24.11 |
| Cellulose | 20.10 | 23.81 | 9.34 | 29.80 |
| Permanganate lignin | 6.39 | 7.03 | 0.80 | 8.60 |
| Silica | 0.60 | 0.65 | 0.40 | 1.10 |
| IVOMD % | 67.50 | 58.10 | 70.70 | 40.50 |

Surprisingly the mature green pods had a higher crude protein content than tender green pods (Table 3), but the IVOMD of mature pods was lower. The crude protein and IVOMD values of mature velvet bean seeds were 26.4% and 70.7%, respectively. These values compare closely to those reported for the common food legumes grown in the Asian continent.[7] Recent reports suggest that the velvet bean seeds have potential as a protein source both in human and animal nutrition.[17,18] The data show pod husks to be a poor quality roughage. The crude protein content and IVOMD value of pod husks are similar to those generally quoted for paddy straw.[10]

On the strength of these preliminary results, the forage potential of velvet bean appears good. The data indicate that the velvet bean forage should be harvested around 90 days. Harvested at this age, velvet bean would provide a yield of 3100 kg/ha of moderate quality (20.6% crude protein and 55.3% IVOMD) forage dry matter. The productivity of velvet bean forage is sufficiently encouraging to warrant further field trials. The persistency of the legume under repeated harvesting and its compatibility with companion grasses are aspects that need be evaluated in future studies.

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**Acknowledgement**

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# A NEW ISOLATE OF *SCLEROTIUM ROLFSII* SACC. CAUSING BULB ROT IN ONION (*ALLIUM CEPA* L. VARIETY POONA RED)

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*(Date of receipt : 03 March 1987)*
*(Date of acceptance : 25 February 1988)*

**Abstract :** The causative fungus of a hitherto unreported bulb rot disease of onion (*Allium cepa* L. variety Poona red) was found to be *Sclerotium rolfsii* Sacc. The vegetative propagative structures of the fungus known as sclerotia, were able to germinate within 24h on a suitable substratum and produce new colonies. The fungus took about ten days to produce fresh sclerotia from germinating sclerotia, under laboratory conditions. The fungal isolate obtained from infected onion plants (*Sclerotium rolfsii* – Isolate 5) had a temperature optimum of 35°C for germination of sclerotia. It showed luxurious growth and maximum amount of sclerotial production at temperatures between 30 and 35°C. The fungus was able to survive in a wide range of pH conditions (pH 3 to 9) but had an optimum pH of 5.5 for germination of sclerotia and radial growth.

## 1. Introduction

*Sclerotium rolfsii* Sacc. is a versatile soil borne pathogen attacking several crop plants.[7] It is widespread throughout the moist tropics and warmer region of the temperate zone and has an extensive and varied host range.[2] It has been found to cause a variety of diseases namely damping off of seedlings, collar or stem rot,[1] foot rot, crown rot, Sclerotium wilt and blight.[3] The sequence of events during the maceration or death and decay of host tissue resulting in the formation of symptoms has been found to be complicated as suggested by Punja *et al.*[5]

The fungus forms sclerotia during unfavourable conditions and remains in the soil for long periods without being affected by adverse soil and environmental conditions. When favourable conditions intervene and if suitable substrata are available, the sclerotia germinate producing clusters of hyphae around them.[4]

In July, 1986 our attention was drawn to a disease of Bombay onion *(Allium cepa. L.)* in the Arali area of Jaffna. The onion plants of the variety Poona red were attacked by a fungus. The disease occurred in the seed bed when the plants were about 45 days old. Symptoms were associated with bulb rot. However, the initial symptoms appeared as yellowing of tips of onion scales or leaves. The discolouration was found to spread down the leaves and the tips became necrotic and brown. The above symptoms were

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followed by wilting of scathes. Groups or patches of plants showing the above symptoms appeared in the field. On close examination of uprooted diseased plants, the bulbs were found to have withered with the complete loss of their root system. Strands of white fan shaped hyphae were seen on the bulbs. These clusters of hyphae also enveloped the base of the stem near soil level. The rotten plant material and the soil samples taken from around these plants when examined showed presence of numerous, tiny, white or yellow-brown sclerotia at various developmental stages and in the form of strands. Each of the hyphal strands found on the surface of bulbs consisted of 4 — 10 hyphae. From the characteristic growth of hyphae and the size and shape of sclerotia, the fungus was identified as *Sclerotium rolfsii* Sacc. The identity of the fungus was confirmed by Commonwealth Mycological Institute (CMI Report No: H107/86/YS18).

The fungus was constantly associated with the symptoms produced on onion bulbs in the field. This is in fact the first record of the disease caused by *S. rolfsii* on onion variety-Poona red in Sri Lanka. The fungus obtained from affected bulbs was referred to as *Sclerotium rolfsii* — Isolate 5.

## 2. Experimental

Infected onion bulbs (variety Poona red) were collected from the field and brought to the laboratory. These were washed with tap water, surface sterilized by dipping in 0.5% sodium hypochlorite solution for 1 min and then washed in 3—4 changes of sterile distilled water. Small pieces from surface sterilized bulbs were transferred on to freshly poured Potato Dextrose Agar (PDA) plates and incubated at room temperature (30+ $4^{0}$C) for seven days.

The morphology of the colony and of the fungus on PDA was noted. Measurements of length and breadth of primary, secondary and tertiary hyphae were made under a light microscope from a 3 day old colony of the fungus. Hand sections of mature sclerotia were also made and examined under the microscope.

Following observations on the morphology of the fungus, studies were made on germination of sclerotia, radial growth of mycelium and sclerotial production after different periods of incubation on PDA. For studies on germination of sclerotia mature brown sclerotia were dislodged from the surface of a ten day old colony with a soft brush and transferred on to fresh PDA plates. Ten sclerotia were transferred on to each of 5 PDA plates and incubated at room temperature under continuous illumination (7 kl). The percentage sclerotial germination was determined at 24h intervals during incubation. The diameter of colonies formed from the germinating sclerotia were also measured at 24h intervals along two predetermined diameters at right angles to each other. Radial growth of the fungus was also studied by inoculating 10 fresh PDA plates at the centre with 0.8cm diameter mycelial discs cut from the advancing region of a 6 day old colony of *Sclerotium*

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*rolfsii* — Isolate 5. These plates were also incubated as before for 7 days and the diameter of colonies was measured daily. Sporulation was measured by counting the number of sclerotia produced per plate and then converting the value to number of sclerotia produced per $1cm^2$ of the colony.

With the basic information on the morphology and various developmental phases in the life cycle of *S. rolfsii*, the effects of some environmental factors on the above developmental stages were studied.

To study the effect of temperature on sclerotial germination, PDA plates inoculated with mature brown sclerotia harvested from a ten day old colony of *S. rolfsii* were incubated at 10,15,20,25,30,35 and $40^0$C under continuous illumination for 5 days. The sclerotial germination was determined at 24h intervals at the above temperatures. Five plates were used for each temperature.

Similarly, PDA plates were inoculated with 0.8cm diameter mycelial discs cut from a 6 day old colony and incubated at the above temperatures for 7 days. The diameters of colonies were measured as before on each day during incubation. Ten plates were used at each temperature to give a total of 20 diameter measurements.

To study the effect of temperature on sclerotial production, PDA plates were inoculated with mycelial discs as described earlier and incubated at room temperature for 3 days. They were then kept at 10,15,20,25,30,35 and $40^0$C for further 4 days. Sclerotial production was estimated at 24h intervals from the third day after initial incubation. Ten plates were used at each temperature.

The effect of pH was studied only on the germination of sclerotia and the growth of the fungus. PH of the medium was adjusted to 3.0,4.0,5.0,5.5, 6.0,7.0,8.0 and 9.0 with a buffer mixture of citric acid/potassium dihydrogen orthophosphate, before being poured into sterile petri dishes. Sclerotia of *S. rolfsii* — Isolate 5 were transferred to PDA plates of different pHs and were incubated at room temperature for 3 days. The mean percentage germination of sclerotia was determined each day during incubation. Each set up was replicated 5 times. Similarly, mycelial discs were transferred to PDA plates at different pHs and were incubated for 7 days at room temperature. The diameters of colonies were measured at 24h intervals as described earlier.

## 3. Results

Colonies of *S. rolfsii* -- Isolate 5 on PDA were pure white with narrow mycelial strands radiating on the colony surface. The sclerotial initials appeared as tiny white knots of mycelia which later turned pale yellow, pale brown and finally dark brown in colour while they increased in size. Mature brown sclerotia were spherical but slightly flattened below measuring up to

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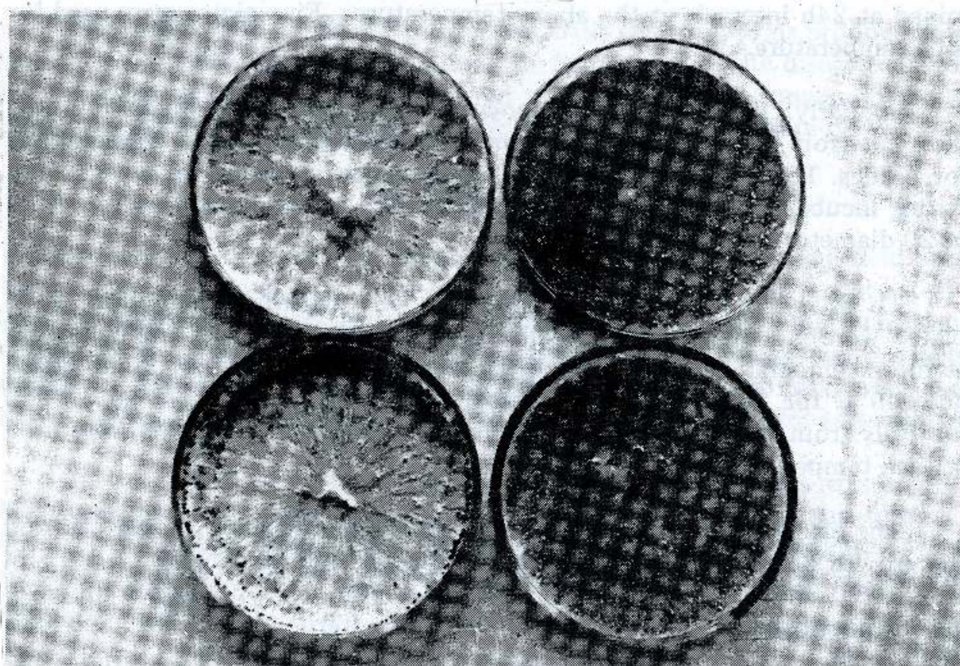
about 2mm across when fresh. The outer wall of sclerotia was shiny and smooth. Primary hyphae of the fungus were 5.0 — 8.2 m in breadth and grew along the surface of the medium while the secondary hyphae were usually narrow, about 2—4 m in breadth and about 350 m in length and often grew adpressed to the primary hyphae. Tertiary hyphae and other subsequent branches were also narrow and had comparatively short cells. The branching was of tertiary hyphae occurred in a wide angle and was not associated with septation. The tertiary hyphae were usually about 190 m in length and 1.0 — 1.8 m in breadth. Clamp connections were only present on the primary hyphae.



Photograph 1 : The mycelium (left top), formation of sclerotial initials (right top), maturation of sclerotia (right bottom) and a mature colony (left bottom) of *Sclerotium rolfsii* on PDA (X⅙)

Hand sections of the mature sclerotia of *S. rolfsii* — Isolate 5 showed a sharply differentiated outer rind about 1.2 — 2.0 $\mu$m in thickness with strongly pigmented cells. Cortex of the inner region had fairly pigmented small cells and the innermost medulla had large and almost hyaline cells. Reserve materials were found as globules in the cells of cortex and medulla.

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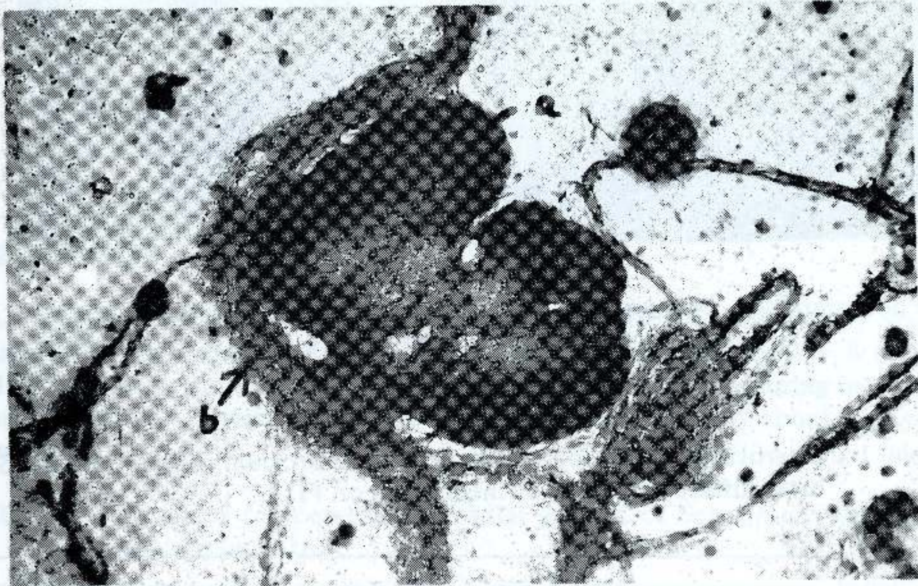
### 3.1 Sclerotial germination

Sclerotia germinated within 24h on PDA. The mode of germination was hyphal according to Punja[4] and the hyphae originated from cells of the medulla in all directions. Mean percentage germination of sclerotia increased significantly at 24h intervals during incubation and reached 100% on the third day after inoculation (Table 1).




