Australian Government

Australian Centre for International Agricultural Research

Final report

project

Microbial contaminants associated with sago processing and storage in Papua New Guinea

project number	ASEM/2001/016
date published	June 2009
prepared by	Dr Jeffrey Warner, Microbiology and Immunology, James Cook University
co-authors/ contributors/ collaborators	Dr Andrew Greenhill, Microbiology and Immunology, James Cook University, Townsville Dr Mary Fletcher, Biosecurity Queensland, Department of Primary Industries and Fisheries
approved by	Dr Caroline Lemerle
final report number	FR2009-18
ISBN	978 1 921531 98 9
published by	ACIAR GPO Box 1571 Canberra ACT 2601 Australia

Contents

1 Acknowledgments

The project was supported and led by a number of people throughout its life who have now departed and deserve mention. To Prof. Warren Shipton, the foundation project leader for his tireless effort to establish the project as one ACIAR would consider supporting and his honest and effective stewardship. He and Dr Andrew Greenhill undertook the majority of the work contained in this report and deserve the majority of the credit. Dr Betty Amoa, recently retired from Unitech, for her strong in-country leadership of the recent extension phase of the project. Dr Greg Johnson, our initial project manager for his enthusiasm for the project. Mr Asiak Pue, who with collaborators was the first to consider sago as a topic of research ACIAR might be willing to support; all power to him in his new role as a PhD student. We look forward to further collaboration.

To Barry Blaney and his laboratory staff for their invaluable contribution to the work and the ongoing publication of results. Without Barry's willingness and enthusiasm I doubt much of the work on mycotoxins could have been possible.

In PNG, to Missionary Aviation fellowship (MAF) for going beyond the call of duty in supporting logistics throughout the WP (WP), deserve many thanks. To the personnel of the Evangelical Church of Papua New Guinea (ECPNG) health service for kind help and Daniel Pelowa in particular.

The village people of rural WP and ESP who were bothered by us through data collection and logistical needs, it is hoped that a more safe and secure staple is their reward.

2 Executive summary

Sago starch from the palm Metroxylon sagu is an important staple carbohydrate for many rural lowland Papua New Guinean communities, particularly those where soil fertility is poor and where access to alternatives is limited. The safety and security of sago starch has been questioned with gastrointestinal disease and the presence of the enigmatic Sago Haemolytic Disease (SHD), a haemolytic syndrome with high mortality rates, linked to sago consumption. This project was established to determine the extent and significance of microbial contamination in village-derived sago starch with the aim to elucidate the relationship between sago processing and storage and microbial populations.

Sago starch was found to harbour a variety of typical food borne bacterial pathogens and haemolytic fungi of the genera Aspergillus, Penicillium, Trichoderma and Fusarium. Post harvest storage methods were compared and those that resulted in rapid lactic acid bacteria fermentation were found the least contaminated. Optimising fermentation was found to be critical for the safe storage of sago starch for human consumption.

Upon analysis, no common mycotoxins were detected. The haemolytic properties of all fungi analysed were characterised as free fatty acids. It is hypothesised that when sago starch is stored in ways that does not result in rapid and protective fermentation, levels of free fatty acids from fungal biomass are sufficiently high that when ingested by individuals with protein malnutrition or a co-morbidity which results in low circulating albumin (which would normally be protective) act as detergents on red cell membranes causing intravascular haemolysis. Sago not properly fermented also results in higher numbers of bacterial pathogens which may contribute to the burden of gastrointestinal disease in communities where the sago is consumed.

A village applicable hazard analysis and critical control point (HACCP) analysis was undertaken and presented as the “Six Steps to Safe Sago Starch” program. This was widely disseminated throughout the WP to village communities and health centres. It is hoped that through more optimal post harvest production of village derived sago starch, in terms of decreasing harmful microbial populations, will result in a safer and more secure staple for rural Papua New Guineans.

But also, as the resource is culturally significant, under utilised and vast its potential for commercialisation should not be discounted. Through a more thorough understanding of the microbial ecology of palm derived sago starch, its optimal storage conditions can be established for bulk production and safe export. Through further agri-economic and microbiological analysis its role as a substrate for biofuel production (ethanol) could be established. These initiatives could contribute to the sustainability and development of rural communities.

3 Background

The health and nutritional status of the people of Papua New Guinea (PNG) remains one of the biggest challenges faced by that country. Statistics from relevant organizations point to one of the lowest life expectancies, highest infant and child mortality rates and lowest health care expenditures in the Pacific region (PNG Ministry of Health, 2000; WHO, 2004). Basic needs such as access to safe water and food security are yet to be adequately met. Diarrhoea is one of the leading causes of morbidity and mortality in the country, and typhoid and malnutrition also feature in the leading 15 causes of death (PNG Ministry of Health, 2000).

Undoubtedly, improved access to safe water, which currently stands at about 40% (PNG Ministry of Health, 2000) would help reduce the incidence of diarrhoea. However, the importance of foodborne illness should not be underestimated. Based on the findings of two World Health Organisation (WHO) reports, Ehiri and Prowse (1999) suggested that worldwide over 70% of cases of diarrhoea in children under five years old are attributable to the consumption of contaminated food. Other factors that may be less tangible than the incidence of diarrhoea also contribute to food safety, and should not be overlooked. Although it is difficult to relate to national health statistics, exposure to mycotoxins, for example, is likely to have numerous detrimental health effects, particularly in developing countries (FAO, 2001), such as PNG.

One of the most important food crops in PNG is the endemic sago palm, Metroxylon sagu Rottboell. The palm covers an estimated one million hectares of the country (Power, 1999; McClatchey et al., 2004), and is an important source of carbohydrates for approximately 10% of the population (Rhoads, 1980; Sopade, 2001). Dependence on sago starch is greatest in lowland areas prone to intertidal flooding, where very few other sources of carbohydrate will grow. In such areas, life is based on subsistence agriculture, household income is very low, infrastructure is poor and access to health services is limited.

Sago starch is extracted from the macerated pith of M. sagu using traditional methods, and is typically stored for up to two months. In the 1970s, Taufa (1974) described an illness that was linked to the consumption of old stored sago. This illness, known as sago haemolytic disease (SHD), was initially thought to be confined to the Maprik region of the ESP , but two years later, two outbreaks were reported in the WP (Donovan et al., 1976). Sporadic outbreaks continue to occur, with high mortality rates, but to date little is known about the aetiology, epidemiology or pathophysiology of the disease.

On the basis of the current knowledge of SHD (Taufa, 1974; Donovan et al., 1976; Donovan et al., 1977), it seems likely that the as yet unidentified aetiological agent of the disease is microbial. The purpose of this project was to establish detailed basal knowledge of the microbiology of sago starch, which will form a foundation for further investigation. Specifically, the presence of pathogenic microbes and associated toxins in sago starch will be determined, and the conditions under which their presence can be limited will be established.

4 Objectives

Key Objective:

To determine the causes and extent of health risks to consumers caused by contamination in village-produced sago in PNG and identify options for reducing those risks and improve marketability of the processed sago.

Objective 1: Conduct surveys to document production and processing and storage technologies in ESP and WP

1.1 Survey instrument designed and implemented

Objective 2: Conduct analysis of the microbial contamination of good and poor quality sago samples

2.1 Mycological survey

2.2 Bacteriological survey

Objective 3: Assess mycotoxin contamination and assessment of their potential to cause haemolytic disorders

3.1 Mycotoxin survey of sago starch

3.2 Haemolytic activity of microorganisms isolated from sago starch and methods for their detection

3.3 Characterisation of haemolytic compounds for Penicillium (steckii) citrinum

3.4 Assay remaining fungal isolates that have demonstrated haemolytic compounds upon screening, particularly P. brevicompactum and Trichoderma spp.

3.5 Determine the role of haemolytic fungi in SHD

3.6 Establish techniques to demonstrate Koch's postulates

3.7 Clarify the inoculum source of the haemolytic fungi

3.8 Determine the extent of colonisation of the fungi under varying conditions

3.9 Develop methodologies for organism and toxin identification and where possible transfer technology to staff at Unitech, Lae and the Central Public Health Laboratory, Port Moresby

Objective 4: Hazard Analysis at Critical Control Point (HACCP) of selected sites to devise practical measures for reducing contaminant risks for consumers

4.1 Develop critical control point recommendations

4.2 Enhancement of HACCP training for Unitech staff

4.3 Advise national and provincial health authorities on how sago starch sample collection and processing could be undertaken in the event of an outbreak investigation

Objective 5: Recommend practical measures for reducing contaminant risks for consumers

5.1 Communicate findings to village communities

5.2 Communicate findings to health authorities

5.3 Communicate findings to national health fraternity

5 Methodology

5.1 Study sites and sampling

Two study regions were established in lowland areas of PNG. In the north of the country in the ESP , samples of sago starch and sociological data were collected predominantly from Ambunti and Angoram villages, and surrounds. Some samples were also collected in the Wewak region. In the south of the country samples were collected from throughout the southern and central regions of the WP. Many of the samples were collected from villages along the Aramia River in the Balimo district. Further samples were collected from the Morehead, Suki, Mabawe and Lake Murray districts. Sample collection was based around the ESP and WP primarily because both s have regions of high sago starch dependence, and they are the only two s from which cases of SHD have been documented in the scientific literature (Taufa, 1974; Donovan et al., 1976; Donovan et al., 1977).

In the study sites, a sociological survey, developed by Dr Elizabeth Kopel, University of PNG, was used to gain insight into the management and cultivation of sago palms (particularly gender roles), harvesting of sago palms and subsequent extraction of the starch, storage of sago starch, preparation and consumption, and social and cultural significance. The outcomes of this survey were used to develop and test hypotheses.

The survey data and sago samples were collected during five field trips between November 2002 and June 2005. Sago samples were storage at 8 ˚C in a portable field refrigerator and transported to Townsville and Lae for analysis as soon as possible. After microbial analysis the sample were stored at −20 ˚C for mycotoxin analysis.

Samples were divided with half sent to Unitech in Lae and the others brought to James Cook University (JCU). The subsequent analysis was duplicated. Scientists based at JCU, principally Prof Shipton and Dr Greenhill, supporting Unitech's needs. This was undertaken in attempt to secure microbiological expertise, in terms of sago sample analysis, in PNG.