Photograph 2 : The T.S. of a developing sclerotial initial (a) and cluster of hyphae (b) taken from a 3 day old colony of *S. rolfsii* on PDA, X40

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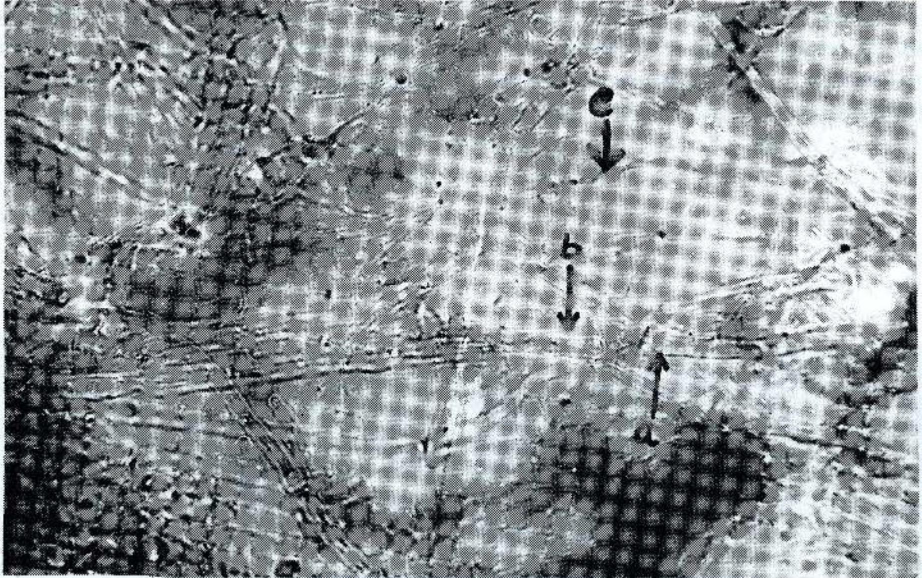


Photograph 3 : Primary (a), secondary (b), and tertiary (c) hyphae taken from a 3 day old colony of *S. rolfsii* on PDA, X400

Table 1. Sclerotial germination and growth of *Sclerotium rolfsii* — Isolate 5 after different periods of incubation on PDA.

| Period of incubation (hours) | Mean % germination of sclerotia | Mean diameter of colony in mm. |
|---|---|---|
| 24 | 40 a | 3.00 |
| 48 | 75 b | 8.50 |
| 72 | 100 c | 21.50 |
| 96 | 100 | 32.00 |
| 120 | 100 | 41.50 |

Note: Means after different periods of incubation were compared by simple t-test and the values denoted by different letters were found to be statistically different.

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The germinated sclerotia formed tiny white and circular colonies, consisting of sparse hyphae, which become visible after 24h of incubation. The colony size increased with the period of incubation (Table 1). The above colonies developed sclerotial initials on the aerial hyphae on the fifth day after inoculation.

Table 2. Effect of temperature on sclerotial germination of *Sclerotium rolfsii* — Isolate 5 on PDA.

| Period of incubation | Mean % germination of sclerotia Temperature ($^0$C) | | | | | | |
|---|---|---|---|---|---|---|---|
| (hours) | 10 | 15 | 20 | 25 | 30 | 35 | 40 |
| 24 | 0 | 0 | 0 | 30 | 60 | 65 | 65 |
| 48 | 0 | 0 | 0 | 45 | 85 | 100 | 100 |
| 72 | 0 | 0 | 0 | 90 | 100 | 100 | 100 |
| 96 | 0 | 0 | 0 | 100 | 100 | 100 | 100 |
| 120 | 0 | 0 | 0 | 100 | 100 | 100 | 100 |

### 3.2 Radial growth of the fungus

The fungal mycelium showed luxurious growth on PDA. The colony was pure white and fluffy with dense hyphal strands consisting of 4 – 5 individual hyphae per strand. The colonies increased in diameter during incubation and almost covered the entire 9.5cm diameter plate in about 5 days (Table 3).

Table 3. Effect of temperature on radial growth of *Sclerotium rolfsii* — Isolate 5 on PDA.

| Period of incubation (hours) | Mean diameter of colony in cm Temperature ($^0$C) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 10 | 15 | 20 | 25 | 30 | 35 | 40 |
| 0 | 0.80 | 0.80 | 0.80 | 0.80 | 0.80 | 0.80 | 0.80 |
| 24 | 0.80 | 0.80 | 0.80 | 0.80 | 1.00 | 1.30 | 1.10 |
| 48 | 0.80 | 0.80 | 0.80 | 0.90 | 4.20 | 4.25 | 3.25 |
| 72 | 0.80 | 0.80 | 0.80 | 1.50* | 8.15* | 8.35* | 5.20 |
| 96 | 0.80 | 0.80 | 0.90 | 3.15 | 9.05 | 9.35 | 6.25 |
| 120 | 0.80 | 0.80 | 1.10 | 5.00 | 9.50 | 9.50 | 7.55 |
| 144 | 0.80 | 0.80 | 1.70 | 7.15 | 9.50 | 9.50 | 8.90 |
| 168 | 0.80 | 0.80 | 2.20* | 8.75 | 9.50 | 9.50 | 9.50 |

*Formation of sclerotial initials

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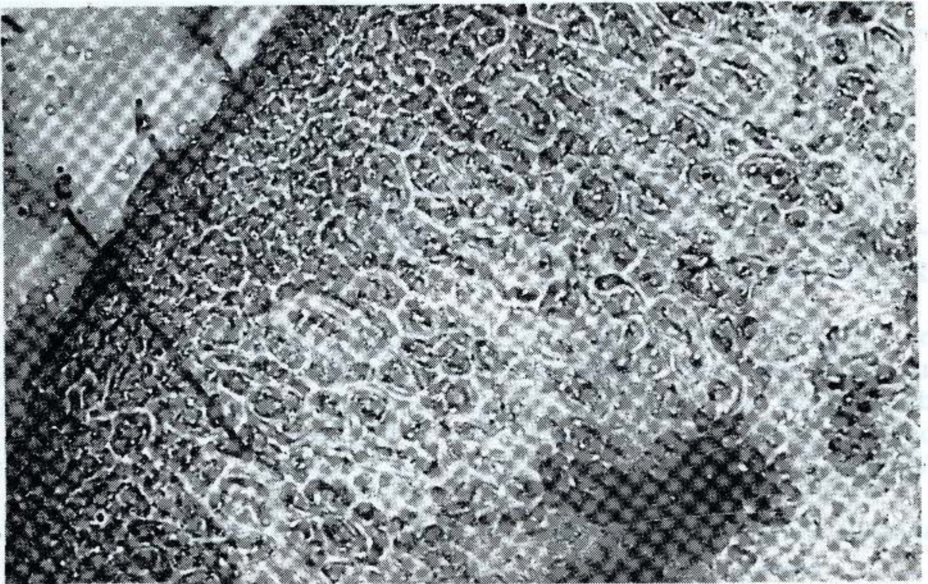
### 3.3 Production of sclerotia

Sclerotial initials appeared on the third day after inoculation and turned yellow -brown within two days. The number of immature sclerotia produced increased gradually during the incubation and mature brown sclerotia were seen only on the seventh day after incubation (Table 4).



Photograph 4 : A portion of T.S. through a mature sclerotium obtained from a 7 day old colony of *S. rolfsii* on PDA. (a) Rind, (b) Cortex, (c) Medullary region X400

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Table 4. Effect of temperature on production of sclerotia of *Sclerotium rolfsii* — Isolate 5 on PDA.

| Days after inoculation | Number of sclerotia produced $cm^{-2}$ of colony Temperature in $^0C$ | | | | | | |
|---|---|---|---|---|---|---|---|
| | 10 | 15 | 20 | 25 | 30 | 35 | 40 |
| 4 | 0 | 0 | 0 | 0 | 10 | 12 | 6 |
| 5 | 0 | 0 | 0 | 11 | 26 | 32 | 14 |
| 6 | 0 | 0 | 0 | 19 | 35 | 35 | 28 |
| 7 | 0 | 0 | 0 | 36 | 46 (13) | 38 (9) | 36 (7) |
| 8 | 0 | 0 | 0 | 38 (4) | 68 (18) | 42 (12) | 36 (10) |

Note: The numbers given within brackets indicate the number of mature brown sclerotia.

### 3.4 Effect of temperature on the germination of sclerotia

Sclerotia failed to germinate at temperatures below $25^0$C (Table 2). Optimum temperature for germination of sclerotia was around $35^0$C. Mean percentage germination improved with increase in period of incubation.

### 3.5 Effect of temperature on the radial growth of the fungus

Results which appear in Table 3 show that the fungus failed to grow at low temperatures below $15^0$C while the growth was slow at 20 and $25^0$C. Best radial growth was observed at temperatures between 30 and $35^0$C. White knots of sclerotia appeared in the colony on the third day at 30, 35 and $40^0$C and on the sixth day at $25^0$C.

### 3.6 Effect of temperature on production of sclerotia

The total number of sclerotia and sclerotial initials formed on the colonies was counted on all the plates at different temperatures after different periods of incubation. From the above data the number of sclerotia and sclerotial initials formed on $1cm^2$ of the colony was determined.

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No sclerotia production was observed at temperatures below $25^0$C and maximum amount of sclerotia were produced at temperatures between 30 and $35^0$C (Table 4). The mean number of sclerotial initials produced per $cm^2$ area of colony increased with increase in period of incubation. Mature brown sclerotia were observed on the seventh day after initial inoculation at 30, 35 or $40^0$C and on the eighth day at $25^0$C.

### 3.7 Effect of pH on sclerotial germination

Sclerotia germinated at all pHs tested in this experiment (Table 5). Germination occurred best at pH 5.5. Germination also improved with increase in period of incubation and was maximum on the third day after inoculation at pHs 4 to 7.

Table 5. Effect of pH on sclerotial germination of *Sclerotium rolfsii* — Isolate on PDA.

| pH | Mean % germination of sclerotia period of incubation in hours | | | | |
|---|---|---|---|---|---|
| | 24 | 48 | 72 | 96 | 120 |
| 3.0 | 30 | 39 | 62 | | |
| 4.0 | 33 | 58 | 100 | 100 | 100 |
| 5.0 | 40 | 75 | 100 | 100 | 100 |
| 5.5 | 60 | 85 | 100 | 100 | 100 |
| 6.0 | 38 | 52 | 100 | 100 | 100 |
| 7.0 | 35 | 50 | 100 | 100 | 100 |
| 8.0 | 30 | 42 | 78 | 80 | 80 |
| 9.0 | 28 | 35 | 58 | 60 | 60 |

### 3.8 Effect of pH on the radial growth of the fungus

The fungus showed luxurious growth at pHs between 4—7 but growth occurred best at pH 5.5 (Table 6). The fungus was able to survive a wide range of pH conditions.

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Table 6. Effect of pH on radial growth of *Sclerotium rolfsii* — Isolate 5 on PDA.

| pH | Mean diameter of colony in cm. Period of incubation in hours. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 24 | 48 | 72 | 96 | 120 | 144 | 168 |
| 3.0 | 0.80 | 0.82 | 2.86 | 4.22 | 6.96 | 8.15 | 9.06 | 9.50 |
| 4.0 | 0.80 | 0.90 | 3.23 | 7.50 | 8.25 | 9.50 | 9.50 | 9.50 |
| 5.0 | 0.80 | 0.95 | 3.65 | 7.86 | 8.70 | 9.50 | 9.50 | 9.50 |
| 5.5 | 0.80 | 1.00 | 4.20 | 7.94 | 8.95 | 9.50 | 9.50 | 9.50 |
| 6.0 | 0.80 | 0.93 | 4.10 | 7.16 | 8.65 | 9.50 | 9.50 | 9.50 |
| 7.0 | 0.80 | 0.87 | 3.41 | 6.09 | 8.15 | 8.90 | 9.40 | 9.50 |
| 8.0 | 0.80 | 0.80 | 8.00 | 6.00 | 7.45 | 8.30 | 8.78 | 9.20 |
| 9.0 | 0.80 | 0.80 | 2.65 | 5.53 | 6.25 | 6.18 | 7.50 | 8.15 |

## 4. Discussion

An aerial mycelium composed of both single hyphae and hyphal strands consisting of a few hyphae has been observed on the surface of onion bulbs infected with *S. rolfsii* which has been described as a plant pathogenic soil - borne fungus by Aycock.[2]

The active spread of the fungus through the soil following the seasonal rain has been observed in the onion fields in the Arali area of the Jaffna region. The infected bulbs became partially or entirely covered with thick wafts of hyphae which radiate upwards from the base of the stem.

The tiny but macroscopic spherical sclerotia produced in large numbers on and around the rotten bulbs form the outstanding characteristic of the organism. The mature sclerotia obtained from diseased bulbs germinated readily on PDA without any period of dormancy. The fungus took about ten days to produce fresh sclerotia from germinating sclerotia on artificial medium. The observations that sclerotial initials arise from the aerial strands of a colony tally with those made by Townsend and Willetts.[6]

Sclerotia of *S. rolfsii* — Isolate 5 germinated best at $35^0$C while other stages in the fungal development such as radial growth and sclerotial production required an optimum temperature slightly less than $35^0$C. This is in accordance with the observation that the soil temperature in the Arali area during the period of disease incidence was around $35^0$C.

Although the fungus was found to establish itself in a wide range of pH,

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best performance was obtained at pH 5.5. The severity of infection in the affected area may be therefore due to the acidic nature of soil (pH around 6.0). Further, studies are underway to control the establishment of the fungus in the field and the spread of the disease.

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# DRIED BIOGAS SLURRY AS A NUTRIENT SOURCE IN GROWING PIG DIETS

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**Abstract:** Two feeding experiments were conducted to evaluate the effects of including dried biogas slurry (DBS) on the performance of growing pigs. In each experiment, 36 growing pigs were limit-fed diets containing 0,5,10, and 20% DBS substituted (w/w) for coconut oil cake and rice bran. The results suggest that DBS is potentially useful as a nutrient source in growing pig diets. The inclusion of up to 10% DBS in diets had no adverse effect on gain and feed efficiency, whereas 20% DBS inclusion depressed performance which may be attributed to its high fibre and low available energy contents. The merits of biogas slurry over the raw manure and the practical limitations to its use are discussed.

## 1. Introduction

The need to develop renewable sources of energy has generated much interest during the recent decades in biogas production from animal manures. The slurry (solid by-product) from biogas digesters is generally utilized as a fertilizer for crops as in fish ponds. Because the biogas slurry contains moderate amounts of protein,[7] it is possible that the dried slurry could be used as an animal feeding stuff.

Extensive reviews are available on the refeeding of various forms of animal manure,[3,4,5] but corresponding data on the feeding value of biogas slurry is almost non-existent. The experiments reported herein were carried out to evaluate dried biogas slurry (DBS) as a source of nutrient in growing pig diets.

## 2. Materials and Methods

### 2.1 Source of DBS

The DBS samples used in this study originated from a continuosly-fed Indian-type field scale biogas digester maintained at the piggery of the University of Peradeniya. Fresh manure was washed daily into the digester from pigs housed in semi-open concrete-floored pens. The pigs were fed on a low-quality swill (8-10% crude protein, 10-16% crude fibre) and limited quantities of a mixture of rice bran and coconut oil cake. The solid effluent from the digester was removed, initially sun-dried on a concrete floor to 86-88% solids content and finally dried overnight in an Unitherm oven at

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$60^0$C. The resultant hard crumbs were ground into a semi-powdery form before incorporation into the diets.

Prior to the commencement of each feeding trial, the DBS was bulked and representative samples were taken for the analysis of proximate components.[1] Representative samples of rice bran and coconut oil cake were also subjected to similar analyses.[1]

## 2.2 Feeding experiments

The study consisted of two separate feeding experiments. In each experiment, thirty-six crossbred barrows were divided into three groups on the basis of weight. The average weight of pigs in Experiments 1 and 2 were 15.5 and 18.3 kg, respectively. Pigs within each weight group were assigned to one of twelve pens, so that there were three pigs per pen. The four treatments were then randomly allocated to three pens each.

The composition of the experimental diets is shown in Table 1. They were designed to contain 0,5,10 and 20% DBS, the DBS replacing coconut oil cake and rice bran in the control diet. Each five parts of DBS replaced (w/w) three parts of coconut oil cake and two parts of rice bran. The diets were iso-nitrogenous but not iso-caloric since the digestible energy value of DBS was not available.

Pigs were housed in concrete-floored pens. The diets were limit fed twice daily in equal amounts. The amount of feed to be given was determined by the animal group consuming the least during a 30-minute feeding period at each weighing. Feeding levels were held constant until next weighing. Body weights were recorded at weekly intervals. The experiments were of eight weeks duration. Diets were mixed with water immediately prior to feeding to improve consumption and to reduce wastage. Water was freely available at all times.

Average daily gain and feed to gain ratio were computed on a pen basis. The data were subjected to analysis of variance and the treatment means were compared using the Tukey's procedure.[8]

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Table 1. Percentage composition of pig grower diets used in the feeding trials

| | Control | Level of DBS (%) | | |
|---|---|---|---|---|
| | | 5 | 10 | 20 |
| Maize | 25 | 25 | 25 | 25 |
| Coconut oil cake | 40 | 37 | 34 | 28 |
| Rice bran | 25 | 23 | 21 | 17 |
| DBS | - | 5 | 10 | 20 |
| Fish meal (50% CP) | 6 | 6 | 6 | 6 |
| Skim milk powder | 2 | 2 | 2 | 2 |
| Bone meal | 1.5 | 1.5 | 1.5 | 1.5 |
| Vitamin-trace mineral premix[a] | 0.25 | 0.25 | 0.25 | 0.25 |
| Salt | 0.25 | 0.25 | 0.25 | 0.25 |
| Analysis (dry matter basis) | | | | |
| Crude protein (%)[b] | 18.2 | 18.1 | 18.3 | 18.2 |
| Digestible energy (Mcal/kg)[c] | 2.88 | - | - | - |

[a]Supplied (per kilogram of premix): 6,600,000 IU vitamin A, 528,000 IU vitamin $D_3$, 880 IU Vitamin E, 1.98 g riboflavin, 4.4 g calcium panthothenate, 8.8 g niacinamide, 22.0 g choline chloride , 8.8 g vitamin $B_{12}$, 1.32 g vitamin K, 5.28 g manganese sulfate, 1.98g copper carbonate, 0.99 g calcium iodate, 0.55 g cobalt sulfate and 0.22 g zinc oxide.

[b]Determined value.

[c]Calculated value.

## 3. Results and Discussion

The compositional data of DBS confirm its moderate nutritional potential for non-ruminants (Table 2). The crude protein content of DBS samples used in Experiments 1 and 2 were 16.6 and 14.7%, respectively. The crude fibre contents of the samples were however, high averaging 18.5%. The high ash content was related to sand contamination, as indicated by the high levels of acid-insoluble ash. The blocking of biogas digesters by sand is a common practical problem and contamination with sand may be expected to be more severe under field situations. But preliminary observations have shown that considerable reduction in contamination could be achieved by 'scoop shovelling' instead of directly washing the manure into the biogas digester.

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Table 2. Average chemical composition of dried biogas slurry, coconut oil cake and rice bran (dry matter basis)

| Constituent | Dried biogas slurry | | Coconut oil cake | Rice bran |
|---|---|---|---|---|
| | Trial 1 | Trial 2 | | |
| Crude protein (Nx6.25) | 16.6 | 14.7 | 21.0 | 10.5 |
| Ether extract | 3.1 | 3.3 | 8.6 | 11.2 |
| Crude fibre | 18.5 | 18.4 | 12.1 | 16.8 |
| Ash | 11.4 | 13.5 | 6.9 | 12.5 |
| Nitrogen-free extract | 50.4 | 50.1 | 51.4 | 49.0 |
| Acid-insoluble ash | 6.1 | 8.2 | 0.6 | 5.1 |

The potential of biogas slurry as a non-ruminant feedstuff has been questioned by Summers *et al.*[9] on the grounds that a well digested slurry will have minimal 'potentially' digestible carbohydrates left. The DBS samples used in the present study contained an average of 50.2% nitrogen-free extracts, which is generally assumed to be indicative of the digestible carbohydrate fraction.

The variable nature of biogas slurry needs special mention at this point. As could be expected, the composition of slurry would vary depending on the source of manure (animal species) which, in turn, would differ widely according to the type of diet fed to the donor animals. For example, preliminary evaluation of biogas slurry from cattle manure have shown it to be a poor quality feed for non-ruminants containing crude protein contents of less than 9% and crude fibre contents as high as 31%.[7]

In Experiment 1, the performance of animals fed diets containing 5 and 10% DBS were similar to those on the control diet (Table 3). But the performance tended to be non–significantly($P > 0.05$) higher at 5% DBS. The significance of this slight growth response is unclear. Although pigs fed diets with 20% DBS gained 11% slower and required 12% more feed per unit of gain than did controls, the differences were not statistically significant.

In Experiment 2, the performance of pigs were unaffected by the dietary inclusion of up to 10% DBS (Table 3). Gain and feed efficiency of pigs fed diets with 20% DBS, however, were significantly poorer ($P < 0.05$) when compared to other treatments.

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Table 3. Performance of growing pigs fed diets containing varying levels of DBS

| | Avg. initial weight(kg) | Avg. final weight(kg) | Avg. daily gain(kg) | Feed per gain |
|---|---|---|---|---|
| Experiment 1 | | | | |
| Control | 15.4 | 31.1 | 0.28[ab] | 3.79[ab] |
| 5% DBS | 15.6 | 32.6 | 0.30[a] | 3.53[a] |
| 10% DBS | 15.4 | 31.1 | 0.28[ab] | 3.79[ab] |
| 20% DBS | 15.5 | 29.5 | 0.25[b] | 4.24[b] |
| SEM* | 0.31 | 0.60 | 0.02 | 0.14 |
| Experiment 2 | | | | |
| Control | 18.2 | 37.2 | 0.34[a] | 3.37[a] |
| 5% DBS | 18.2 | 37.8 | 0.35[a] | 3.27[a] |
| 10% DBS | 18.4 | 38.0 | 0.35[a] | 3.37[a] |
| 20% DBS | 18.3 | 35.1 | 0.30[b] | 3.82[b] |
| SEM* | 0.42 | 0.72 | 0.02 | 0.12 |

* Standard error of mean.

[a,b]Within each trial, means in the same column with different superscripts are significantly ($P<0.05$) different.

The weight gains of animals in Experiment 1 were markedly lower than those in Experiment 2. Reasons for this difference are unclear, but may have been due to the extremely hot weather experienced during the experimental period.

The overall results suggest that DBS could be used as a nutrient source in growing pig diets up to 10% level without any adverse effect on growth performance. The pigs appear to have the ability to extract the benefits of microbial protein and vitamin B-complex up to this level of inclusion. A very high content of vitamin $B_{12}$ (3000 mcg/kg) in DBS has been reported.[2] The growth depression observed on 20% DBS diets is probably related to the low available energy content, resulting from its high crude fibre content. It is known that animal manures have a low digestible energy content.[3,4,5] The available energy could be expected to further lowered during the production of biogas.[6] Thus the low energy content of DBS would be a major factor limiting its high level usage in non-ruminant diets. From the energy point of view, ruminants are the most desirable species for feeding biogas slurry because of the ability of the rumen microbes to utilize fibre components.

The digested slurry has several advantages over the raw manure. The slurry has only a faint odour and is greatly improved in this respect over the

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manure. The slurry is also stable on long-term storage. A potential health hazard due to the survival of pathogens in DBS does exist, but studies have demonstrated that anaerobic digestion considerably reduces the number of pathogens present in animal manures.[9] No health problems were observed in pigs in the present study.

While DBS is potentially useful as a feedstuff for growing pigs, utilization of this material requires some artificial dehydration. Even under sunny and dry weather conditions, sun-drying was found to reduce the moisture content of the slurry to only 12-14%. Cheaper ways of drying the slurry need be evolved before its practical use can be encouraged. An additional factor of concern is the possible wide variability in the chemical composition of the slurry. Considerable variations have been reported with regard to the crude protein (14.9-18.2%), crude fibre (17.4-26.4%) and ash (11.2-20.0%) contents of DBS samples.[7] This would prevent any general recommendation regarding the potential use of slurry in animal diets.

**Acknowledgement**

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# CARBOHYDRATE CONSTITUENTS OF THE MARINE ALGAE OF SRI LANKA. PART III. COMPOSITION OF THE CARBOHYDRATES EXTRACTED FROM THE BROWN SEAWEED *TURBINARIA CONOIDES.*

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**Abstract:** The brown seaweed *Turbinaria conoides* was subjected to sequential extraction with 80% ethanol, aqueous 2% calcium chloride, dilute hydrochloric acid and aqueous 3% sodium carbonate solution. Mannitol and eight polysaccharide fractions I - VIII were separated. The neutral sugar composition and the uronic acid content of each fraction was determined. Results indicate the presence of laminaran in fractions I, II and IV, while fractions III, V, VI and VII are rich in 'fucans.'

## 1. Introduction

The brown seaweed *Turbinaria conoides* is found in the coastal regions of Sri Lanka, India and Japan. Carbohydrates which have been isolated from the brown seaweeds include low molecular carbohydrates such as mannitol, while laminaran, 'fucans' and alginates comprise a characteristic range of polysaccharides which have been found in all species of brown seaweeds investigated.

The sugar alcohol mannitol acts as a food reserve carbohydrate[12] and also as a substrate for respiration.[5] Laminaran is a β–D–(1⟶3) linked glucan found in all brown algae and occasionally in the green algae.[7] It is the food reserve material of the brown seaweeds which, unlike red and green algae, do not synthesize starch-like polysaccharides. Fucose containing sulphated polysaccharides have been described under different names such as fucoidan, fucoidin, ascophyllan, sargassan, glucuronoxylofucans and fucans. These are polymers of fucose (6–deoxy–L–galactose) sulphate containing xylose, glucuronic acid and in some species galactose and / or mannose as the major constituents. 'Fucans' are water soluble and present in the intercellular tissue of brown seaweeds. It is also found in the mucilage which exudes from the surface of fronds. Alginic acid is a mucilaginous polyuronide which is an important cell wall constituent of the brown seaweeds, where it occurs as a mixed salt of sodium, calcium and magnesium.

Seasonal variations in the growth and contents of alginic acid and mannitol in *T. conoides* from the Gulf of Mannar (India) have been studied earlier.[13] In a previous report we have discussed the composition and sequence of uronate residues in alginates extracted from three species of brown algae including *T. conoides.*[8]

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## 2. Experimental

### 2.1 Analysis of dried seaweeds

Fronds of *T. conoides* were collected at Mankumban in the Northern coast of Sri Lanka. These were washed in fresh water, sun dried and ground on a Wiley mill to pass a 1 mm screen. The contents of dry matter, ash and crude protein were analysed by standard methods.[4] The ground seaweed was extracted successively with 80% ethanol and chloroform in a Soxhlet apparatus and the residue analysed for starch,[3] Klason lignin,[2] uronic acids[16] and glycosyl composition[2] following acid hydrolysis.

### 2.2 Sequential extraction of dried seaweeds

Dried and milled seaweeds (200 g, 85.2% dry matter) were extracted sequentially with (a) aqueous 80% ethanol, (b) aqueous 2% $CaCl_2$, (c) dilute HCl (pH 2, 0.01M) and (d) aqueous 3% $Na_2CO_3$ as shown in Scheme 1.

#### *2.2.1 Ethanol extraction*

Dried and milled seaweeds were extracted with aqueous 80% ethanol (2 x 100ml) for 2 x 6h at room temperature. The extracts obtained were combined and concentrated to give a precipitate and an ethanolic solution. The ethanolic solution was partitioned between toluene—n—butanol—water (1:1:1) and the ethanol water solution was poured into ethanol to give a white precipitate and an ethanol—water layer (Scheme 1). The residual seaweed was extracted with aqueous 80% ethanol (2 x 1000ml) for 2 x 4h at $70^0$C and the extract was again treated as described above. The precipitates obtained were recrystallized from ethanol—water giving a white crystalline substance, m.p. $166^0$C. (lit.[17] m.p. of mannitol $166^0$C). The identity of mannitol was confirmed by paper chromatography and comparison with an authentic sample of mannitol. Paper chromatography was carried out on Whatman No. 1 chromatographic paper using the solvent systems *n*-butanol-ethanol-water (40:11:19) and ethyl acetic acid-water (3:1:1) with aniline hydrogen phthalate as the spray reagent.