The pH of samples was determined by emulsifying sago in distilled water and measuring with a pH meter. Water activity was determined using a dew point microvoltmeter.

5.2 Microbiological procedures

Sago samples were tested for the presence of microbial pathogens associated with food-borne disease at the North Queensland Food Testing Laboratory, James Cook University. General bacteriological procedures followed relevant Australian Standards: general methods for food microbiology pertaining to preparation of dilutions (AS 1766.1.2); pour plates (AS 1766.1.3 – 1991); colony counts (AS 1766.1.4 – 1991); most probable number (MPN) (AS 1766.1.6 – 1991); standard plate counts (AS 1766.2.1 – 1991) (Standards Australia, 1991c; Standards Australia, 1991d; Standards Australia, 1991e; Standards Australia, 1991a; Standards Australia, 1991b).

Methods used to enumerate Bacillus cereus were based on AS 1766.2.6 – 1991 (Standards Australia, 1991g); Clostridium perfringens, AS 1766.2.8 – 1991 (Standards Australia, 1991h); Non-Salmonella enterobacteriaceae, AS 1766.2.3 – 1992 (Standards Australia, 1992). Salmonella sp, AS 1766.2.5 – 1991 (Standards Australia, 1991f). other enterobacteriaceae of interest included Citrobacter freundii which was identified after augmenting AS 1766.2.3 – 1992 and testing for citrate and urease with those positive confirmed using Vitek II (bioMerieux Australia Pty Ltd). Enterobacter sakazaki was identified using methods of Kandhai et al. (2004) with confirmation determined by Vitek II (as above). Listeria monocytogenes was detected based on AS/NZS 1766.2.16.1:1998

(Standards Australia, 1998); Staphylococcus aureus based on AS 1766.2.4 – 1994 (Standards Australia, 1994b).

Similarly, enumeration and identification of yeasts and moulds was based on Australian Standards AS 1766.2.2 – 1994 (Standards Australia, 1994a). When required, specific organisms were further identified based on published methods (Pitt, 1979; Barron, 1968; Zycha and Siepmann, 1969; von Arx, 1970; Bissett, 1991; Gams and Bissett, 1998). Actinomycetes were cultured and isolated using methods of Waksman (1967). To determine the overall fungal biomass in sago samples, egosterol analysis was also undertaken using high performance liquid chromatography (HPLC)

Statistical analysis, to determine significance differences in microbial populations, was performed using a one way ANOVA or Fisher's exact test. A T-test and Pearson correlation was also used to examine the relationship between yeast and mould numbers and ergosterol concentration.

5.3 Mycotoxin analysis

Samples were screened for common mycotoxins at the Animal Research Institute in Yeerongpilly by Dr Greenhill under the stewardship of Barry Blaney and Dr Mary Fletcher. A 25 g representative sample of the sago collected (5.1) was screened. The extraction process was based on that of Blaney et al. (1984). Using two-dimensional thin layer chromatography (TLC), samples were tested for the presence of aflatoxin B1, B2, G1, G2, ochratoxin A, cyclopiazonic acid and sterigmatocystin. Citrinin and zearalenone were tested for using one-dimensional TLC. Plates were visualised at ambient light and with ultraviolet (UV) light at either 254 nm or 365 nm. In addition to screening samples, specific fungal isolates (5.2) were also tested for the presence of mycotoxin production; cultures being established on grain and then extracted as above.

5.4 Haemolytic micro-organisms

Sago samples were screened for the presence of haemolytic micro-organisms and haemolytic extracts were obtained. Samples were prepared in serial dilutions based on AS 1766.1.2 – 1991 (Standards Australia, 1991a). Initial screening was conducted by culturing the samples on blood (sheep and human) agar with sterile sago processed and tested as a control. Yeasts and mould were isolated and tested for haemolysis individually. Organisms that were screened as haemolytic were inducted as candidates for further testing. Extracts were obtained using various techniques depending on the organism's growth medium (including an in-house developed sago agar), including freeze-thaw of growth media with filtration and sonication of culture material (Elek and Levy, 1954). Filtrates were extracted through agitation in an ethanol-based extraction buffer. The supernatant was freeze-dried and resuspended in isotonic saline to eliminate non-specific haemolysis. The extract was filter sterilised and stored at −20 ˚C until required. These fractions were subjected to a haemolysis bioassay, using human red blood cells, in order that the haemolytic activity could be quantified.

Seven fungal isolates were selected for further study based on the intensity of haemolysis (100%) and the rate over time they were able to achieve this intensity incubated with human red cells in the bioassay (typically the isolates tested achieved 100% haemolysis in < 6 hours), the collection included species of Pencillium, Fusarium and Trichoderma.

5.5 Characterisation of haemolytic fractions

Preparation and characterisation of the haemolytic fractions from fungal cultures and sago starch samples was conducted at the Animal Research Institute in Yeerongpilly by Dr Greenhill and Asiak Pue, with the support of Barry Blaney and Dr May Fletcher. Partial separation of the haemolytic components from other fungal metabolites was undertaken.

The methods were based on those of Blaney et al. (1984) with minor modifications resulting in two fractions, one hexane rich the other dichloromethane rich. The fractions of each extract were brought to dryness and resuspended in methanol and tested for haemolytic activity using the red cell bioassay, using appropriate reagent, negative and positive controls.

Preparative thin layer chromatography (Prep TLC) was used to further separate the haemolytic component(s) of the fungal extracts. The resultant segments were separated clear from citrinin (if it was present) and were suspended in 50 ml of methanol. The extracts were aliquoted, taken to dryness and stored at −20 ˚C. Aliquots were resuspended in methanol and diluted and tested in the bioassay.

A large scale methanolic extract of the fungal culture was partitioned between hexane and methanol, and the haemolytic hexane extract was separated by C18 Solid Phase Extraction (SPE), Prep TLC and High Performance Liquid Chromatography-Evaporative Light Scattering Detector (HPLC-ELSD) to provide semi-purified haemolytic fractions. These fractions were analysed by liquid chromatography-mass spectrometry (LCMS) and Nuclear Magnetic Resonance (NMR). The identity of the resultant fatty acids was determined by fatty acid methyl ester (FAME) analysis by GC and GC-MS, with confirmation by comparison with fatty acid standards

Further, field collected sago starch samples considered fit for consumption and one implicated in SHD were directly extracted by methanol and the haemolytic activity determined based on the techniques used for fungal cultures.

In an attempt to determine the significance of the fraction obtained from P. citrinum an assay was developed based on Dodge (1963) to identify its activity on the red cell membrane proteins. Red cell membrane proteins were harvested from washed human red cells. Packed cells were resuspended in lysis buffer at a ratio of at least one part cells into 20 parts buffer, centrifuged (20,000 g, 4 °C, 20 minutes) and the supernatant discarded. The washing step with lysis buffer was repeated approximately seven times until erythrocyte membrane proteins appeared free from haemoglobin as determined by visual inspection against a white background. The resultant “ghost cells” were resuspended into 2 ml of lysis buffer from which aliquots of 200 µl were removed to 1.5 ml micro-centrifuge tubes for storage at –20°C until required.

Aliquots of the red cell ghosts were exposed to the haemolytic fractions. After incubation the red cell membranes (with appropriate controls) were separated using sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE). A molecular marker was used to indicate relative sizes of the erythrocyte protein bands and therefore their identity. Gels were stained with 0.2% Coomassie blue R–250, silver stain and with periodic acid Schiff’s stain (PAS) to visualise bands. Cleaving of bands was indicative of activity. The location of the cleaving dictated the mode of action of the compound and aided in a functional classification. The clinical significance of the compound could be inferred by the specific function (for example, if the compound was found to be active against major structural membrane proteins, this could be indicative of intracellular haemolysis and consistent with the clinical findings of SHD).

5.6 Comparison of the effect of selected traditional storage techniques on microbial communities

Based on the outcome of the sociology survey (5.1), three methods of sago storage were identified as commonly used and therefore chosen to be tested: sago wrapped in leaves (bundles) – typical of the WP; sago dried and smoked - sometimes used in ESP and other coastal communities but rarely in the WP; sago in clay pots covered with water – the most common method in ESP but rarely in WP. Sago sourced from Balimo, WP (three examples purchased from a village market, < 3 days old), were prepared and stored using the methods described above in replicates of three. The experiment was undertaken at

JCU; the sago was transported from PNG on the day of purchase and prepared the next day.

All three samples representing the three storage methods were sampled weekly for 4 weeks and then once more at 7 weeks. At each time period total viable bacteria, lactic acid bacteria (LAB), yeasts, moulds, mucoraceous fungi and haemolytic organisms were enumerated (based on the Australian standards referenced previously).

Known bacterial pathogens (B. cereus, E.coli, L. monocytogenes, Salmonella sp. and S. aureus) were seeded into samples of sago freshly harvested (sourced from Balimo, WP) and sago which had been previously stored for 2 weeks (sourced from Wewak, ESP). Approximately 104 cfu/g of each pathogen was added and thoroughly mixed through. To decrease the likelihood of competition between species, separate sub-samples were used for Salmonella and E.coli, Bacillus and Staphylococcus and Listeria. The study was undertaken in triplicate at 30 ˚C. The samples were tested twice weekly for new sago and once weekly for old sago. The pH and aw were determined in the fresh sago.

5.7 Investigations into fermentation

The fermentation potential of sago starch was determined by the enumeration of LAB and the determination of acid using gas chromatography on samples collected from village sources. The volatile fatty acids tested for were acetic acid, propionic acid, iso-butyric acid, n-butyric acid, iso-valeric acid, n-valeric acid, n-caproic acid and n-heptonic acid. The organic acids tested for were pyruvic and lactic acid. Regression analysis of LAB numbers, pH and acid content was conducted using SPSS 12.0.1. Further analysis was conducted on sago freshly obtained and where fermentation was conducted in laboratory conditions. The inhibition effects of LAB were tested using a deferred spot on lawn technique using a selected number of LAB (isolated from experiments above) against known pathogens as targets (E.coli, Enterobacter aerogenes, Salmonella salford, B. cereus, L. monocytogenes and S. aureus).

5.8 Hazard analysis and critical control point

A systematic approach to the identification and assessment of risk concerning sago processing and storage was conducted. A hazard analysis and critical control point (HACCP) process was used and consisted of seven principles (NACMCF, 1992; NACMCF, 1998).

- Conduct a hazard analysis. Prepare a list of steps in the process where significant hazards occur and describe the preventative measures
- Identify the critical control points in the process
- Establish critical limits for the preventative measures associated with each identified critical control point
- Establish critical control point monitoring requirements. Establish procedures for using the results of monitoring to adjust the process and maintain control
- Establish corrective action to be taken when monitoring indicates that there is a deviation from the established critical limit
- Establish effective record keeping procedures that document the HACCP system
- Establish procedures for the verification that the HACCP system is working correctly.

The preliminary steps and principles of HACCP were applied to sago starch production and storage as applicable.

5.9 Application of HCCAP at the village level: community feedback

The principles of the HACCP analysis outcome were converted to a village-based communication tool called the “Six Steps to safe Sago”. Posters for villagers and pamphlets for health care centres were distributed and community meetings were undertaken. Communities that were involved with the project and where SHD had been reported were targeted. The text and pictures were refined by Dr Betty Amoa through feedback with local Papua New Guineans. The pictures were created by Mr. Heso Kiwi of the PNG National Department of Health.

6 Achievements against activities and outputs/milestones

Objective 1 Conduct surveys to document production and processing and storage technologies in ESP and WP s

no.	Activity	outputs/ milestones	completion date	Comments
1.1	Survey instrument designed and implemented	A sociological survey instrument was constructed jointly by JCU (A) and UPNG (PC) and conducted in 49 villages throughout the ESP and WP, two field trips each (PC). Surveys were conducted WP 03/03; 04/04 and ESP 11/02; 06/03.	Final data analysis was completed 09/04	Valuable data was collected which enabled the detection of control points likely to be involved in microbial contamination. The diversity of sago processing and storage methodology was documented which enabled for further hypothesis testing (e.g. does storage method influence microbial populations and is this correlated with the incidence of SHD?). Thus providing insights into the fermentation potential of sago and its protective action in terms of controlling microbial populations. Surveying the Gulf was originally planned, although unforseen cost and logistical problems prevented this being conducted. It is anticipated that, although there is considerable variation in sago processing practices through PNG, communities of the Gulf share many similarities with those of WP.

PC = partner country, A = Australia

Objective 2: Conduct analysis of the microbial contamination of good and poor quality sago samples

no.	Activity	outputs/ milestones	completion date	Comments
2.1	Mycological survey	Identification of fungal isolates was completed on 58 sago samples collected from the WP and ESP during the field trips listed above (1.1). All Isolates were identified to genus level and those that demonstrated haemolytic activity further identified to species level. Determination of fungal biomass was determined through ergosterol analysis and a correlation was sought with fungal culture. The mycological populations of sago stored differentially was assessed and compared as was the influence of storage time.	04/03; 05/04; 1/03; 07/03	Most sago samples collected and tested were considered fit for consumption. Late in the project a SHD implicated bundle became available; time did not permit its thorough investigation although mycological analysis did reveal haemolytic organisms of the same genus as those cultivated from other samples – investigation was focused on these moulds. A newly funded project has just been implemented that, at its core, will be seeking out and identifying SHD outbreaks and retrieving sago samples for analysis, this should aid in clarifying the outcomes of this objective in this project. It was considered reasonable that sampling prolonged stored sago mimicked the situation where SHD-implicated organisms thrive. Fungal content rose with increased age. Sago stored in leaves or woven baskets tended to support higher numbers of filamentous fungi than those stored by other methods. This finding pointed to the potential for fermentation as a variable in sago starch safety and led to further hypothesis testing.
2.2	Bacteriological survey	Isolation and identification of a number of different bacterial groups was pursued. Three groups were focused upon: those implicated in food safety; those that demonstrated haemolysis and those linked with the processes of fermentation. A detailed analysis was undertaken to investigate the bacteria and conditions associated with effective fermentation.	04/03; 05/04; 1/03; 07/03	Sago was found to harbour typical food borne pathogens that are associated with diarrheal disease such as Bacillus cereus and faecal coliforms. Although a number of haemolytic bacteria were isolated the project was guided down a mycology path in the assumption that mycotoxins were more likely to be associated with SHD. In light of the later findings (3.3) and upon reflection on the clinical manifestations of SHD, phospholipase activity should be investigated; these compounds have been associated with bacteria. This hypothesis will be pursued when more SHD implicated samples are sourced in the years to come. Regardless, important information was gathered which highlighted the processes of fermentation. This was shown to be responsible for the reduction of potentially pathogenic bacteria and filamentous fungi and was associated with sago storage methods which kept the starch wet and anaerobic, as was typical when sago was stored underwater. Sago stored in weaved baskets was less likely to encourage effective fermentation and prolonged stored samples were likely to dry with the resultant reduction of fermentative bacteria and the resultant protection. The findings of this objective were to aid in the development of a HACCP-based analysis of storage methods.

PC = partner country, A = Australia

Objective 3: Assess mycotoxin contamination and assessment of their potential to cause haemolytic disorders

no.	Activity	outputs/ milestones	completion date	Comments
3.1	Mycotoxin survey of sago starch	Selected samples (51) were analysed for common mycotoxins using dichloromethane extraction and thin layer chromotography: aflatoxin B1, B2, G1 and G2; ochratoxin A; cyclopiazonic acid; sterigmatocystin; citrinin and zearalenone. Also, 21 fungal strains isolates as part of activity 2.1 were tested for production of ochratoxin A, cyclopiazonic acid, sterigmatocystin, citrinin, penicillic acid and patulin. The sago samples from which fungal strains were derived represented a diversity of storage age and storage methods. Samples were collected from the study sites described earlier (1.1).	04/03; 05/04; 1/03; 07/03	Although the fungal biomass in sago samples was often high, upon analysis no mycotoxins were demonstrated from the samples tested. This included two samples sourced from SHD outbreak situations. However, 13 fungal isolates when tested directly demonstrated the production of citrinin (P. citrinin, P. brevicompactum and Pencillium sp. and one sterigmatocyctin (A. versicolor). These results point to the complexities associated with secondary metabolite production – clearly although fungi isolated from sago starch are capably of producing mycotoxins, the environmental conditions represented in the samples chosen to be tested, were not conducive to their production. This maybe linked to the poor availability of trace elements in sago and/or the protective capacity of fermentation which was demonstrated in 2.2. Most samples of sago in this study were aged 1 month or less (fit for consumption). A thorough study on aged and SHD implemented sago starch is required to further elucidate the role common mycotoxins play in sago-associated mycotoxicosis.

3.2	Haemolytic activity of microorganisms isolated from sago starch and methods for their detection	Further analysis was directed to haemolytic activity. Sago sampled (1.1) was subject to direct screening using blood agar; implicated organism were isolated. Direct screening was found not be suitable for moulds or yeasts, in these cases individual strains were isolated and screened independently. Methods were optimised and candidate isolates were chosen based on the extensiveness of haemolytic clearing in the agar-based methods. These organisms were subject to an extraction process (5.4 above) and the activity was accessed quantitatively using a bioassay. Those isolates that achieved haemolytic activity of 100% in the shortest period of time (typically <6 hrs) were considered candidates for detailed compound characterisation.	11/03; 01/04	Methods to determine the haemolytic activity of fungi and bacteria screened on blood agar were developed. This bioassay was used to semi-quantitate and compare haemolytic activity. In only two of the 30 samples tested levels of haemolytic bacteria reached 1 × 105 cfu/g. Of note though, was that one sample was implicated in SHD. Preliminary identification was made of these bacteria and identification revealed the majority were Bacillis spp. Of all the bacteria tested in the bioassay, B. cereus demonstrated the most significant haemolytic activity. The significance of haemolytic bacteria was not pursued. Clinical presentations and limited pathology undertaken on individuals implicated with SHD suggest that phospholipase activity (typically produced by bacteria) should be investigated. The assumption was made that a novel mycotoxin was more likely to be the aetiological agent of SHD and therefore all subsequent activities were directed towards fungi. Of the collection of fungi (moulds and yeasts) that were considered likely candidates upon screening, strains of P. steckii demonstrated the most aggressive haemolysis as determined by the bioassay. These organisms were subsequently sent to a reference laboratory and identified as P. citinum. Other fungi including Trichoderma spp and P. brevicompactum also demonstrated haemolysis and like P. citrium were frequent isolates from sago. As such, they made up a collection of isolates that were subject to further analysis.

3.3	Characterisation of haemolytic compounds from Penicillium (steckii) citrinum	Initially, simple chromatographic techniques were used, using the bioassay as an indictor of haemolysis, to further fractionate and characterise the active component(s). Seven fungal isolates obtained from 3.2 were tested (3 × P. citrinum,). Two resultant fractions, one rich in hexane and one rich in dichloromethane were tested. It was found that the hexane-rich fraction was the most haemolytic, so this fraction was further separated using Prep TLC. The hexane fraction of a strain of P. citrium (initially thought to be P. steckii) was subject to further separation using C18 SPE HPLC-ELSD to provide semi-purified haemolytic fractions. These fractions were analysed by LCMS and NMR. The identity of the resultant fatty acids was determined by FAME analysis by GC and GC-MS,	04/06; 12/07	The direction of the experimental work was focused on the detection on novel mycotoxins. Therefore, initial extraction methods were development based on standard methods of mycotoxin analysis. This avenue of investigation precluded the discovery of other compounds, such as phospholipases from non-fungal sources. These directions will be explored by other on-going investigations. Haemolytic strains of P. citrinum were consistently isolated from sago starch. It demonstrated significant haemolysis in-vitro. Characterisation of the active compound was finalised late in the project and found to be a fatty acid. Notwithstanding the narrowness of the investigations to date, given albumin provides a transport and detoxifying role for free fatty acids, it is possible that malnourished individuals (with high carbohydrate but low fat and low protein diet – typical of rural PNG) could eat sago infected with fungi with associated haemolytic free fatty acids, and experience the affects of haemolysis. This avenue of investigation will be followed through Dr Miila Gena's PhD program.
3.4	Assay remaining fungal isolates that have demonstrated haemolytic compounds upon screening, particularly P. brevicompactun and Trichoderma spp.	Strains of P. brevicompactum, F. semitectum, A. flavipes and T. virens which demonstrated haemolysis after initial extraction (as above) were subject to further characterisation	Begun 12/07, incomplete.	As a result of the time required to characterise the compound(s) from P. citrinum, further analysis was not possible within the timeframe of the project. This analysis though, will be a component of Mr Asiak Pue's PhD project and is ongoing.