#### *2.2.2 Aqueous $CaCl_2$ extraction*

The residue obtained after ethanol extraction was air dried and pre-treated with 40% formaldehyde solution overnight. The air dried pre-treated residue was extracted with aqueous 2% $CaCl_2$ (1000ml) at room temperature for 8h. The extract on centrifugation gave a supernatant which was dialysed and freeze dried to give the dry extract A. The residual seaweed was re-extracted with aqueous 2% $CaCl_2$ (1000ml) for 8h at $70^0$C and treated similarly to yield dry extract B.

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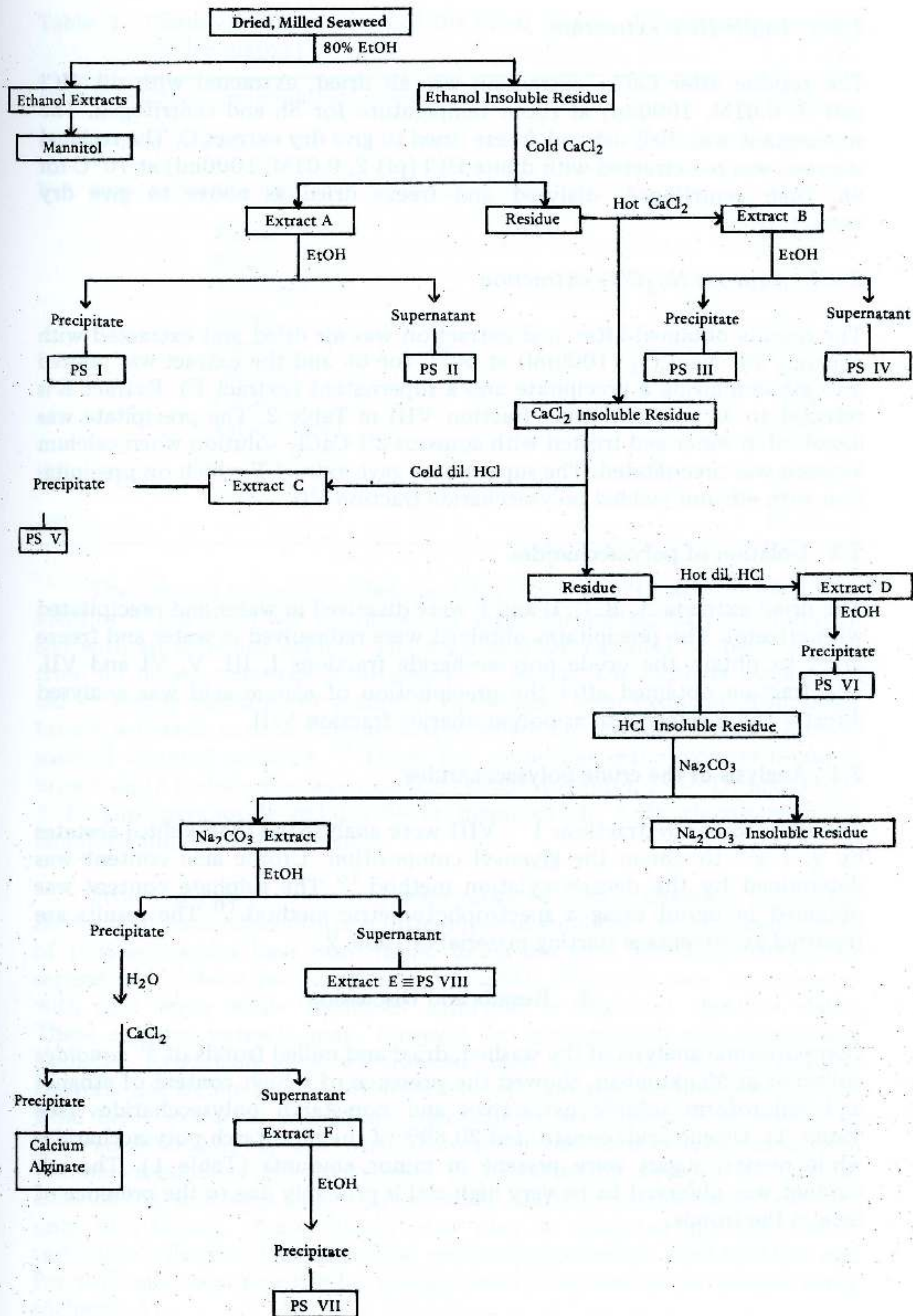


Scheme I.

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*2.2.3 Dilute HCL extraction*

The residue after $CaCl_2$ extraction was air dried, extracted with dil. HCl (pH 2, 0.01M, 1000ml) at room temperature for 8h and centrifuged. The supernatant was dialysed and freeze dried to give dry extract C. The residual seaweed was re-extracted with dilute HCl (pH 2, 0.01M, 1000ml) at $70^0$C for 8h, then centrifuged, dialysed and freeze dried as above to give dry extract D.

*2.2.4 Aqueous $Na_2CO_3$ extraction*

The residue obtained after acid extraction was air dried and extracted with aqueous 3% $Na_2CO_3$ (1000ml) at $50^0$C for 6h and the extract was poured into ethanol giving a precipitate and a supernatant (extract E). Extract E is referred to as polysaccharide fraction VIII in Table 2. The precipitate was dissolved in water and treated with aqueous 2% $CaCl_2$ solution when calcium alginate was precipitated. The supernatant gave extract F which on precipitation with ethanol yielded polysaccharide fraction VII.

**2.3 Isolation of polysaccharides**

The dried extracts A, B, C, D and F were dissolved in water and precipitated with ethanol. The precipitates obtained were redissolved in water and freeze dried to obtain the crude polysaccharide fractions I, III, V, VI and VII. The fraction obtained after the precipitation of alginic acid was analysed directly and is referred to as polysaccharide fraction VIII.

**2.4 Analysis of the crude polysaccharides**

The polysaccharide fractions I — VIII were analysed as their alditol acetates by g. l. c.[1] to obtain the glycosyl composition. Uronic acid content was determined by the decarboxylation method.[16] The sulphate content was obtained in ug/ml using a spectrophotometric method.[10] The results are reported as percentage starting material in Table 2.

## 3. Results and Discussion

Compositional analysis of the washed, dried and milled fronds of *T. conoides* collected at Mankumban, showed the presence of a high content of ethanol and chloroform soluble extractives and non-starch polysaccharides (see Table 1). Uronic acids constituted 20.89% of the non-starch polysaccharides while neutral sugars were present in minor amounts (Table 1). The ash content was observed to be very high and is probably due to the presence of salts in the fronds.

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Table 1. Chemical Composition of the dried fronds of *Turbinaria conoides* (% dry matter)

| | |
|---|---|
| Ethanol and chloroform soluble extractives | 38.06 |
| Crude protein (N x 6.25) | 8.60 |
| Non–starch polysaccharides | 31.83 |
| Fucose | 1.45 |
| Ribose | 0.09 |
| Arabinose | 0.14 |
| Xylose | 0.45 |
| Mannose | 1.66 |
| Galactose | 1.25 |
| Glucose | 5.90 |
| Uronic acids | 20.89 |
| Klason lignin | 19.27 |
| Ash | 34.30 |

The ethanol extracts yielded mannitol (m. p. 165$^0$C) in 9.3% yield. The identity of mannitol was confirmed by paper chromatography and comparison with an authentic sample. The sugar alcohol mannitol has been isolated from all brown seaweeds investigated[6,14] except *Dictyopteris plagiogramma.*[15] Higher amounts of mannitol (25%) have been extracted from other brown seaweeds such as *Laminaria* spp. where the mannitol content showed marked seasonal variation.[14] Other low molecular carbohydrates found in brown algae include the seven carbon polyol volemitol, 1–*O*–D–mannitol–β–D–glucopyranoside and 1,6–*O*–D–mannitol–di–β–D–glucopyranoside, laminitol and *C*–methyl inositol.[11]

Brown seaweeds contain a wide range of polysaccharides. Sequential methods of extraction utilizing differences in solubility of the various types of polysaccharides have been found to be the most effective method for separation of these polysaccharides. 'Soluble' laminaran may be extracted with cold water while 'insoluble' lamiraran is extracted with hot water. These aqueous extracts may, however, be contaminated with fucans and alginates. Extraction of brown seaweeds with aqueous 2% calcium chloride converts the alginic acid in the fronds to calcium alginate thus preventing it from being extracted with dilute hydrochloric acid. Hence it is possible to get a good yield of alginates when the seaweed residue is later extracted with sodium carbonate. Laminarans may be extracted by aqueous 2% calcium chloride together with some 'fucans' while dilute hydrochloric acid extracts only the 'fucans'. Some 'fucans' may also be extracted into the sodium carbonate solution. The sequential extraction procedure used by Mian and Percival[9] and more recently by Venegas Jara[19] was used in the present study (Scheme 1).

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Sequential extraction of the dried fronds of *T. conoides* resulted in the separation of several polysaccharide fractions. In the present study the sugar composition of each fraction was analysed by g.l.c. of the derived alditol acetates.

The seaweed residue after extraction with ethanol was treated with aqueous 40% formaldehyde to polymerise phenolic constituents which may otherwise contaminate the polysaccharide fractions.[9] Pre-treatment with formaldehyde was followed by extraction with aqueous 2% $CaCl_2$ to give extract A, which on precipitation with ethanol gave polysaccharide fractions I and II (Scheme 1). Fraction I was found to contain fucose, glucose, galactose and uronic acids as the major constituents with small amounts of the other sugars (Table 2). Fraction II also contained fucose, glucose and galactose as the major sugar constituents but contents of rhamnose and mannose were higher than in Fraction 1. The sugar composition of fractions I and II suggest that the two fractions are probably mixtures of laminarans and fucans.

Table 2. Percentage composition of the crude polysaccharide fractions I – VIII isolated from *Turbinaria conoides*

| | I | II | III | VI | V | VI | VII | VIII |
|---|---|---|---|---|---|---|---|---|
| Yield(%) | 0.12 | 0.07 | 1.47 | 0.03 | 0.07 | 0.20 | 0.15 | 2.80 |
| Non starch polysaccharides | 35.4 | 52.0 | 43.1 | 27.2 | 43.3 | 52.0 | 46.0 | 8.8 |
| Rhamnose | 1.8 | 3.6 | – | 0.9 | 3.1 | – | 0.5 | 0.1 |
| Fucose | 9.9 | 18.6 | 27.4 | 1.2 | 21.9 | 25.1 | 14.0 | 2.5 |
| Arabinose | 0.2 | 0.3 | 0.2 | 17.3 | 0.2 | 0.2 | 0.2 | 0.1 |
| Xylose | 1.2 | 1.8 | 1.0 | 0.3 | 1.7 | 3.0 | 3.2 | 0.4 |
| Mannose | 1.5 | 2.5 | 1.3 | 0.5 | 2.6 | 2.8 | 5.1 | 1.0 |
| Galactose | 4.4 | 8.5 | 8.3 | 0.7 | 10.9 | 10.7 | 6.6 | 1.5 |
| Glucose | 8.4 | 13.2 | 2.0 | 6.3 | 2.2 | 2.1 | 2.4 | 0.2 |
| Uronic acids | 8.0 | 3.5 | 2.9 | – | 0.7 | 8.1 | 14.0 | 3.0 |
| Crude protein | 16.5 | * | 5.9 | * | 3.7 | 2.8 | 3.6 | 1.1 |
| Sulphate$(SO_4)^{2-}$ | 15.3 | * | 60.9 | * | 34.8 | 38.4 | 38.8 | * |

*Not determined: – Not detected

Extraction of the seaweed residue with aqueous $CaCl_2$ at 70°C gave extract B which on precipitation with ethanol yielded polysaccharide fractions III and IV. Fraction III was composed mainly of fucose and galactose

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(Table 2) with small amounts of the other sugars including uronic acids. Hence fraction III consists mainly of fucans. Aqueous calcium chloride is known to extract both fucans and laminaran. Fraction IV was rich in arabinose and contained little of fucose and galactose. Therefore this fraction contained a lower amount of fucans. The glucose content of fraction IV is significant and indicates the presence of some laminaran. The high content of sulphate in fraction III is probably an overestimation.

Extraction of the seaweed residue after $CaCl_2$ extraction with dilute hydrochloric acid at room temperature and at $70^0C$ gave extracts C and D respectively. Precipitation of extract C with ethanol gave fraction V which was composed mainly of fucose and galactose with small amounts of rhamnose, mannose and glucose. Fraction VI obtained by precipitation of extract D had a high content of fucose and substantial amounts of glucose and galactose. Rhamnose was found to be completely absent while the uronic acid content was quite high. The sulphate content of both fractions V and VI was high. Fractions III, V and VI had a low content of glucose suggesting that these fractions had little laminaran.

The seaweed residue after the acid extraction was extracted with aqueous 3% sodium carbonate solution. The sodium alginate obtained (Scheme 1) was converted to calcium alginate by treatment with 2% calcium chloride. The alcohol supernatant gave extract E which is referred to as fraction VIII in Table 2. Fraction VIII had a very low content of non—starch polysaccharides of which 3.0% was uronic acids. The supernatant obtained after the precipitation of calcium alginate gave extract F which on precipitation with ethanol yielded polysaccharide fraction VII. Fraction VII had high contents of uronic acid, fucose, galactose and sulphate. The neutral sugar composition of 'fucans' is known to vary from species to species.

## 4. Conclusion

Compositional analysis of the polysaccharide fractions I—VIII indicate the presence of laminaran in fractions I, II and IV while fractions III, V, VI, and and VII are rich in 'fucans'. Analysis of the alginate fraction has been reported elsewhere.[8]

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# ANTIMICROBIAL ACTIVITY OF SOME MARINE ALGAE OF SRI LANKA

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**Abstract:** Thirty five species of seaweeds were screened for antibacterial (against *Staphylococcus aureus* and *Escherichia coli*) and antifungal (against *Cladosporium cladosporioides* and *Candida albicans*) activity. Twenty six species exhibited antibacterial and/or antifungal activity. Extracts with pronounced activity were obtained from *Chondrococcus hornemanni* (Rhodophyta) and the active component was found in a mixture containing dihalogenated monoterpenes. Acrylic acid appears to be responsible for the antimicrobial activity of *Gracilaria corticata* (Rhodophyta) and *Ulva lactuca* (Chlorophyta).