3.5	Determine the role of haemolytic fungi in SHD	Red cell membranes of human origin were subject to the effects of the fractionated compounds derived from activity 3.3. The specific activity of red cell membranes could be visualised by separating the proteins on SDS-PAGE and observing cleavage. The significance of the compound could the inferred through analysing this activity.	10/07	The compound derived from P. citrium demonstrated activity against the major structural red cell membrane protein (band 3 and α spectrin). This activity, if replicated in vivo is consistent with red cell destruction. Interestingly, given band 3 deletion (in the form of Melanesian ovalocytosis) is a common inherited disorder in PNG, it may play a role in either protection or predisposing to the effects of the compound. The work is ongoing through the initiatives Dr Miila Gena (PhD candidate with JCU).
3.6	Establish techniques to demonstrate d Koch's postulates	The implicated compound was not able to be manipulated into an assay which could have been used to test hypotheses of origins and exposure. Also, further work on other haemolytic organisms requires completion before this work can continue.	12/07 - onging	The development of ELISA detection methods for the detection of free fatty acids has not been pursued due to the ubiquitous nature of FFA and the ready availability of other detection methods. Commercial NEFA (non-esterified fatty acid) colorimetric kits are already available for the detection of free fatty acids in plasma. The work is ongoing through the initiatives of Asiak Pue (PhD candidate with UQ) and Dr Miila Gena (PhD candidate with JCU). Serum albumin is detectable by standard clinical chemistry methods. It is anticipated that individuals with a history of SHD would have both low serum albumin levels and high plasma NEFA levels. Monitoring both serum albumin and plasma NEFA levels in patients affected by SHD would enable the hypothesis that SHD is caused by high plasma NEFA to be proved or disproved.
3.7	Clarify the inoculum source of the haemolytic fungi	This activity was not completed.		Time constraints have not allowed this objective to be further investigated, but now that we have a better understanding of the haemolytic toxins involved, this microbiological study will be completed as part of Asiak Pue's PhD project. A lack of implicated sago samples prevented the project be able to determine a definitive aetiological agent, therefore clarifying the inoculum can be only speculated. Through ongoing clinical studies (Dr Miila Gena's PhD), this should be rectified.

3.8	Determine the extent of colonisation of the fungi under varying conditions	Preliminary data at the stage only	Ongoing	Sago collected from villages and markets was extracted and analysed for haemolytic activity. Such activity was then correlated with recorded age, storage conditions and water content of collected sago. Preliminary data suggests the greatest haemolytic activity is in aged sago, stored dry with limited water content. Only low haemolytic activity was measured in sago samples stored wet or under water. Weathering trials have been planned, whereby sago will be stored under different storage conditions – under water in buckets, tightly wrapped in leaves, in open leaves and in plastic bag – at village locations in Madang. These sago samples are being tested for haemolytic activity on a regular basis over a 6 month period. This work is being undertaken as part of Aisak Pue's PhD study in collaboration with local PNG Institute of Medical Research (PNGIMR) field staff.
3.9	Develop methodologies for the organism and toxin identification and where possible transfer technology to staff at Unitec, Lae and the Central Public Health Laboratory, Port Moresby.	As part of Mr Asiak Pue's PhD program, skills and methodologies are being developed that will be transferred to Unitec. This will be completed in 2010.	11/07	Techniques for haemolytic blood bioassay have been demonstrated by Aisak Pue (AUSAID PhD student and Unitech lecturer) to other Unitech staff in Lae as a qualitative assay of haemolytic activity. This technique has been used in Lae to qualitatively assess village collected sago for haemolytic activity, and can be used in the future to assess any sago implicated in SHD outbreaks in PNG. Unitech has a suitable plate reader with associated software for quantitative assays but currently lack the 540nm filter used in our project work (closest wavelength filter held at Unitech is 490nm). Aisak Pue (AUSAID PhD student and Unitech lecturer) is now proficient in techniques required for fatty acid analysis, and will be able to transfer this technology to Unitech Lae at the completion of his PhD. This analysis only requires suitable standards for comparison and identification and access to a GC equipped with an FID detector. Unitech Lae has a Hewlett Packard instrument suitable for this analysis. Such technology can then be used to assess free fatty acid content of any sago implicated in SHD outbreaks in PNG.

Objective 4: Hazard Analysis at Critical Control Points (HACCP) of selected sites to devise practical measures for reducing contaminant risks for consumers

No.	Activity	outputs/ milestones	completion date	Comments
4.1	Develop critical control point recommendations	A HACCP analysis was undertaken using the guidelines of the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) and guided by the outcomes of activities1.1 – 2.2.	12/05	With the exception of tree felling, where no food safety hazards were identified, potential hazards were identified, appropriate control measures determined and CCPs established for each step in the sago making process. Many of the CCPs identified could not be activated in the environment in which the work was conducted. Consequently, CCPs were characterised as notional or practicable, based on their suitability to application in village-based production of sago starch in PNG. Notional CCPs were identified in all six steps where hazards were identified, namely tree selection, trunk storage, pith maceration and removal, starch extraction, starch storage and fermentation, and food preparation. A total of six practicable CCPs were identified, occurring in all of the aforementioned stages of the process except starch extraction. Two of the six practicable CCPs were in the storage and fermentation stage, suggesting that this stage is particularly important in determining the food safety of sago starch. Following the determination of the critical control points, critical limits, monitoring procedures and corrective actions were established for all practicable CCPs. The establishment of verification and documentation procedures was beyond the practical extent of the HACCP analysis of village based sago production and storage process. However, a tool – based on HACCAP - to be used to communicate finding to individuals in villages and to health care workers in rural health centres was developed (see 5.1 and 5.2)
4.2	Enhancement of HACCP training for Unitech staff	Dr Betty Amoa and Mr Asiak Pue, were chosen for HACCAP training. Training in Food Safety Management Systems Auditor/Lead Auditor Course was provided for Dr Amoa by NCSI Training and Development Pty Ltd on 29 May – 2 June 2006.	06/06	It was recommended that the HACCP training for Mr Pue be held back until the final year of his PhD program. This would ensure that he would be better able to pass on skills upon returning to Unitech. The course has been planned for mid 2010. Unfortunately, Dr Amoa has resigned from Unitech, although she was able to include the training in teaching programs at Unitech prior to departure.

4.3	Advise national and provincial health authorities on how sago starch sample collection and processing could be undertaken in the event of an outbreak investigation	Presentations were held at the 2006 National PNG Medical Symposium. Senior health officials of the National Department of Health (NDOH) were presented with publications.	09/06; 12/07	Details of outbreak investigation are to be followed up and better prepared by Dr Miila Gena as part of her PhD program. She will establish a WP outbreak surveillance network for SHD involving OTML. After the death of the director of the CPHL, collaboration with this institute became difficult. Dr Andrew Greenhill will be taking up an appointment at the PNGIMR where this aspect of the program will be developed.

Objective 5: Recommend practical measures for reducing contaminant risks for consumers

no.	Activity	outputs/ milestones	completion date	Comments
5.1	Communicate findings to village communities	A “Six Steps to Safe Sago Starch” program – based on HACCP analysis - was created and implemented in the WP. A series of community meetings were held in the villages where sago was sampled and where SHD has been reported and an A3 poster was distributed and explained.	12/07	Particular attention was given to women and children and mostly, the meetings were held in market environments. Both Dr Greenhill and Dr Gena were involved in this program, ensuring some continuity between the ACIAR project and the newly established and ongoing clinical project (Dr Gena’s PhD project).
5.2	Communicate findings to health authorities	A pamphlet version of the Six Steps to Safe Sago Starch program was developed which provided more detail for health workers in the hope that further questions of the village community could be dealt with by hospital staff.	12/07	The program was well received and will be the subject of a sociological analysis as part of Dr Gena’s project – measuring the effectiveness of the program and its outputs.

5.3	Communicate findings to national health fraternity	A mini symposium was conducted where findings of the project were communicated. Present was senior NDOH officers and national and local politicians. As a result of this symposium, the National Secretary of Health invited investigators to present at the 2005 PNG medical symposium. During 2007 and after the WP based field trip where local health authorities and villages were informed of project outcomes, investigators made representation to the NDOH and provincial governor.	09/05; 12/07	Every effort has been made to ensure stakeholders and policy makers are informed of the project outcomes and the ongoing project objectives.

7 Key results and discussion

7.1 Sociology survey of sago production and consumption

The analysis of survey results demonstrated that there is a dependence on both cultivated sago palms and naturally growing palms, with no sago consumers dependant on naturally occurring sago palms alone. In the ESP 15% (5–35%) of respondents use only cultivated palms for starch extraction, with the remaining 85% (65–95%) using a combination of cultivated and naturally occurring palms. In the WP the figures were 36% (17–59%) and 64% (41–83%) respectively.

In the ESP , the majority of respondents (85%; 66–96%) indicated that they typically harvest the palm during flowering, with the remaining 15% (4–34%) harvesting the palm either just before, or during, flowering. In the WP, responses were varied, with no trends evident. Approximately 27% (11–50%) of respondents fell the palm before it flowers and a further 14% (3–35%) harvest before, or during, flowering. Many of the respondents (32%; 14–55%) harvest at around the time of flowering, regardless whether it is slightly before, during, or slightly after flowering.

Most tasks associated with sago cultivation and starch production are shared among both men and women (Table 11.01, Appendix 11.1). In the ESP the sago palms are felled by men predominantly, but both men and women do all other tasks. Starch extraction, as a whole, is done by both men and women, but roles within the process are gender specific. In the majority of responses, males were said to extract and macerate the pith, while women wash the pith to extract the starch. In the WP, most jobs are shared between the men and women, as is the case for the ESP. However, men do not extract starch as commonly as women.

The transfer of knowledge from one generation to the next is influenced by gender roles (Table 11.02, Appendix 11.1). In the ESP, transfer of knowledge for planting and growing sago palms, and for the sago extraction process, is from parent to child through observation and participation. None of the respondents stipulates gender in the transfer of knowledge. In the WP transfer of knowledge for both tasks is most commonly non-gender-specific, from parent to child. However, just under half the respondents identified mother to daughter as the most important route of knowledge transfer for starch extraction in the WP, whereas no respondents reported father to son transfer of knowledge for that task.

7.1.1 Extraction of sago starch and factors that can contribute to microbial contamination of the starch

The equipment and practises used to extract sago starch are similar in the two regions surveyed. Some of the practises could potentially contribute to microbial contamination of the sago starch. Following the felling of the sago palm, it is common to store the trunks prior to maceration of the pith and extraction of sago starch. In the ESP 93% (76–99%) of respondents store trunks prior to extraction, mostly dry (on the ground), but occasionally underwater. A similar trend was observed in the WP =, with 73% (50–89%) of respondents claiming to store sago trunks at least some of the time. Trunks might be stored in water in the wet season, but are more commonly stored on dry land. The remaining 27% (11–50%) of respondents from the WP generally do not store trunks between felling the palm and processing the sago starch.

Direct comparison of cleaning regimens of extraction equipment between the ESP and the WP is difficult, given the different level of detail obtained in the two s. In both s equipment is cleaned more often than not, although the difference is not statistically significant in the WP (Table 11.03, Appendix 11.1). In the ESP, equipment is usually cleaned to some degree.

The source of water used in the sago extraction process varies greatly, and is largely dependent on seasonal availability and proximity to the sago palm being processed. The vast majority of respondents in both s listed at least two common sources of water. In the ESP bore water and river water were commonly identified as the primary water source, with creek water and rainwater also used regularly. In the WP swamp water and river water are the most commonly used water sources, followed by lagoon water. When using swamp water a small well is dug to access the water just below ground level (Figure 7.01).

Figure 7.01: Example of a well dug in a sago swamp to access water for starch extraction. Note that the water is very close to the ground surface (within 50 cm).

In most villages pit toilets are employed as the primary mode of human faecal waste disposal (Table 11.04, Appendix 11.1), although some villages that are situated in close proximity to water have toilets that empty straight into the water. When at 'camping places' processing sago starch, the people of the WP rely heavily on promiscuous defaecation.

7.1.2 Storage of sago starch

The sociological survey demonstrated differences in sago storage preferences between the ESP and the WP (Table 11.05, Appendix 11.1). In the ESP the preferred storage method is in watertight containers. Traditionally clay pots have been used, but more recently saucepans and buckets have come into use. A total of 19% (6–38%) of all respondents from the ESP stated a preference for clay pots, and 19% (6–38%) for saucepans, buckets or similar. A further 47% (29–68%) of respondents did not disclose an individual preference for clay pots, saucepans or buckets. In total, 85% (66–96%) of respondents from the ESP region stated a preference for the aforementioned storage vessels. The remaining 15% (4–34%) of people from the ESP preferentially store sago starch wrapped in leaves.

In contrast, the vast majority (85%: 65–97%) of respondents in the WP preferentially store sago wrapped in leaves, with the remaining 15% (3–35%) opting for bags or baskets woven from natural fibres such as tree bark. The majority of people in the WP use woven bags, woven baskets and plastic bags as their second storage preference. In the ESP sago that is produced for sale at the market is usually stored in baskets, leaves or plastic bags.

In the ESP, lemon is used as an additive to sago starch during storage by 33% (17–54%) of respondents. It is said to keep the sago white. No data were collected on how commonly lemon is used, or how much is added.

Sago starch is typically made when required, and as such consumption of sago starch commences very soon after extraction, within a day in most cases. In both s surveyed, sago starch is typically kept for 2–4 weeks, but sometimes up to 2–3 months.

Sago starch that is no longer considered suitable for human consumption is commonly thrown out in both the ESP and the WP. Starch that is thrown out in the ESP is often fed to animals. Interestingly, almost one quarter of respondents from the WP stated that they only discarded stale sago if they had sufficient food supply. In times of food shortage old sago is likely to be consumed.

The most commonly used characteristic to determine suitability of sago starch for consumption is change of colour (Table 11.06, Appendix 11.1). However, the colour of sago starch varies considerably, so there is no definitive colour that signifies sago is unsuitable for consumption. A vast array of colours was used to describe stale sago in both s, including various shades of grey, brown, black, red and yellow. In the WP, 72% (50–89%) of respondents described stale sago as having some degree of yellow colouration, often in conjunction with other colours. One respondent from the same suggested black and brown spots were indicative of stale sago. Anecdotal evidence suggests that visible mould growth is more commonly used as an indicator of poor quality sago starch in the ESP than in the WP.

7.1.3 Sago starch preparation and consumption

In both the WP and ESP surveyed, a variety of methods are used to cook sago starch, but nonetheless, a clear trend is evident. In the ESP, the preferred method of cooking sago is by boiling, followed by cooking ‘flat bread’ in a frying pan. In the WP the frying pan method is clearly favoured, followed by sago wrapped in leaves and cooked over the fire. Only rarely is sago prepared by boiling in the WP. Other methods of preparation used include: placed inside bamboo or tree bark and cooked, rolled into balls and cooked in hot ashes, in a soup, or mixed with banana and served sweet as a cake.

Importance of sago starch as a food source

Sago starch remains the staple carbohydrate for the majority of respondents throughout lowland areas of the ESP and WP (Table 7.01). In most villages there has been no change in dependency upon sago starch as the dietary staple, but some villages described a decrease in dependency. In the ESP, this decrease was solely attributed to an increase in consumption of garden foods and purchased foods, namely rice and flour. While the same reason was given for a decrease in sago consumption by some respondents in the WP, equally as important was the decreased availability of sago palms.

Table 7.01: The significance of sago starch as a food source and changes in dependency in recent years. Results are presented as percentage of respondents, with 95% confidence limits in parenthesis.

	ESP (n = 27)	WP (n = 22)
Significance as food source		
Staple carbohydrate	81% (62–94%)	95% (77–100%)
Highly used carbohydrate source	19% (6–38%)	0% (0–13%)
Minor carbohydrate source	0% (0–11%)	5% (0–23%)
Change in dependency		
No change	70% (50–86%)	68% (45–86%)
Less consumed now than 10 years ago	22% (9–42%)	27% (11–50%)
More consumed now than 10 years ago	8% (1–24%)	5% (0–23%)

People of all ages eat sago starch, from the very young to the very elderly. In the ESP all respondents stated that infants from six months of age eat sago starch, whereas many respondents in the WP stated that infants are two years or older when they start eating sago starch. Differences in answers may be in part attributable to different personnel assisting with the survey in the different s, and differences in weaning ages of infants. Regardless, it is evident that young children commonly eat sago starch.

Cultural importance of sago starch

For some sago consumers, sago is an important food source, but is not highly regarded in a cultural sense. In the ESP the majority of respondents stated that sago was not culturally significant, but when asked about the role sago plays in ceremonies, usually stated that it was important, particularly at weddings and funerals. Many respondents from the WP acknowledged the cultural importance of sago starch, where it also plays an important role in ceremonies. Selected responses to cultural aspects of sago usage are given in Table 11.07, Appendix 11.1. These responses are not necessarily representative of all opinions and cultural beliefs in the respective, but provide an insight into the beliefs of some sago users. In the WP multiple respondents stated that the traditional prayers asking for “more than enough sago” are not as common as they once were, or are restricted to the older generation, as Christianity has taken over from traditional beliefs.

7.2 Microbial contamination and microbial ecology of sago starch

7.2.1 Bacteria

Total culturable aerobic bacteria

Standard plate counts were conducted to enumerate total culturable aerobic counts in sago starch. Bacteria were isolated from the vast majority of sago samples analysed.

Statistical analysis was conducted to determine whether any relationships existed between the log10 of total viable bacterial numbers and various parameters that might influence microbial growth in sago starch. Storage duration, storage pH and starch pH were considered. The data set did not lend itself to analysis for aw using ANOVA as there was at least one group with less than two cases. Table 7.02 shows the level of significance using ANOVA least significant difference post hoc analysis. The analysis shows an association between older samples and total viable bacteria, and also between low pH and total viable bacteria. There was no association between total culturable bacteria and province (analysis not shown).

Table 7.02: Statistical analysis of the influence of pH and storage duration on numbers of total culturable bacteria isolated from sago starch.

Parameter	Group 1	Group 2	Mean difference	Significance
Storage duration	< 1.0 week	≥ 5.0 weeks	-1.296	0.003
Storage duration	1.0–2.9 weeks	≥ 5.0 weeks	-2.187	0.011
Storage duration	3–4.9 weeks	≥ 5.0 weeks	-1.034	0.072
pH	< 4	4.00–4.99	1.589	0.005
pH	< 4	5.00–5.99	1.336	0.037
pH	< 4	6.00–6.99	1.274	0.065
pH	< 4	≥ 7.00	1.063	0.018

Total bacterial counts in sago starch were generally very high, typically in the range of 107 to 108 cfu/g, but up to 5.9 × 109 cfu/g. The vast majority of samples tested exceed the Indonesian standard for sago starch, as stated by Sopade (1999), of 106 cfu/g. The high numbers of total aerobic bacteria is indicative of the high level of contamination of sago

starch from the environment, and from the water used to extract sago. Statistical analysis suggests that the high counts might also be attributable to fermentation of sago starch. The analysis shows an association between low pH and high bacterial numbers. There is also an association between storage duration and bacterial numbers, with sago five weeks or older having higher total bacterial counts than samples that had been stored for a lesser duration.

It is difficult to assess the suitability of sago starch based on total bacterial counts alone, as such a count might represent potential pathogens, indicator organisms, harmless organisms and potentially beneficial organisms. However, tests for specific bacteria of public health significance suggest that pathogenic bacteria are present in sago starch. Statistical analysis suggests that in many cases there is an association between pH of the sample and growth of certain bacterial organisms. Although there was no association between storage method and pathogen numbers, storage method might influence other parameters.

Bacillus cereus

Bacillus cereus was present in 32 of the 43 sago samples tested. However, numbers were typically low, with less than 1.0 × 102 cfu/g being detected in 58% (25/43) of samples. A further 23% (10/43) of samples had between 1.0 × 102 cfu/g and 1.0 × 103 cfu/g. Thus, over 80% of all samples tested had less than 1.0 × 103 cfu/g of B. cereus.