## 1. Introduction

Marine flora are known to contain biologically active substances including antimicrobial compounds. A variety of marine algae collected mostly from Caribbean and Pacific waters have been screened for antimicrobial activity.[2,6]

Marine algae have been found abundant particularly along the Northern and Southern coastal regions of Sri Lanka. The algal vegetation along the coasts shows distinct floristic associations and seasonal variations. In a floristic survey in 1961, the presence of 315 species of algae distributed among the orders Chlorophyta, Phaeophyta and Rhodophyta has been recorded from the coastal areas in Sri Lanka.[7] Here we present the results of screening of 35 marine algae collected in Sri Lanka for their antimicrobial activity against *Cladosporium cladosporioides*, *Candida albicans*, *Staphylococcus aureus* and *Escherichia coli* and chemical fractionation of the extracts from *Chondrococcus hornemanni*, which was found to be the most active seaweed.

## 2. Materials and Methods

### 2.1 Algal Specimens

Algal specimens were collected from shallow waters of Mandativu, Mankumban, Keeramalai and Thiruvadinilayam in the Jaffna peninsula (Northern Coast) and also at Kirinda and Tangalla along the Southern Coast of Sri

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Lanka in 1980 (May, Oct.), 1981 (June, Oct.), 1982 (July, Aug) and 1983 (Feb, Sept.) (Table 1). Voucher specimens were deposited at the University Herbarium, Department of Botany, University of Peradeniya, Sri Lanka. The plant material was washed in fresh water, transferred into bottles (10 1.— 20 1.) and sufficient methanol added to completely cover the plant material. The bottles were transported to the laboratory.

## 2.2 Extraction

Methanol was decanted from the bottles which contained the plant material 1 to 4 weeks after collection and storage. The algal material was reduced in size by chopping and fresh methanol was introduced into the bottle to completely cover the plant material. The mixture was allowed to stand at room temperature for two days. This was repeated twice with fresh methanol. The plant material was then extracted successively with cold light petroleum (40—60$^0$C) and cold chloroform for 2 days, three times. With the algae collected in 1980 and 1981, dichloromethane was used as the solvent instead of chloroform, as a shortage of chloroform was experienced during this time. The methanol, petrol and chloroform/dichloromethane solubles were combined separately, and concentrated to dryness on a rotavapor at temperatures below 40$^0$C. A separate extraction of *C. conoides* was carried out with light petroleum (40—60$^0$C) under reflux conditions for two days and the petrol solubles were concentrated on a rotavapor at temperatures below 40$^0$C. The resulting solvent-free extractives were subjected to antimicrobial screening tests as described below.

## 2.3 *Cladosporium*-TLC-Bioassay

The foregoing solvent-free extracts (2 mg) were spotted on TLC plates (silica gel 60 $PG_{254-366}$, 0.5mm x 20cm x 20cm) and the plates were developed in ethyl acetate: light petroleum (1:1). The plates, after air-drying overnight, were sprayed with a suspension of conidia of *Cladosporium cladosporioides* in Czapek-Dox nutrient solution and incubated in a moist chamber at 26 ± 2$^0$C for 48 h.[11] The regions in which the growth was inhibited, appeared light coloured against a background of green mycelia. The diameter of the zones of inhibition was measured in mm; the zones of inhibition were usually approximately circular. The extracts which showed inhibition are given in Table 1 with $R_f$ value (distance travelled by the spot/distance travelled by the solvent front) and the diameter of the zone of inhibition. Benlate (0.2 mg in MeOH; 50% active ingredient methyl 1—(butylcarbonyl)-2-benzimidazolecarbamate, Du Pont, USA) was spotted on each TLC plate as the standard and the diameter of the resultant inhibition area was measured. Some of the extracts were not tested for *Cladosporium*-TLC bioassay (Table 1).

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Table 1. Antimicrobial activity of marine algae

| | Collection | | Activity[a] | | | |
|---|---|---|---|---|---|---|
| | Site | Date | *Cladosporium cladosporioides*[b] | *Candida albicans*[c] | *Staphyloccocus aureus*[c] | *Escherichia coli*[c] |
| *CHLOROPHYTA* (Green algae) | | | | | | |
| *Caulerpa chemitzia* (Esper)Web.V.Bosse (Caulerpaceae) | Mankumban | Sept. 1983 | +(petrol) [0.68,20] | — | — | — |
| *Caulerpa racemosa* (Forssk.)Web.V.Bosse (Caulerpaceae) | Mandativu | Sept. 1983 | +(petrol) [0.97,5] | — | — | — |
| *Caulerpa taxifolia* (Vahl)Ag. (Caulerpaceae) | Mandativu | Oct. 1980 | NT | — | — | — |
| *Cladophora* sp. (Cladophoraceae) | Mandativu | Oct. 1981 | NT | +(petrol) [12] | +(MeOH) [19] | +(MeOH) [14] |
| *Codium adherens* Anderson (Codiaceae) | Mankumban | Sept. 1983 | — | — | — | — |
| *Dictyophaeria favulosa* (Ag.)Decaisne (Valoniaceae) | Mandativu | Oct. 1980 | NT | — | — | — |
| *Halimeda macroloba* (Ag.)Decaisne (Codiaceae) | Kirinda | Feb. 1983 | +(MeOH) [0.73,18] +(petrol) [0.57,12] | — | — | — |

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Table 1 contd.

| | | | | | | |
|---|---|---|---|---|---|---|
| *Microdictyon agardhianum* Decaisne (Valoniaceae) | Mankumban | Sept. 1983 | +(MeOH) [0.84,12] +(petrol) [0.84,10] | – | – | – |
| *Ulva fasciata* Delile (Ulvaceae) | Kirinda | Feb. 1983 | +(MeOH) [0.94,15] +(petrol) [0.63,14] | – | – | – |
| *Ulva lactuca* Linnaeus (Ulvaceae) | Keerimalai | Oct. 1981 | NT | – | +(MeOH) [40] | +(MeOH) [20] |
| *Valoniopsis pachynema* (Martens)Boergesen (Valoniaceae) | Mankumban | Sept. 1983 | +($CHCl_3$) [0.82,8] | – | – | – |
| | Mandativu | Sept. 1983 | +(petrol) [0.81,5] +($CHCl_3$) [0.81,10] | – | – | – |
| | Kirinda | May 1980 | NT | +(petrol) [12] | +(MeOH) [16] +($CH_2Cl_2$) [14] | +(MeOH) [20] |
| *PHAEOPHYTA* (Brown algae) | | | | | | |
| *Chnoospora fastigiata* J. Agardh (Chnoosporaceae) | Kirinda | Feb. 1983 | +(MeOH) [0.83,20] +(petrol) [0.58,25] | – | – | – |

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Table 1 contd.

| | | | | | | |
|---|---|---|---|---|---|---|
| *Cystoseira trinodis* (Forssk)C.Ag. (Cystoseiraceae) | Mandativu | Sept. 1983 | – | – | – | – |
| *Cystoseira triquetra* L. (Cystoseiraceae) | Mandativu | Sept. 1983 | – | – | +(MeOH) [13] +(petrol) [12] | – |
| *Stoechospermum marginatum* J.Ag. (Dictyotaceae) | Mandativu | Oct. 1980 | NT | – | +(MeOH) [14] | – |
| | | Sept. 1983 | – | – | – | – |
| *Sargassum cristaefolium* J.Ag. (Sargassaceae) | Kirinda | Feb. 1983 | – | – | – | – |
| *Sargassum* sp. (Sargassaceae) | Mandativu | Oct. 1980 | NT | +(MeOH) [12] | +(MeOH) [14] | +(MeOH) [12] |
| *Turbinaria conoides* Kuetz (Sargassaceae) | Mandativu | Oct. 1981 | NT | +($CH_2Cl_2$) [18] +(petrol)[d] [50] | +($CH_2Cl_2$) [18] +(petrol) [20] | +($CH_2Cl_2$) [11] +(petrol) [11] |
| | Mandativu | Sept. 1982 | +(petrol) [0.75,15] | – | – | – |
| | Mandativu | Sept. 1983 | – | – | – | – |
| | Mankumban | Sept. 1983 | – | – | – | – |

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Table 1 contd.

| | | | | | | |
|---|---|---|---|---|---|---|
| *Turbinaria ornata* J.Ag. (Sargassaceae) | Mandativu | Oct. 1980 | NT | +($CH_2Cl_2$)[e] [11] +(petrol) [12] | +(MeOH)[e] [14] +(petrol) [15] +(CH2Cl2) [12] | +(petrol)[e] [11] |
| | Mankumban | Sept. 1983 | – | – | +(petrol) [15] | – |
| Tangalla | | Feb. 1983 | – | – | – | – |
| *RHODOPHYTA* (Red Algae) | | | | | | |
| *Acanthophora delilei* Lamaroux (Rhodomelaceae) | Mandativu | Aug. 1982 | +(petrol) [0.68,30] | – | – | – |
| | Mandativu | Oct. 1981 | NT | – | – | – |
| *Bryocladia thwaitesii* Harvey (Rhodomelaceae) | Kirinda | June 1981 | NT | – | – | – |
| *Chondrococcus hornemanii* (Mert)Schmitz (Rhizophyllidaceae) | Thiruvadini–layam | Aug. 1982 | +(MeOH) [0.87,35; 0.70,14] | +(MeOH) [27] | +(MeOH) [26] | +(MeOH) [20] |
| | | | +(petrol) [0.87,38; 0.70,15] | +(petrol) [22] | +(petrol) [20] | +(petrol) [18] |
| | | | +($CHCl_3$) [0.87,26] | +($CHCl_3$) [19] | +($CHCl_3$) [17] | +($CHCl_3$) [18] |

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Table 1 contd.

| | | | | | | |
|---|---|---|---|---|---|---|
| *Chrysemania uvaria* Boergesen (Rhodymeniaceae) | Kirinda | June 1981 | NT | – | – | – |
| *Corynomorpha prismatica* J.Ag. (Halymeniaceae) | Kirinda | Feb. 1983 | +(petrol) [0.71,20] | – | +(MeOH) [13] | – |
| *Gellidiella acerosa* (Forssk.)Felman et Himel (Gellidiellaceae) | Mandativu | Oct. 1980 | NT | +(MeOH) [12] | +(MeOH) [14] | +(MeOH) [11] |
| *Gracilaria corticata* J.Ag. (Gracilariaceae) | Kirinda | Feb. 1983 | +(MeOH) [0.83,18; 0.27,10] +(petrol) [0.83,25] | – | +(MeOH) [13] | +(MeOH) [13] |
| *Gracilaria edulis* (Gmel)Silva (Gracilariaceae) | Mandativu | Oct. 1980 | NT | – | – | – |
| *Gracilaria fergusonii* J.Ag. (Gracilariaceae) | Kirinda | May 1980 | NT | – | – | – |
| | Kirinda | Feb. 1983 | +(petrol) [0.74,20] | | | |

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Table 1 contd.

| | | | | | | |
|---|---|---|---|---|---|---|
| *Gymnogonyrus pygmaeus* (Greville)J.Ag. (Phyllophoraceae) | Kirinda | June 1981 | NT | – | +(MeOH) [20] | +(MeOH) [23] +($CH_2Cl_2$) [13] |
| *Laurencia papillosa* (Forssk.)Greville (Rhodomelaceae) | Mandativu | Aug. 1982 | – | – | – | – |
| *Liagora* sp. (Helminthocladiaceae) | Mandativu | July 1982 | +(petrol) [0.68,20] +($CHCl_3$) [0.67,10] | – | +(MeOH) [16] +($CHCl_3$) [13] | – |
| *Polyopes ligulata* (Harv.)Schmitz (Grateloupiaceae) | Kirinda | Feb. 1983 | +(MeOH) [0.77,35; 0.26,10] +($CHCl_3$) [0.26,10] | – | – | – |
| *Sarcodia ceylanica* Harv. (Sarcodiaceae) | Mandativu | Oct. 1981 | NT | – | – | +(MeOH) [18] |
| | Kirinda | Feb. 1983 | +(MeOH) [0.93,35] +($CHCl_3$) [0.55,40] | – | +(MeOH) [14] | – |

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Table 1 contd.

| | | | | | | |
|---|---|---|---|---|---|---|
| *Spyridia aculeata* J.Ag. (Ceramiaceae) | Kirinda | Feb. 1983 | +(petrol) [0.48,35] | +(petrol) [18] | +(MeOH) [14] +(petrol) [16] | — |
| *Vancoorstia spectabilis* Harv. (Delesseriaceae) | Mandativu | Oct. 1980 | NT | +($CH_2Cl_2$) [18] +(petrol) [20] | +($CH_2Cl_2$) [14] | — |
| Benlate | | | + [0.15,38±2] | | | |
| Nyastatin | | | | + [36±2] | | |
| Penicillin | | | | | + [32±2] | + [30±2] |

*a* + indicates extract derived using the solvent given in parenthesis is active; - , all extracts tested found inactive; NT, not tested.
*b* TLC bioassay; $R_f$ value(s) of the active spot(s) and the diameter of the active spot (in mm) are indicated within [ ]
*c* Filter paper disc bioassay; diameter of the zone of inhibition around the paper disc (in mm) are indicated within [ ]
*d* hot petrol extract.
*e* 6 mm discs used.

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### 2.4 Screening against *Staphylococcus aureus, Escherichia coli* and *Candida albicans*

Aqueous ethanolic solutions (1 ml) of the extracts (15 mg) were absorbed into ten 6 mm paper discs. After ovendrying ($42^0$C) the discs were aseptically placed on Muller Hintant Agar (MHA) plates seeded with *S. aureus* (Oxford stain) and *E. coli*, and on Saboured Agar plates seeded with *C. albicans*. Tablets of Penicillin (10 i.u., DIFCO, Detroit, U.S.A.), standard for *E. coli* and *S. aureus* assay, and Nystatin (25 $\mu$g, DIFCO, Detroit, U.S.A.), standard for *C. albicans* assay, were also placed on a separate set of seeded MHA and Saboured Agar plates, respectively.[1] The plates were incubated at $38^0 \pm 2^0$C for 48h and the diameter of the Zone of inhibition on agar around the paper disc/tablet was measured. The results are given in Table 1.