Sago starch less than one week old had significantly lower numbers of B. cereus than that of older sago. Fresh sago has significantly less growth of B. cereus than sago stored in traditional woven containers or wrapped in leaves. There are significantly higher numbers of B. cereus in sago with a pH below 4.00 than in sago with a pH greater than 5.00. No correlation between province of origin and numbers of B. cereus was observed. Bacillus cereus was not detected in the implicated sago (Table 11.09, Appendix 11.2)

Clostridium perfringens and saccharolytic clostridia

Of the 16 samples tested for C. perfringens and saccharolytic clostridia, only three tested positive for C. perfringens and one positive for saccharolytic clostridia. The remaining 13 samples of C. perfringens had < 3 organisms/g, the lower limit of detection for the MPN method used. Saccharolytic clostridia were detected in only one sample, W0605-01 (the sample of sago implicated in the Suki outbreak of SHD). All other samples had <100 cfu/g, the lower limit for detection using the spread plate technique. All samples in which clostridia were detected had only low numbers. The data set was not suitable for the application of statistical analysis.

Enterobacteriaceae

Members of the family Enterobacteriaceae were commonly isolated from sago starch. Total coliforms, which comprise members of the family Enterobacteriaceae, along with bacteria of some other families, were commonly isolated from sago starch at the upper limits of detection. Faecal coliforms were also commonly isolated, and often at the upper limits for detection. In samples with high faecal coliform numbers, E. coli was common. Of the 69 samples tested for the presence of Salmonella spp, the analysis of five demonstrated the presence of the organism. None of the 13 samples tested was positive for E. sakazakii, and only one of the 13 samples, W0405-11, was demonstrated C. freundii after culture.

Test results from the analysis of the samples collected on the first four sample collection expeditions (samples S1102-01 to S1102-10, M1102-01, W0303-01 to W0303-20 and S0603-01 to S0603-20) demonstrate that total and faecal coliform numbers were often at the upper limit of detection (1.1 × 103 cfu/g). In surveying the samples collected on the subsequent collection expeditions, further dilutions were conducted, resulting in the upper

limit of detection being 1.1 × 104 cfu/g for samples W0404-01 to W0404-22 and 1.1 × 105 cfu/g for samples W0405-01 to W0405-12.

Statistical analysis was done to determine whether an association existed between the presence or absence of total coliforms, faecal coliforms, E. coli and Salmonella spp. with parameters such as the following: the the sago was collected from (WP compared to ESP), the age of the sago sample at time of collection (< 3 weeks compared to ≥ 3 weeks), the pH of the sample (< 6 compared to ≥ 6), the aw of the sample (< 0.997 compared to ≥ 0.997) and storage of the samples (stored in leaves or woven bags compared to stored in plastic bags and other less common methods). All analysis was done using Fisher's exact test. Table 7.03 demonstrates the results of the analysis that was conducted. An association exists between the age of the sample and total coliforms and E. coli, and between the pH of the sago and faecal coliforms/E. coli. There were no other statistically significant correlations (P < 0.05) between organisms and the parameters tested.

Table 7.03: Significance levels using Fisher's exact test of the relationship between presence/absence of total coliforms, faecal coliforms, E. coli and Salmonella spp. with of origin for sago starch, the age of the sample at time of collection, the pH of the sample, and the water activity of the sample.

	Total coliforms	Faecal coliforms	E. coli	Salmonella
	P = 0.606	P = 0.583	P = 0.276	P = 0.375
Age of sample	P = 0.032	P = 0.080	P = 0.007	P = 0.605
Sago pH	P = 0.053	P = 0.037	P = 0.007	P = 0.197
Water activity	P = 0.366	P = 0.275	P = 0.486	P = 0.792
Storage	P = 0.557	P = 0.447	P = 0.509	P = 0.087

Listeria monocytogenes

Listeria monocytogenes was not detected in any of the 57 samples tested.

Coagulase positive staphylococci

Coagulase positive Staphylococcus spp (S. aureus) were isolated from 12 of 57 (21%) sago samples. The remaining 45 samples had < 1.0 × 102 cfu/g, the lower detection limit of the plate count method used. Appendix 3.5 demonstrates the number of colony forming units of coagulase positive staphylococci per gram of sago starch.

Statistical analysis was done to determine whether an association existed between the presence or absence of coagulase positive staphylococci with parameters such as the province the sago was collected from, the age of the sago sample at time of collection, the pH of the sample and the aw of the sample. Using Fisher's exact test (P < 0.05), no association was detected between the presence/absence of S. aureus and age (P = 0.543), water activity (P = 0.596), (WP compared to ESP , P = 0.099) or storage (stored in leaves or woven bag compared to plastic bags P = 0.425). An association was found for the presence of S. aureus with 'neutral' pH (for pH > 6.00, P = 0.043).

7.2.2 Yeasts, moulds and actinomycetes

Enumeration of yeasts and moulds and statistical analysis

Of the 69 sago samples analysed (excluding the implicated sago), yeasts were isolated from all but 2 samples, while moulds were present in all but 5 samples. In general yeast numbers were higher than mould numbers (t = 3.925; df = 70; P = 0.000).

Analysis of numbers (log10) suggested that yeast numbers increased over the first few weeks of storage, then decreased in sago stored for over five weeks. Yeast numbers were significantly lower in samples greater than five weeks compared to samples aged one to three weeks (F = 2.32, df = 3, P = 0.014). However, there was no significant difference

between yeast numbers in samples one to three weeks old compared to samples three to five weeks old (F = 2.32, df = 3, P = 0.170), or in sago samples one to three weeks old than in samples less than one week old (F = 2.32, df = 3, P = 0.067).

Sago samples stored in natural woven containers had significantly more yeasts than fresh sago (F = 4.44, df = 4, P = 0.020) and sago categorised as 'other and unknown' (including in earthenware pots and saucepans covered with water and smoked samples) (F = 4.44, df = 4, P = 0.000). Sago samples in the 'other and unknown' category also had lower yeast numbers than sago starch stored in leaves (F = 4.44, df = 4, P = 0.004) and plastic bags (F = 4.44, df = 4, P = 0.023).

Yeast numbers were lower at pH < 4.0 compared to all other pH categories, although the only value that reached statistical significance (to P = 0.05) was the comparison between the pH < 4.0 and pH 4–4.99 (F = 2.68, df = 4, P = 0.002). Yeast numbers were greatest within the pH range 4–4.99, although there was no significant difference between numbers in this category compared to higher pH categories (5.00–5.99; 6.00–6.99; > 7.00).

Mould numbers increased in old sago. Samples greater than five weeks old had significantly higher mould numbers than samples less than one week old (F = 3.76, df = 3, P = 0.004) and samples one to three weeks old (F = 3.76, df = 3, P = 0.016). Sago samples aged between three and five weeks also had significantly higher mould numbers than sago less than one week old (F = 3.76, df = 3, P = 0.046).

Analysis of mould numbers subjected to different storage techniques shows significantly higher mould numbers in sago stored in natural fibre woven containers compared to the other storage techniques (F = 4.52, df = 4; fresh P = 0.018; leaves P = 0.003; plastic P = 0.010; other, unknown P = 0.001).

Statistical analysis suggested that starch pH had little effect on mould numbers. Mould numbers were highest in sago starch with pH < 4, but there was no statistical significance between this low pH category and the other categories.

Analysis of the effect of aw on yeast or mould numbers could not be done using analysis of variance, as there was at least one group with fewer than two cases for both yeasts and moulds.

Identification and prevalence of mould genera

Moulds were identified to genus level or beyond where possible. Table 7.04 demonstrates the prevalence of the fungal genera identified in sago starch. The genus Penicillium Link was present in over 60% of sago samples tested. Other genera of moulds to occur in numerous sago samples were Scytalidium Pesante (30 %) and Aspergillus Link (24 %). Numerous genera, including Aureobasidium Viala and Boyer, Acremonium Link, Fusarium Link, Cladosporium Link, Trichoderma Pers. and Cylindrocarpon Wollenw. were isolated from 10% or more of samples.

Table 7.04: Prevalence of individual mould genera from sago samples collected from the ESP (S1 and S2) and the WP (W1,W2 and W3).

Genus	S1 (11)	W1 (16)	S2 (15)	W2 (16)	W3 (12)	Total (70)	Prevalence (%)
Penicillium	6	10	7	11	9	43	61
Scytalidium	5	6	7	0	3	21	30
Aspergillus	7	3	2	3	2	17	24
Aureobasidium	2	0	4	9	0	15	21
Acremonium	3	2	4	4	1	14	20
Geotrichum-like	1	4	7	1	1	14	20
Fusarium	4	3	0	4	2	13	19
Cladosporium	3	3	4	0	2	12	17
Trichoderma	1	2	2	4	1	10	14

Cylindrocarpon	0	5	0	0	2	7	10
Paecilomyces	0	3	1	1	0	5	7
Curvularia	1	0	1	0	1	3	4
Aphanocladium-like	0	0	0	2	1	3	4
Scopulariopsis	0	0	0	1	1	2	3
Gliomastix	1	1	0	0	0	2	3
Phialophora	0	1	0	0	1	2	3
Zythia	1	0	0	0	0	1	1
Pestalotia	1	0	0	0	0	1	1
Chrysosporium	1	0	0	0	0	1	1
Moniliella	1	0	0	0	0	1	1
Stachybotrys	0	1	0	0	0	1	1
Helicostylum	0	1	0	0	0	1	1
Talaromyces	0	1	0	0	0	1	1
Humicola	0	0	1	0	0	1	1
Absidia	0	0	0	1	0	1	1
Cephalosporiopsis	0	0	0	1	0	1	1
Neurospora	0	0	0	1	0	1	1

Legend: S1: Samples collected from the ESP, November 2002; S2: Samples collected from the ESP, June 2003; W1: Samples collected From the WP, March 2003; W2: Samples collected From the WP, April 2004; W3: Samples collected From the WP, April 2005

Although both Aureobasibium and Geotrichum-like are yeasts, they commonly exist in a filamentous state, so were enumerated with the moulds. Aureobasibium spp. were isolated from approximately 21% of samples. Geotrichum and Geotrichum-like organisms were isolated from 20% of samples surveyed using DRBC agar.

Other genera isolated from sago starch that could to be identified to genus level include: Paecilomyces Bain.; Curvularia Boedijn; Scopulariopsis Bain.; Gliomastix Guéguen; Phialophora Medlar; Zythia Fr.; Pestalotia de Not.; Chrysosporium Corda; Moniliella Stolk and Dakin; Stachybotrys Corda; Helicostylum Bain.; Talaromyces Benjamin; Humicola Traaen; Absidia van Tieghem; Cephalosporiopsis Moreau; and Neurospora Shear and Dodge.

Statistical analysis was conducted using Fisher's exact test to determine whether there was an association between the presence/absence of the most commonly occurring moulds (those with a prevalence of ≥ 10%) and storage duration, storage technique, starch pH, starch aw and the from which the sago sample was collected. In general, there was no association between any of these factors and the presence/absence of any of the moulds analysed. The only exceptions were for Geotrichum spp. and Geotrichum-like fungi, which were not so commonly isolated from the WP (P = 0.037, one sided) and Cylindrocarpon spp., which was isolated only from the WP (P = 0.033, one sided).

7.2.3 Enumeration of mucoraceous moulds

Growth was observed on some SMA, but no mucoraceous moulds were isolated that were not also isolated from DRBC agar.

7.2.4 Enumeration of Geotrichum species

Numbers of Geotrichum spp. were generally low, being below the limit of detection (1.0 × 102 cfu/g) for the majority of sago samples. Geotrichum spp. were detected in nine sago samples using Geotrichum selective agar; four of which were from the ESP and five from the WP . The highest count was 7.4 × 104 cfu/g.

7.2.5 Enumeration of actinomycetes

No actinomycetes were isolated from sago starch using actinomycete agar.

7.2.6 Ergosterol analysis of sago starch

Ergosterol was detected in all but two sago samples analysed. The lower limit for detection was 0.10 µg /g of sago starch. A Pearson correlation test was conducted between yeast and ergosterol and mould and ergosterol. The correlation at the P = 0.001 level was 0.742 for yeast and 0.629 for moulds.

An ANOVA was conducted to determine whether correlations between ergosterol and storage duration, storage technique, pH, and aw existed. Samples greater than five weeks old had significantly higher ergosterol levels than all the fresher categories (F = 0.221, df = 4; < 1 week: P = 0.025; 1–3 weeks P = 0.019; 3–5 weeks P = 0.042). There was negligible association between ergosterol levels and storage technique, the only statistically significant difference (P = 0.05) occurred between sago starch stored in leaves compared to sago in the other and unknown category (F = 1.71, df = 4, P = 0.029). Analysis could not be conducted for pH or aw due to the fact that for both parameters at least one group had fewer than two cases.

7.2.7 Overview of fungal contamination of sago starch

Sago starch stored in bags or baskets made of natural woven fibre had significantly higher mould numbers than sago stored by other techniques. The small holes in the bags and baskets favour colonisation by moulds in two main ways. They allow the flow of air into the sago starch, making it a more hospitable environment for moulds, and they allow airborne spores to colonise the starch. Moreover, bags and baskets are often reused, so they may act as a source of inoculum. Sago starch that is wrapped tightly in leaves or packed tightly in a plastic bag is more likely to remain anaerobic and have a barrier to airborne contamination by filamentous fungi.

The statistical difference between mould numbers in woven containers compared to the ‘other and unknown’ category was highly significant. Many of the samples in the ‘other’ component were stored in pots and covered with water. The majority of the ‘unknown’ samples were collected in the ESP, where storage in pots is most common. It is expected that this storage technique would be the most successful at inhibiting mould growth, providing both a good physical barrier to spore contamination, and highly anaerobic conditions. Due to the undefined nature of the category ‘other and unknown’ it can only be speculated at this stage that the ESP method of storage in pots and immersion in water is optimal for prevention of mould growth. This theory will be further explored in Chapter 9. As with yeast numbers, the lower number of moulds in fresh (un-stored) sago is indicative of lack of opportunity for the fungi to grow and sporulate.

Penicillium spp. and Aspergillus spp. were among the most commonly isolated moulds in sago starch, present in 61% and 24% of samples respectively. Fusarium spp. were present in approximately 19% of samples tested. These genera are ubiquitous in distribution and are the dominant fungi associated with food contamination and mycotoxin production (Hocking and Pitt, 2003). Other genera isolated from sago starch have also been associated with mycotoxin production, including Trichoderma, Cylindrocarpon, Stachybotrys and Talaromyces (Frisvad and Thrane, 2000; Bennett and Klich, 2003). While the presence of mycotoxigenic genera does not necessarily equate to the presence of mycotoxins, such contamination is still undesirable and constitutes a potential health risk.

7.2.8 Microbial ecology of sago starch

Comparison of the effect of selected traditional storage techniques on microbial communities

The effect of three traditional storage techniques on numbers of total viable bacteria, LAB, yeasts, moulds, mucoraceous fungi and haemolytic organisms in sago starch was studied over seven weeks. No mucoraceous fungi were isolated from sago stored under any of the three conditions tested. The enumeration of haemolytic microorganisms proved to be difficult in mixed culture, as was found to be the case in other experiments (see Section 7.3.1.1). Haemolytic bacteria were noted in some samples, but numbers were low (generally 102–103 cfu/g).

Figure 7.02 shows the number of total viable bacteria isolated from sago starch stored using three different traditional techniques. The smoked sago starch initially had considerably lower total bacterial numbers, but after 1 week numbers were comparable to the other two storage methods. After 6 weeks total bacterial numbers decreased slightly in smoked sago and in sago stored in earthenware pots with water, while bacterial numbers in sago stored wrapped in sago leaves remained high.

Figure 7.02: Number of total viable bacteria (cfu/g) over 6 weeks in sago stored wrapped in sago leaves (sago bundle), smoked sago and sago stored in an earthenware pot covered with water.

A similar trend (as observed for total viable bacteria) was evident for LAB (Figure 7.03). Initially, numbers of LAB were low in the smoked sago, but after 1 week all storage techniques gave rise to comparable numbers. Numbers of LAB remained comparable in all storage techniques over the duration of the experiment.

Figure 7.03: Number of LAB (cfu/g) over 6 weeks in sago stored wrapped in sago leaves (sago bundle), smoked sago and sago stored in an earthenware pot covered with water.

As for total bacteria and LAB, yeast numbers were initially low in the smoked sago, but had increased after 1 week (Figure 7.04). There were few differences among yeast numbers in any of the storage methods between 1 week and the conclusion of the experiment.

Figure 7.04: Number of yeasts (cfu/g) over 6 weeks in sago stored wrapped in sago leaves (sago bundle), smoked sago and sago stored in an earthenware pot covered with water.

The trend observed for moulds was different to the trends observed for other microorganisms. Initially, mould numbers were similar (between 102 and 104 cfu/g) for all storage techniques (Figure 7.05). However, over the following 2 weeks mould numbers increased in sago wrapped in leaves and smoked sago, but decreased in sago stored in earthenware pots with water. In the latter storage method, mould numbers remained low at week 3, but by week 6 had increased by approximately three log cycles to have a similar level of growth as sago stored under the other two methods.

Figure 7.05: Number of moulds (cfu/g) over 6 weeks in sago stored wrapped in sago leaves (sago bundle), smoked sago and sago stored in an earthenware pot covered with water.

Further work is required to gain greater understanding on the influence of traditional storage methods on both beneficial (fermentative) and undesirable microbial populations in sago starch.

7.2.9 Survival of bacterial pathogens in sago starch

The MPN method was used to determine the survival of all bacterial pathogens in sago starch except S. aureus. Consequently, in the cases where the most probable number was outside the upper or lower limit of detection, it is not possible to determine mean or standard deviation of the triplicate samples. Table 7.05 show the results of pathogen survival in both fresh and previously stored sago as accurately as possible given the aforementioned limitation.

In fresh sago there was a decrease in pathogen numbers throughout the course of the experiment. Escherichia coli was able to survive in sago starch for the duration of the experiment, with numbers remaining above the upper limit of detection for the first 10 days. Numbers gradually decreased on days 14 and 21. Salmonella sp. was still detectable at very low levels on day 7, but was not detectable thereafter. Bacillus cereus did not survive well in sago starch: numbers were low on the day of inoculation and remained low on day 3. It could not be detected from day 7 onwards. Despite high numbers of S. aureus being detected on the day of inoculation, the organism was not detectable on day 3 of the experiment, or subsequent days. Listeria monocytogenes survived in low numbers until day 3, but was not detected from day 7 onwards.

None of the organisms tested survived well in previously stored sago. Both E. coli and S. aureus were detected in low numbers after 2 weeks, despite being undetectable after 1 week. Salmonella sp. and L. monocytogenes were not detected after the first day. Bacillus cereus was not detected on the day of inoculation, but was detected in very low numbers over the following 2 weeks. None of the pathogens was detected in previously stored sago starch at day 21.

Table 7.05: Estimated bacterial counts per gram of sago for various bacterial pathogens in sago starch over three weeks. Sago starch was seeded with approximately 1.0 × 104 cfu/g of each pathogen on day 0.

	Day 0	Day 3	Day 7	Day 10	Day 14	Day 21
Fresh sago						
E. coli	>1.1 × 104	>1.1 × 104	>1.1 × 104	>1.1 × 104	~8.9 × 103	6.0 × 102
Salmonella sp.	~3.9 × 103	2.9 × 102	<3.6 × 101	ND	ND	ND
B. cereus	<3.6 × 101	<3.6 × 101	ND	ND	ND	ND
S. aureus	2.1 × 105	ND	ND	ND	ND	ND
L. monocytogenes	~8.1 × 103	<2.3 × 102	ND	ND	ND	ND
Stored sago						
E. coli	1.2 × 101		ND		1.4 × 102	ND
Salmonella sp.	1.0 × 101		ND		ND	ND
B. cereus	ND		1.3 × 101		< 4	ND
S. aureus	1.5 × 103		ND		1.0 × 102	ND
L. monocytogenes	3.1 × 102		ND		ND	ND

Legend: > All triplicate samples were above the upper limit of detection; ~ One of the triplicate samples was above the upper limit of detection, but the other two samples were within the detection range; < One or two of the samples were below the detectable limit and the remaining samples were within the detectable range; ND Not detected. All triplicate samples below the lower limit of detection

The water activity of fresh sago starch remained high throughout the course of the experiment, with the lowest recorded value being 0.997. The pH steadily decreased as the experiment progressed. At the commencement of the experiment the sago starch was pH 6.60, but the mean pH dropped to 4.64 ± 0.31 on day 21.