### 2.5 Fractionation of the extractives of *Chondrococcus hornemanni*

A large sample (dry wt. 1.5 kg) of *C. hornemanni* was collected in Thiruvadinilayam and stored in chloroform:methanol (1:1) mixture for 2 weeks. The extract was filtered and the filtrate on standing separated into two layers and these were treated separately. The aqueous layer was washed with chloroform and the washings were combined with the organic layer. The combined organic layer was washed with water, dried over anhydrous $MgSO_4$ and concentrated to obtain a brown oil (14.1 g). Flash chromatography (silica gel $GF_{254}$ Merck, light petroleum and $CHCl_3$) of the brown oil afforded a white crystalline solid (57 mg), m.p. 52-55$^0$C, $[\alpha]_D$;+18.7$^0$ (*c*, 1.34, $CHCl_3$). IR $\nu^{KBr}_{max}$ cm$^{-1}$: 2920, 1620, 1450, 1100, 900, 700. $^1$H-NMR (60 MHz, $CCl_4$): $\delta$ (ppm) 5.82(IH,D, J = 3HZ), 5.62(IH,d, J = 3Hz), 4.03 (IH,d, J = 10Hz), 3.80(2H,s), 2.9-1.9(4H,m), 1.80(3H,s), 1.68(3H,s). MS: *m/z* (relative intensity) 366($M^+$-Cl, 6%), 285(26), 249(16), 205(18), 203 (22), 169(15), 167(29), 127(32), 125(34), 113(19), 105(20), 103(26), 91(30), 79(36), 77(100). Further elution of the column gave a colourless oil (766 mg) which was homogeneous on TLC. IR $\nu^{neat}_{max}$ cm$^{-1}$: 2920, 1440, 1250, 1200, 675. $^1$H-NMR ($CCl_4$): $\delta$ (ppm) 5.83-5.50(m), 5.10(s,br), 4.90 (s,br), 4.17(s), 4.10(s), 4.04(s), 3.97(s), 3.91(s), 2.30(s), 2.25(s), 1.83(s), 1.80(s). MS: *m/z* 252, 250, 248, 209, 207, 205, 191, 171, 169, 133, 129, 127, 103, 102, 79, 75, 69, 68, 67, 65.

## 3. Results and Discussion

Cold methanol, petroleum and chloroform/dichloromethane extracts of 35 algae belonging to Chlorophyta (11 species), Phaeophyta (8 species) and Rhodophyta (16 species) were examined for antimicrobial activity against *C. cladosporioides, C. albicans, S. aureus* and *E. coli*. The results (Table 1) show that 26 species exhibited activity against one or more microorganisms.

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### 3.1 Chemical fractionation of the extractives of *Chondrococcus hornemanni*

The extracts of *C. hornemanni* inhibited the growth of all four microbes tested, and the observed activities were comparable to those of a standard antibiotic Penicillin and standard fungicides, 'Benlate' and 'Nystatin'. The active extract of *C. hornemanni* was partitioned on a silica gel column using combinations of light petroleum and $CHCl_3$ with increasing polarity. A white crystalline solid (*1*) and a colourless oil (*2*) were obtained. The IR spectrum of the solid showed a band at 1620 $cm^{-1}$ corresponding to an olefinic double bond. The $^1H$-NMR spectrum of this compound indicated the presence of two tertiary methyl groups ( $\delta$ 1.80 and 1.68), three hydrogens on halogen bearing carbon atoms ( $\delta$ 4.03,IH,d, J = 10 Hz and $\delta$ 3.80,2H,s) and two olefinic hydrogens which are geminally coupled ( $\delta$ 5.83,IH,d, and J = 3Hz and $\delta$ 5.62,IH,d, J = 3HZ). The mass spectrum showed two peaks at *m/z* 366 and 285 corresponding to M-Cl and M-Cl-HBr, respectively. High resolution mass spectral data indicated the molecular formula of the ion at *m/z* 285 to be $C_{10}H_{14}Cl_2Br$. This suggested that compound *1* was identical to 6-bromo-3-bromomethyl -2,3,7-trichloro-7-methyl-1-octene previously isolated from *C. hornemanni.*[3] This compound was found to be inactive against *C. cladosporioides.*

The colourless oil (*2*) obtained from the above column was homogeneous on TLC although GLC and HPLC analysis suggested the presence of more than one compound in the oil. The oil was not fractionated into more than one component by either silica gel/$AgNO_3$ impregnated silica gel chromatography (plates or column) eluted with *n*-hexane or HPLC (Whatman Partisil-10 ODS 2, aqueous MeOH). The oil (*2*) corresponded to the inhibition zone at $R_f$ 0.87 in the *Cladosporium*-TLC-bioassay. Mass spectral fragments at *m/z* 252, 250, 248($M^+$), 209, 207, 205(M-43), 171, 169(M-Br), 133(M-Br-Cl-H), 129, 127(M-Br-43+H) and 69(base peak) suggested that the active fraction contained mainly dihalogenated monoterpenes.[10] The presence of several halogenated monoterpenes in *C. hornemanni* has been reported previously.[3,10,15] Structurally related compounds isolated from *Delisea fimbriata* (Rhodophyta) have also displayed antimicrobial activity.[12,13]

### 3.2 Screening for antimicrobial activity

The extracts of *Cladosphora* sp , *Valoniopsis pachynema, Turbinaria conoides, Corynomorpha prismatica, Gracilaria corticata, Liagora* sp., *Sarcodia ceylanica* and *Spyridia aculeata* also showed antibacterial and antifungal activity but to a lesser extent than *C. hornemanni*. The methanol extract of *G. corticata* produced two inhibition areas in the *Cladosporium*-TLC-bioassay plate (petrol:EtOAc:1:1) corresponding to low polar ($R_f$ 0.83) and high polar ($R_f$ 0.27) active constituents. The $R_f$ value of the inhibition area produced by the high polar compound was identical to that of acrylic acid.

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It is noteworthy that the antimicrobial principle of *G. folifera* and *G. varrucosa* has been reported to be acrylic acid.[9]

The extracts of *Caulerpa chemitzia, Caulerpa racemosa, Halimeda macroloba, Ulva fasciata, Microdictyon agardharanum, Chnoospora fastigiata, Acanthopora delilei, Gracilaria fergusonii* and *Polyopes ligulata* inhibited only *C. cladosporioides*. In a previous survey on fungistatic properties of marine algae,[14] *C. racemosa* was, however, found to be particularly active against *Candida albicans* and *Cryptococcus neoformans*.

Only antibacterial properties were detected in the extracts of *Ulva lactuca, Cystoseira triquetra, Stoechospermum marginatum, Turbinaria ornata, Cymnogyrus pygmaeus* and *Sarcodia ceylanica*. Our results of *U. lactuca* are in agreement with those of a previous study,[2] and the active compound is probably (TLC) acrylic acid.[8] The antibacterial constituents of *S. marginatum* has been identified as a spatol acetate.[5]

The extracts of *Caulerpa taxifolia, Codium adherens, Dictyosphaeria favulosa, Cystophyllum muricatum, Sargassum cristaefolium, Bryocladia thwaitesii, Chrysemania uvaria, Gracilaria edulis* and *Laurencia papillosa* did not inhibit the growth of the microbes tested. A homogenized preparation of *L. papillosa* has, however, been found to be a potent inhibitor of some pathogenic fungi in a previous survey.[14]

Varying activities of the extracts were observed with different collections of algal specimens, e.g. *V. pachynema, S. marginatum, T. conoides, T. ornata* and *S. ceylanica.* This may be attributed partly to seasonal variations[2] and ecotypic variations from different locations.[4]

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# NON–STARCH POLYSACCHARIDES OF SEEDS OF SOYBEAN [*GLYCINE MAX.* (L)]

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*(Date of receipt : 07 June 1988)*
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**Abstract:** Mature soybean seeds (variety FFR 559) were analysed for the non-starch polysaccharide composition. Non-starch polysaccharide (NSP) fractions were extracted by two methods, namely, chemical (trichloroacetic acid-TCA) and enzymatic methods. Yields of TCA- soluble, enzyme-soluble, TCA-insoluble and enzyme-insoluble NSP were 1.4, 13.1, 24.9 and 28.8% of the defatted flour, respectively. The differences in yields between the two methods were primarily due to contamination with non-carbohydrate materials (protein, ash and lignin) to the extent of 18.4-30.8%. The results showed the soluble NSP to be an arabinogalactan type of polysaccharide containing arabinose and galactose in approximately 2:1 ratio, and a uronic acid content of 30.4%. Cellulose associated with arabinogalactan, xylan and pectic polymers predominate the insoluble NSP. Mature soybean seeds were found to be free of starch.

## 1. Introduction

Carbohydrates of legumes have been extensively studied for their starch constituents.[17] Cell walls, essentially constituting of non-starch polysaccharides, which together with lignin are now referred to as the dietary fibre,[5] have received comparatively little attention because they are highly insoluble and therefore lack the functional properties exhibited by starch. They are indigestible by the human digestive system and consequently believed to contribute little nutritionally.[18] However, there is current evidence that this indigestible fraction could be nutritionally beneficial in lowering blood cholesterol levels and various intestinal disorders.[18]

The significance of these legume seed components also stems from the fact that they are important in many technological processes. One example is the arabinogalactan of black gram. It has been shown that in the preparation of '*idli*', arabignogalactan is essential for stabilization of the texture created by the protein network.[20]

Some researchers have attempted to characterise the complex polysaccharide in various legume seeds without any special interest in dietary fibre. Morita,[11] and Aspinall and Cottrell[3] have extracted a crude hot water soluble polysaccharide using NaOH and phenol:acetic acid:water, respectively to solubilise proteins. In these studies, components representing only a small proportion of the total polysaccharide were isolated and characterised. This approach tends to yield highly purified fractions which can be chemically well characterised, but are not representative of the total polysaccharide. The objective of this study was to isolate, identify and quantify the constituent monosaccharides in the non-starch polysaccharide (NSP) of mature soybean seeds. In addition, the starch content was also determined.

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## 2. Materials and Methods

### 2.1 Sample Preparation

Mature soybean seeds (variety FFR 559) were obtained from Kentucky Seed Improvement Association, U.S.A. They were ground in a Wiley laboratory mill to pass through a 60 mesh sieve and defatted with hexane for 16 h. Moisture determination was carried out by heating samples at 130$^0$C for 1 h. All yields and composition were calculated on a moisture - free basis. All reagents used were of analytical grade.

### 2.2 Isolation of Non-Starch Polysaccharides

Non-starch polysaccharides were isolated by two different methods. The first method[20] involved extraction of 10g flour with 60 ml 10% (w/v) trichloroacetic acid (TCA) for 6 h at 4$^0$C with continuous stirring and then centrifuging for 30 min at 10,000 x g (4$^0$C). The extraction was repeated thrice. The supernatants were combined, filtered and precipitated with 3 volumes of 75% (v/v) acetone. The precipitate was redissolved in 10 ml TCA and reprecipitated with 3 volumes of acetone. The white precipitate that resulted was thoroughly washed with acetone, dissolved in water, dialysed (72 h) and freeze-dried to obtain the soluble polysaccharide (TCA—S).

Since the residue after TCA extraction contained both the insoluble polysaccharide and the protein precipitated with TCA, the residue was treated (1 h) with 0.2 N NaOH until the pH was 11.5, to solubilise the proteins. The residue after deproteinization was the insoluble polysaccharide (TCA—I).

In the second method, the soluble and insoluble polysaccharides were extracted by the procedures of Hellendoorn,[9] as modified by Schweizer and Wurch.[19] This method involved stepwise degradation by pepsin followed by pancreatin, a preparation which has proteolytic, lipolytic and amylolytic activities and precipitation of soluble polysaccharide (ENZ — S) with 4 volumes of ethanol. This residue forms the insoluble polysaccharide (ENZ—I). Since our analysis showed soybean to be free of starch, autoclaving and the use of glucoamylase were omitted from the original procedure. However aqueous sample suspension was boiled at 95$^0$C for 20 min in order to inactivate the protease inhibitors.

### 2.3 Analytical Methods

The polysaccharide fractions were hydrolysed by a modified Saeman's procedure as reported earlier.[13] Preliminary experiments showed that a 96% recovery of standard sugars is possible by this procedure.

Preliminary identification of sugars in the deionized and filtered

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hydrolysates was obtained by Thin Layer Chromatography.[7] Neutral sugars present in the soluble and insoluble polysaccharides were determined by High Performance Liquid Chromatography (HPLC) as described by Ravindran and Palmer.[13] Uronic acid in the polysaccharide fractions was determined by the method of Ahmed and Labavitch[1] using a galacturonic acid standard (Sigma Chemical Co., St. Louis, MO).

Protein and lignin were determined by the micro-Kjeldahl procedure of Robinson[14] and the permanganate oxidation method of Goering and Van Soest,[8] respectively. The ash in the polysaccharide fractions was determined by weighing the residue after heating at 525$^{0}$C for 5 h.

Starch in the defatted flour was determined by perchloric acid method[12] and the results of which was checked by an enzymatic assay.[16]

## 3. Results and Discussion

There is no generally accepted approach to the extraction and/or analysis of non-starch polysaccharides of plants. In the present study, two methods were employed to extract the NSP fractions from defatted soybean flour. The determination of NSP by purely chemical methods offers some advantages. The major one is their effectiveness in deproteinising the materials. The TCA—S extracted in our study contained only 1% protein. But the chemical methods are obviously less 'biologically' relevant than the use of enzyme(s) *in vitro*[22,23], which simulates normal human digestion. ENZ—S and ENZ—I obtained by this method however, contained 9 and 25% protein, respectively. These high levels may have arisen as a result of incomplete digestion of proteins by the enzymes. The indigestible fraction from some plant sources is known to represent considerable percentage of the total protein.[6] Whether this indigestible protein is of structural cell wall nature or merely refractory to digestion requires clarification. There is however, ample evidence that it is not wholly of a cell wall nature in all cases.

Soluble NSP fractions extracted by both methods contained appreciable amounts of ash (Table 1). Decreased solubility of chelated minerals in alcohol and acetone may have contributed to the presence of such levels of minerals in the soluble fractions. In the ENZ—S, however, since the method involved incubation in relatively high concentration of sodium phosphate buffer, it seems likely that most of the ash came from precipitation of buffer salts. ENZ—S also contained 9.3% unhydrolysed residue and is probably attributable to ligno-cellulose complex. In addition to ash and protein, the insoluble fractions also contained lignin (3.3%).

Yields of TCA—S, ENZ—S, TCA—I and ENZ—I fractions were 1.4, 13.1, 24.9, and 28.8% of the defatted soybean meal, respectively. As noted earlier, the differences in yields between the two methods, were primarily due to contamination with protein and ash to varying extents. The TCA

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procedure includes an extensive dialysis of the soluble fraction and it is possible that some losses of low molecular weight material may have occurred during dialysis. Another possible reason for the observed differences may have been the incomplete precipitation of the soluble fraction with acetone.

TCA and enzyme soluble extracts contained 47.4 and 49.5% neutral sugars and 24.6 and 20.8% acidic polysaccharides, respectively (Table 1). The insoluble polysaccharide fractions contained less uronic acid, 58.3-65.1% neutral polysaccharides and 7.8-9.5% acidic polysaccharides. The data was corrected for the presence of non-carbohydrate material (28.4-30.2% in soluble and 18.4-30.8% in insoluble polysaccharide fractions) and expressed as a percentage of polysaccharides in Table 2. Expressed either as percent of defatted meal (Table 1) or of polysaccharides, the data for the sugar and uronic acid composition of soybean NSP was found to be considerably consistent.

Table 1. Composition of Soluble(S) and Insoluble(I) polysaccharide Fractions of Defatted Soybean Flour (% of dry matter)[a]

| Composition | TCA−S | ENZ−S | TCA−I | ENZ−I |
|---|---|---|---|---|
| Yield | 1.4 | 13.1 | 24.9 | 28.8 |
| Galactose[b] | 26.2±0.8 | 26.6±2.1 | 19.9±0.9 | 18.0±1.7 |
| Arabinose[b] | 14.3±0.4 | 13.7±0.7 | 10.5±1.3 | 9.8±1.0 |
| Xylose[b] | 3.9±0.6 | 4.2±0.8 | 6.1±1.2 | 5.7±0.3 |
| Glucose[b] | 1.5±0.1 | 2.9±0.1 | 28.6±2.2 | 24.8±1.1 |
| Mannose[b] | 1.5±0.1 | 2.1±0.9 | | |
| Rhamnose[b] | trace | trace | trace | trace |
| Uronic Acid[b] | 24.6±0.5 | 20.8±0.3 | 7.8±0.3 | 9.5±0.4 |
| (Total neutral + acidic sugars)[b] | (72.0±2.4) | (70.3±4.9) | (72.9±5.9) | (67.8±4.5) |
| Protein | 1.0±0.0 | 8.9±0.8 | 12.3±1.8 | 24.9±0.9 |
| Ash | 29.2±0.3 | 12.2±0.5 | 2.9±0.1 | 2.5±0.1 |
| Lignin | | | 3.2±0.9 | 3.4±0.6 |
| | | 7.3±0.6[c] | | |
| Recovery | 102.2±2.7 | 98.7±7.8 | 91.3±8.8 | 98.6±7.4 |

[a] Each value represents the mean of three determinations ± standard deviation.

[b] Neutral and acidic sugars are expressed as polysaccharides.

[c] Unhydrolysed residue.

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Galactose (36-39%) and to a lesser extent arabinose (19-20%) are the major neutral sugar constituents. The galactose : arabinose ratio was approximately 2 : 1, suggesting that much of the soluble NSP of soybean may be the arabinogalactan characterised by Morita[11] and Aspinall.[3] Lupins are also reported to have gal/ara ratio of 2.5 : 1 in the cold water soluble polysaccharide.[10] Galacturonic acid (27-34%) was the accompanying acidic sugar. Xylose, glucose and mannose were also present in the soluble NSP fractions in smaller quantities, generally in the range of 2-6% (Table 2). Mannose is reported to be present as a side chain sugar in water soluble polysaccharides[21] and might have given rise to the mannose determined in our samples. Glucose might have arisen from the fraction in the residue. Rhamnose was observed in trace amounts. The presence of rhamnose and galacturonic acid suggests that rhamnogalacturonans might occur as cell wall constituents. Moreover, the predominance of arabinose and galactose which are the major pectic neutral sugars indicates that soybean NSP are mainly of the pectic type.

Table 2. Distribution of Neutral and Acidic Sugars as % of Polysaccharide.

| | TCA–S | ENZ–S | TCA–I | ENZ–I |
|---|---|---|---|---|
| Yield | 1.0 | 9.2 | 18.2 | 19.5 |
| Galactose | 36.4 | 37.8 | 27.3 | 26.5 |
| Arabinose | 19.9 | 19.5 | 14.4 | 14.5 |
| Xylose | 5.4 | 5.9 | 8.4 | 8.4 |
| Glucose | 2.1 | 4.1 | 39.2 | 36.6 |
| Mannose | 2.1 | 3.0 | - | - |
| Uronic acid | 34.2 | 26.7 | 10.7 | 14.0 |
| Galactose: arabinose ratio | 1.8:1 | 1.9:1 | 1.9:1 | 1.8:1 |

Glucose (37-39%) was the predominant sugar in both insoluble NSP fractions and was probably derived from cellulose. The composition of the non-cellulosic polysaccharides in the insoluble fractions was calculated, based on the assumption that all the glucose in the hydrolysates was derived from cellulose. The mean composition of the non-cellulosic polysaccharide in the two fractions was 43.4% galactose, 23.3% arabinose, 13.5% xylose and 19.8% uronic acid. Apparently galactose predominates the insoluble NSP of soybean as they do in the soluble NSP. Xylans and pectic substances are also prominent and the proportion of galactose : arabinose is same as the soluble NSP. Hull polysaccharides of legumes are reported to be mainly arabino- and glucuronoxylans.[4] Hence the high xylose content in the insoluble NSP could be due to the fact that whole seeds were used in the present study.

The results of the present study indicate that there is no sharp borderline between the soluble and insoluble NSP fractions. Polysaccharides of very

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related composition , arabinogalactan for example, are found both as soluble and insoluble components.

Starch determination on defatted soybean flour by the enzymatic and perchloric acid procedures showed the mature soybean to be free of starch, confirming the report of Altschul.[2] Several other legume seeds, sunflower,[4] winged beans[13] and lupins[10] are also reported to be nearly starch-free.

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# LUNG FUNCTION IN YOUNG SRI LANKAN FEMALES

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**Abstract:** A study of respiratory function was carried out in 192 adult Sri Lankan females of age group 19 - 29 years. Vital capacity (VC), Forced vital capacity (FVC), forced expiratory volume in one second ($FEV_1$) and maximum voluntary ventilation ($MVV_F$) were measured by spirometry and the peak flow rate by a mini Wright peak flow meter. The values observed were found to be less than that reported for Europeans and closer to those of North Indian populations. The relationship of the results to height and weight was studied. Prediction formulae based on height were derived for some of the tests.

## 1. Introduction

Measurement of lung function is widely used in the diagnosis and the assessment of progress in patients with cardiopulmonary disease and evaluating the fitness of patients to undergo anaesthesia. Therefore it is important to determine reference ranges for the Sri Lankan population who vary significantly in stature from their European counterparts. Cullumbine's islandwide survey of physical fitness in 1949 states that females of average age and height had a mean vital capacity of 1570 ml. These data are significantly lower than those of Koch[6] where the vital capacity of young adult females was reported as 2160 ml. The fact that Koch's study group consisted of University freshmen of a presumably higher socio-economic status may account for this difference.

The above figures are much lower than those reported for European countries. Kaltreider, *et. al*[5] reported a vital capacity(VC) of 3.14 ± 0.41 1 for females of age 23.1 ± 3.4 years. They were taller (163.4 ± 4.2 cm) and heavier (57.2 ± 9.4 kg) than Sri Lankan females of the same age group. This greater stature may account for the larger vital capacities observed. In this respect, Sri Lankans are closer to Indian populations and it would be more relevant to compare Indian figures with the available Sri Lankan figures. Young North Indian females of age group 15—40 years have a vital capacity of 2625 1[4] and South Indians of age group 17—29 years have a mean vital capacity of 2560 1.[8] They are significantly higher than those reported for a comparable age group by Koch.[6] A preliminary study[9] of Sri Lankan females of age range 19—29 years gave a value of 2698 ± 555.6 ml for vital capacity.

Vital capacity has been related to several body measurements such as body surface area, thoracic volume, body weight, height and age. Prediction formulae have been worked out from these relationships for European populations. The purpose of this study is to examine the relationship of lung

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function tests to body height and weight and to obtain prediction formulae based on these relationships for young Sri Lankan females.

## 2. Experimental

### 2.1 Subjects

192 females of age range 19–29 years were studied. The subjects were selected from among medical students and staff of the University of Peradeniya. They had no history of cardiopulmonary illness in the past or at the time of examination. Height and weight of each subject were measured using a standard laboratory scales (Detectomedic, Detecto scales INC, Brooklyn, N.Y., USA).

### 2.2 Spirometric Measurements

Spirometric measurements were carried out by the methods described previously for adult males.[9] The measurements were vital capacity (VC), forced vital capacity (FVC), forced expiratory volume in one second ($FEV_1$), maximum voluntary ventilation ($MVV_F$), and peak expiratory flow rate (PEF). The terminology for lung function tests put forward by Gandevia and Hugh-Jones[3] was used in this study.

## 3. Results

A total of 192 females of mean age 22.07 ± 2.27 years was studied.

Table 1 gives the means and standard deviations of results. All data were expressed at BTPS.

Table 1. Means and Standard Deviations of Results (n=192)

| | | | |
|---|---|---|---|
| Age(years) | 22.1±2.3 | FVC(ml) | 2435.7±437.9 |
| Height(cm) | 156.6±6.2 | $FEV_1$(ml) | 2354.4±405.9 |
| Weight(kg) | 45.4±6.3 | $MVV_F$(ml) | 673.5±18.6 |
| Vital capacity(ml) | 2574.6±462.2 | PEF(1/m) | 428.9±77.4 |

Results obtained for height and weight compare favourably with previous data.[1]

A matrix of bi-variate correlation coefficients was developed to study the relationship between the different body measurements and is shown in Table 2. Height was seen to have a highly significant correlation with weight, VC, FVC, $FEV_1$ and PEF, as indicated by high values of r. Relationship of the above measurements to weight were seen to be of a lower order although significant as indicated by lower r values. The magnitude of these r values indicate that less than 25% of the variation in results could be accounted

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for by changes in weight. Therefore height was chosen as the parameter to derive prediction formulae for the other five measurements.

Table 2. Measurement of physical characteristics and pulmonary function Correlation coefficients (n=192)

| | Height (cm) | Weight (kg) | VC (ml) | FVC (ml) | $FEV_1$ (ml) | $MVV_F$ (1/m) | PEF (1/m) |
|---|---|---|---|---|---|---|---|
| Height | | 0.5800* | 0.6006* | 0.4547* | 0.4412* | 0.1087 | 0.4452* |
| Weight | | | 0.5048* | 0.5814* | 0.3509* | 0.0865 | 0.2624+ |
| VC | | | | 0.7577* | 0.7349* | 0.2184+ | 0.4920* |
| FVC | | | | | 0.8884* | 0.2771+ | 0.3328* |
| $FEV_1$ | | | | | | 0.2230 | 0.3810* |
| MBC | | | | | | | 0.1007 |

* $p < 0.001$
+ $p < 0.01$

Regression equations are given below.

$$VC = -4403.2 + 42.12\,H \quad (r = 0.60)$$

$$FVC = -2347.3 + 30.97\,H \quad (r = 0.46)$$

$$FEV_1 = -2201.7 + 28.85\,H \quad (r = 0.44)$$

$$PEF = -439.4 + 5.61\,H \quad (r = 0.45)$$

(H = Height)

As $MVV_F$ did not show a high correlation with either height or weight, it was not possible to calculate a prediction formula for these measurements.

## 4. Discussion

The results of lung function tests in the present study are lower than those reported for many Western populations. They are closer to values available for North Indian women reported by Jain and Ramiah.[4] Peak expiratory flow rate too seem to be close to North Indian figures.[7]

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Values for maximum voluntary ventilation did not correlate with height or weight. $MVV_F$ depends more on factors such as compliance of the lungs and the thoracic wall and the efficiency of the respiratory muscles than on body size. This may account for the poor relationship observed with height and weight. An earlier study[9] showed the same lack of correlation between height and weight and $MVV_F$ in the case of males. Therefore it was not possible to derive a prediction formula for this value.

The present survey gives higher values for vital capacity than in Koch's study[6] which reported a value of 2160 ml in the case of females. It is difficult to account for this difference as both groups of subjects were of similar socio-economic backgrounds.

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*SHORT COMMUNICATION*

# CYANOGENBROMIDE FRAGMENTATION OF α–GALACTOSIDASE FROM COCONUT

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α–Galactosidase (EC. 3.2.1.22) plays an important role in plant and animal metabolism.[2,10] The enzyme is used in industry to remove raffinose from soybean products and in beet sugar production.[6] However no studies have been done on the primary structure of α–galactosidase. α–Galactosidase from coconut has been purified to homogenity and the amino acid content determined.[1] Chemical modification of α–galactosidase from coconut indicates the presence of one tyrosine, one tryptophan and two carboxyl groups at or near the active site.[7] Based on these results a mechanism of action for α–galactosidase has been proposed.[8] In this study α–galactosidase from coconut has been fragmented by cyanogenbromide and the resulting peptides characterized so that in later studies the peptide containing the active site could be identified by labelling the tyrosine at the active site.

## Materials

Analytical grade BDH and Sigma Chemicals were used. Mature fresh coconuts were purchased locally.

## Purification of α–galactosidase

α–Galactosidase from coconut was purified as described by Balasubramaniam and Mathew.[1]

## CNBr Fragmentation

Purified α–galactosidase was reduced with dithioerythritol and alkylated using sodium iodoacetate and fragmented as described by Gross and Witkop.[4]

## Sephadex G–50 chromatography

Peptides obtained by CNBr fragmentation were dissolved in 20% acetic acid and eluted through a Sephadex G–50 column (1.5 x 155 cm) using 20% acetic acid. Flow rate 6 ml/h. Fraction volume 3 ml.

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**Separation of peptides by HPLC**

Peptides obtained from Sephadex G—50 chromatography were separated using an Altex Model 110 chromatography system. Peptides were chromatographed on a Lichrosorb RP 8 (7$\mu$) column (4.5 x 250 mm) equilibrated with 5 mM ammonium acetate buffer of pH 6.4 and eluted with a linear gradient of acetonitrile or 80% propanol. Appropriate fractions were pooled and samples were taken for amino acid analysis.

**Amino acid analysis**

Samples were hydrolyzed at 110$^0$C with 6 N HCl containing 2 mg/ml phenol and analysed using Durrum D 500 analyzer. Serine and threonine values were calculated using the standard recovery factors of 0.90 and 0.96 respectively.

**N—terminal analysis**

Manual Edman degradation was carried out[3] and phenylthiohydantoin (PTH) derivatives of the amino acids released were identified by HPLC using a Nucleosil C 18 (5$\mu$ ) column (0.4 x 30 cm) equilbrated with 5mM sodium acetate buffer (pH 5.0). A linear gradient was used with acetonitrile. The conditions used to separate the PTH amino acids were a 27 min run with a 12 min linear gradient from 20 to 48% acetonitrile at 35$^0$C.

Sephadex G—50 gel chromatography separated the peptides into six fractions which were labelled A, B, C, D, E & F (Figure 1). Peptides in the above fractions were purified by HPLC varying the hydrophobicity of the solvent to obtain optimum separation. Single peptides were obtained from fractions A, B, D and F which were labelled A1, B1, D1 and F1 respectively. FractionC was separated into two peptides which were labelled C1 and C2 while fraction E was separated into three peptides which were labelled E1, E2 and E3. The large number of peptides obtained by HPLC is probably due to the glycoprotein nature of $\alpha$—galactosidase from coconut.[1]

Amino acid analysis of peptide A1 showed that it does not contain homoserine or homoserine lactone (Table 1). Cyanogenbromide fragmentation produces peptides containing C—terminal homoserine or homoserinelactone.[4] Peptide A1 is probably the C—terminal end of the polypeptide chain. The peptides B1, C1, C2 and D1 have similar amino acid compositions, the main difference being the presence or absence of glucosamine. These four peptides were considered to be a peptide (Table 2). Amino acid analysis also showed that peak E3 was not a peptide. Amino acid analysis agrees with the results expected from cyanogenbromide fragmentation as coconut $\alpha$—galactosidase contains four methionine residues.[1] Thus CNBr fragmentation has produced five peptides (Table 1).

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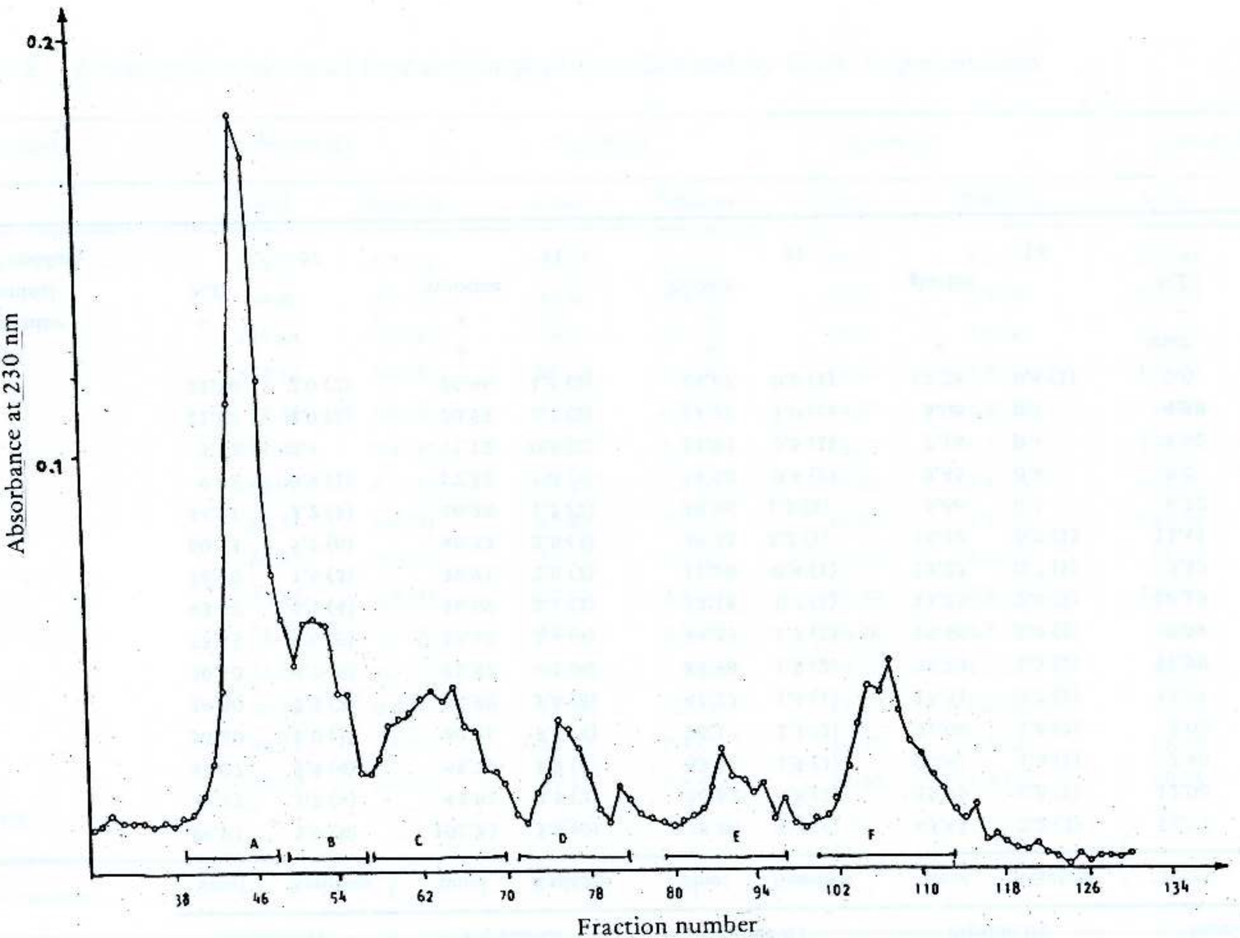


Figure 1. Separation of peptides by Sephadex G—50 gel chromatography.

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Table 1. Amino acid analysis of the peptides obtained by CNBr fragmentation

| Amino Acid | Peptide A1 | | Peptide C2 | | Peptide E1 | | Peptide E1 | | Peptide F1 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | nmol | Residues | nmol | Residues | nmol | Residues | nmol | Residues | nmol | Residues |
| Asp | 94.91 | 8.9 (9) | 102.53 | 5.9 (6) | 138.80 | 4.2 (4) | 43.65 | 2.2 (2) | 12.77 | 1.1 (1) |
| Thr | 39.47 | 3.7 (4) | 43.81 | 2.5 (2) | 26.82 | 0.8 (1) | 15.95 | 0.8 (1) | 12.06 | 1.0 (1) |
| Ser | 42.27 | 3.9 (4) | 68.72 | 3.9 (4) | 63.41 | 1.9 (2) | 20.41 | 1.0 (1) | 7.40 | 0.6 (1) |
| Glu | 53.60 | 5.0 (5) | 66.26 | 3.8 (4) | 76.17 | 2.3 (2) | 31.08 | 1.6 (2) | 9.00 | 0.7 |
| Pro | 24.80 | 2.3 (2) | 31.90 | 1.8 (2) | 47.27 | 1.4 (1) | 13.51 | 0.7 (1) | 13.67 | 1.1 (1) |
| Gly | 50.40 | 4.7 (5) | 71.87 | 4.1 (4) | 58.98 | 1.8 (2) | 28.78 | 1.5 (2) | 11.90 | 1.0 (1) |
| Ala | 75.33 | 7.0 (7) | 59.69 | 3.4 (4) | 44.53 | 1.3 (1) | 19.46 | 1.0 (1) | 5.39 | 0.4 |
| Val | 43.47 | 4.1 (4) | 36.69 | 2.1 (2) | 23.18 | 0.7 (1) | 11.53 | 0.6 (1) | 12.13 | 1.0 (1) |
| Ile | 36.40 | 3.4 (3) | 39.91 | 2.0 (2) | 11.68 | 0.4 (1) | 13.22 | 0.7 (1) | 3.33 | 0.3 |
| Leu | 60.53 | 5.7 (6) | 48.32 | 2.8 (3) | 39.32 | 1.2 (1) | 14.32 | 0.7 (1) | 13.41 | 1.1 (1) |
| Tyr | 34.27 | 3.2 (3) | 26.28 | 1.5 (2) | 48.96 | 1.5 (1) | 6.66 | 0.3 | 6.72 | 0.6 |
| Phe | 9.43 | 0.9 (1) | 17.52 | 1.0 (1) | 14.58 | 0.4 (1) | 8.45 | 0.4 | 0.0 | 0.0 |
| His | 3.72 | 0.4 | 11.12 | 0.6 (1) | 15.63 | 0.5 (1) | 7.16 | 0.4 | 3.92 | 0.3 |
| Lys | 21.87 | 2.0 (2) | 30.53 | 1.7 (2) | 33.33 | 1.0 (1) | 6.08 | 0.3 | 8.58 | 0.7 (1) |
| Arg | 21.20 | 2.0 (2) | 25.46 | 1.5 (2) | 24.87 | 0.8 (1) | 12.28 | 0.6 (1) | 0.0 | 0.0 |
| HSL/HS | – | | + | | + | | + | | trace | |
| Glucosamine | – | | + | | – | | – | | – | |
| N - terminal | N.D. | | tyrosine | | Proline | | glycine | | N.D. | |
| No. of residues | | 57 | | 41 | | 21 | | 14 | | 8 |

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Table 2. Amino acid analysis of four similar peptides obtained by CNBr fragmentation

| Amine Acid | Peptide B1 | | Peptide C1 | | Peptide C2 | | Peptide D1 | |
|---|---|---|---|---|---|---|---|---|
| | nmol | Residues | nmol | Residues | nmol | Residues | nmol | Residues |
| Asp | 178.95 | 5.2 (5) | 143.28 | 5.4 (5) | 102.53 | 5.9 (6) | 207.95 | 4.6 (5) |
| Thr | 84.86 | 2.5 (2) | 65.86 | 2.5 (2) | 43.81 | 2.5 (2) | 111.07 | 2.5 (2) |
| Ser | 135.64 | 4.0 (4) | 98.39 | 3.7 (4) | 68.72 | 3.9 (4) | 156.26 | 3.5 (4) |
| Glu | 132.38 | 3.9 (4) | 99.33 | 3.7 (4) | 66.26 | 3.8 (4) | 171.77 | 3.8 (4) |
| Pre | 61,51 | 1.8 (2) | 45.30 | 1.7 (2) | 31.90 | 1.8 (2) | 84.82 | 1.9 (2) |
| Gly | 151.80 | 4.4 (4) | 114.38 | 4.3 (4) | 71.87 | 4.1 (4) | 149.77 | 3.3 (3) |
| Ala | 145.96 | 4.3 (4) | 119.22 | 4.5 (4) | 59.69 | 3.4 (4) | 165.54 | 3.7 (4) |
| Val | 96.54 | 2.8 (3) | 67.88 | 2.5 (3) | 36.69 | 2.1 (2) | 122.60 | 2.7 (3) |
| Ile | 69.93 | 2.0 (2) | 53.49 | 2.0 (2) | 34.91 | 2.0 (2) | 74.09 | 1.6 (2) |
| Leu | 132.25 | 3.9 (4) | 105.11 | 3.9 (4) | 48.32 | 2.8 (3) | 170.87 | 3.8 (4) |
| Tyr | 71.42 | 2.1 (2) | 58.74 | 2.2 (2) | 26.28 | 1.5 (2) | 93.04 | 2.1 (2) |
| Phe | 51.60 | 1.5 (1) | 41.26 | 1.5 (1) | 17.52 | 1.0 (1) | 86.94 | 1.9 (2) |
| His | 21.72 | 0.6 (1) | 19.76 | 0.7 (1) | 11.12 | 0.6 (1) | 37.51 | 0.8 (1) |
| Lys | 48.21 | 1.4 (1) | 62.77 | 2.4 (2) | 30.53 | 1.7 (2) | 95.03 | 2.1 (2) |
| Arg | 68.57 | 2.0 (2) | 50.27 | 1.9 (2) | 25.46 | 1.5 (2) | 90.36 | 2.0 (2) |
| HSL/HS | + | | + | | + | | + | |
| Glucosamine | – | | + | | + | | + | |
| No. of residues | | 41 | | 42 | | 41 | | 42 |

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N—terminal amino acid of reduced and alkylated ∝— galatosidase was determined to be leucine and the penultimate amino acid was tryptophan. Further analysis was not possible due to the breakdown of the polypeptide chain. However, the N—terminal of ∝—galactosidase from *Vicia sativa*[9] and soybean[5] has been shown to be alanine. The N—terminal analysis of the peptide A1 did not give any results. This could be due to presence of carbohydrate residues near the N—terminal. The small peptide F1 also did not give any results and this could be due to the loss of the peptide during manual Edman degradation. The N—terminal of peptide C2, E1 and E2 were determined to be tyrosine, proline and glycine respectively (Table 1).

## Acknowledgements

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