7.2.10 The fermentation of sago starch

Table 7.06 shows LAB numbers, pH and acid concentrations for the 12 samples of sago starch tested. Lactic acid bacteria were isolated from all 12 samples. Numbers ranged from 3.9 × 104 cfu/g to 6.7 × 107 cfu/g. Acids were also detected in all samples. Acetic acid and propionic acid were present in all 12 samples, while lactic acid was detected in 6 of the samples. Only one sample was positive for iso-butyric acid, and two samples were positive for iso-valeric acid. The values given in Table 7.06 are for the combined iso- and n- forms of these two acids, but more strongly reflect the n- form. Butyric acid was detected in nine samples, in most cases at higher levels than lactic acid. Valeric acid was detected in six samples, but always at low levels. No pyruvic acid was detected in any of the sago samples.

Table 7.06: Enumeration of LAB, pH values and detection of various acids in sago starch.

Sago		LAB	Acid analysis (mM/kg sago starch)				
sample	pH	cfu/g	Acetic	Propionic	Butyric	Valeric	Lactic
W0405-01	4.18	6.7 × 107	18.54	2.79	4.29	0.16	4.09
W0405-02	4.98	1.3 × 107	10.75	7.39	1.04	0.00	0.00
W0405-03	4.94	4.8 × 105	4.52	2.28	11.63	0.35	0.46
W0405-04	4.29	2.0 × 107	8.82	11.59	8.46	0.83	1.69
W0405-05	5.15	1.9 × 107	2.71	3.83	1.66	0.23	0.00
W0405-06	6.16	3.9 × 104	2.25	0.69	0.00	0.00	0.00
W0405-07	6.62	7.3 × 106	1.82	0.56	0.00	0.00	0.00
W0405-08	6.40	5.8 × 105	5.86	1.22	1.52	0.00	0.00
W0405-09	4.63	5.7 × 106	3.74	3.35	18.73	0.88	0.00
W0405-10	4.60	3.2 × 106	7.80	3.98	1.32	0.09	0.35
W0405-11	6.81	3.6 × 104	2.06	0.35	0.00	0.00	0.45
W0405-12	4.18	1.2 × 107	19.34	1.36	0.07	0.00	3.84

Regression analysis was conducted to determine whether any correlations existed between pH, total acid concentration and LAB numbers. Using Pearson's correlation coefficient (two sided), a strong correlation was observed between starch pH and log total acid (n = 12, r2 = 0.846, P = 0.000). Weaker correlations were observed between log LAB and pH (n = 12, r2 = 0.489, P = 0.01) and log LAB and total acid (n = 12, r2 = 0.424, P = 0.02)

The incubation of fresh sago starch at 30 ºC in airtight containers resulted in a steady decrease in pH, and an initial rise in LAB and yeast numbers (Figure 7.06). There were approximately 105 cfu/g of LAB at the commencement of the experiment, rising to approximately 107 cfu/g after 24 hours of incubation. Numbers remained constant at just below 107 cfu/g until day 23. No LAB were detected on day 30 (< 102 cfu/g). A similar trend was noted in yeasts, where numbers increased two log cycles (106–108 cfu/g) within the first 2 days of incubation. Yeast numbers were not as stable as LAB numbers, but remained high (~107 cfu/g) up to day 23 of the experiment. On day 30, the final day of the experiment, yeast numbers had decreased to around 105 cfu/g.

Figure 7.06: Colony forming units of LAB and yeasts in actively fermenting sago starch.

The fermenting sago starch was tested for the presence of various bacterial pathogens and filamentous fungi. Bacillus cereus, L. monocytogenes, Salmonella spp. and S. aureus were not detected. Faecal coliforms and E. coli were present in high numbers for much of

the experiment. During the first 2 weeks numbers of both faecal coliforms and E. coli were over the upper limit of detection, 1.1 × 104 organisms/g. On day 16, E. coli was present in very low numbers only. On days 23 and 30 it was not detected. Numbers of faecal coliforms also decreased late in the experiment, and were not detectable on day 30. Numbers of filamentous fungi remained low throughout the experiment, never exceeding 5.0 × 102 cfu/g (Table 11.10, Appendix 11.2).

Concentrations of propionic acid, iso-butyric acid, iso-valeric acid, n-valeric acid and pyruvic acid were low in fermenting sago. The maximum level of propionic acid was detected at day 12 (7.83 mM/kg), decreasing slightly to 5.09 mM/kg by day 30. For the other minor acids, concentrations reached their maximum at either day 23 (pyruvic acid, 3.33. mM/kg), or day 30 (iso-butyric acid, 1.71 mM/kg; iso-valeric acid, 2.36 mM/kg; n-valeric acid, 6.40 mM/kg). Levels of acetic acid, n-butyric acid and lactic acid were also low in the first week of fermentation, but increased substantially late in the experiment (Figure 7.07).

Figure 7.07: Levels of acetic acid, n-butyric acid and lactic acid in fermenting sago starch.

At the commencement of the experiment, the pH of the sago starch was 4.93 ± 0.06. The pH increased slightly in the early stages of the experiment, and was 5.47 ± 0.63 on day 4. From that point onwards the pH decreased, reaching 3.07 ± 0.15 on day 30. A correlation was observed between log total acid concentration and pH of sago starch (n = 11, r2 = 0.920, P = 0.000).

7.3 Mycotoxin contamination and characterisation of haemolytic compounds

7.3.1 Presence of common mycotoxins in sago starch

Fifty-one samples were tested for the presence of aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2, ochratoxin A, cyclopiazonic acid, sterigmatocystin, citrinin and zearalenone. All samples were negative for all mycotoxins tested.

7.3.2 Production of mycotoxins by fungi in pure culture isolated from sago starch

All 21 fungal isolates tested were negative for the production of ochratoxin, cyclopiazonic acid, penicillic acid and patulin under the test conditions. However, 14 isolates were positive for citrinin production, and 1 isolate was positive for sterigmatocystin. Citrinin concentrations were estimated by visual comparison of intensity of spots with that of standards of known concentrations. The comparison was performed at levels within one order of magnitude of the detection limits, and estimates were repeated and checked between two operators to improve precision (Table 11.11, Appendix 11.3).

Despite evidence of fungal contamination of sago starch none of the common mycotoxins screened for in this study was present in sago starch. There are a number of factors that contribute to the lack of mycotoxins in sago starch. First, only a small percentage of fungal species produce these common mycotoxins. Although many of the commonly isolated genera of fungi from sago are associated with mycotoxin production, mycotoxin synthesis is often species specific. For example, only 3 of approximately 100 species of the genus Aspergillus are known to produce aflatoxins (Hocking and Pitt, 2003). Moreover, the synthesis of any given mycotoxin is dependent not only on the species of fungi, but also the strain (Sweeney and Dobson, 1998).

Secondly, mycotoxigenic fungi do not produce toxic secondary metabolites under all conditions. There are many environmental factors that influence toxin synthesis including temperature, water activity, pH and nutrient availability (Sweeney and Dobson, 1998; Kozlovskii et al., 2000; Pitt et al., 2000). Optimal requirements for mycotoxin production are likely to be dependent on a combination of parameters, but to date most studies have concentrated on the effect of just one or two factors.

From a human perspective, sago starch is generally considered a good source of dietary carbohydrates, but is otherwise nutritionally poor (Vaughan and Geissler, 1997). One may speculate that the low level of trace elements in sago starch might be one factor that contributes to the general absence of classical mycotoxins.

7.3.3 Extraction of the haemolytic component produced by filamentous fungi

Figures 11.01 to 11.10, Appendix 11.2, demonstrate comparisons between the haemolytic activity of hexane-rich extracts and dichloromethane-rich extracts for the various controls, and the seven fungal strains isolated from sago starch.

Wheat used as the culture medium had very little if any haemolytic activity (Figure 11.01). Low levels of haemolytic activity were evident in the dichloromethane-rich extract in which 0.25 g of wheat was assayed, reaching approximately 40% after 4 hours of incubation. Both hexane-rich extracts and the dichloromethane-rich extract with 0.10 g of material were all non-haemolytic over 4 hours, producing very similar haemolysis in the assay as negative controls.

The positive control S. chartarum produced strong haemolytic activity, with 0.1 g culture equivalent in the hexane-rich fraction reaching 80% after 4 hours (Figure 11.02). The dichloromethane-rich fraction was less than half as active, reaching 80% after 4 hours with 0.25 g of culture equivalent. The T. reesei positive control produced a similar pattern and level of activity, also greater in the hexane-rich fraction.

Two P. steckii isolates from sago produced at least twice the haemolytic activity of the positive control isolates (Figures 11.04 and 11.05). Hexane-rich extracts of isolates W1-1101 and W1-1301, containing 0.1 g culture equivalent, reached 100% activity after only 1 hour and dichloromethane-rich extracts of 0.1 g equivalent exceeded 80% haemolysis after 4 hours.

Strong haemolytic activity was also observed in the other fungal strains tested (Figures 11.06 to 11.10), with all but T. virens W4-0119 causing approximately 80% haemolysis or higher within 30 min of incubation with erythrocytes. Hexane fractions were more rapidly haemolytic than dichloromethane fractions except in P. steckii S2-1305 (Figure 11.06) and F. semitectum S2-0207 (Figure 11.09). In the case of these two isolates there was very little difference in the haemolytic activity of hexane-rich and dichloromethane-rich extracts for 0.25 g culture equivalent, but there was more activity in the 0.1 g equivalent dichloromethane-rich extract than the corresponding hexane-rich extract.

Direct comparisons of haemolytic activity of TDS extracts with hexane and dichloromethane extracts were not possible, as different quantities of culture material were used in the TDS assays. However, extrapolation suggested that for equivalent quantities of culture material, hexane and dichloromethane extracts had comparable haemolytic activity to the TDS extracts. Further studies were conducted using solvent extraction rather than TDS in an attempt to garner more information about the chemical properties of the haemolytic compound(s). Moreover, the evaporation of solvents such as hexane and dichloromethane can be carried out with relative ease compared to TDS, and the dried extract resuspended for use in the haemolytic assay.

7.3.4 Separation of haemolytic components of fungal culture extracts using preparative layer chromatography

Due to their greater haemolytic activity in most isolates, hexane-rich extracts were selected for further partitioning (Figures 11.11 to 11.20, Appendix 11.2). In all isolates, there was substantially more haemolytic activity in the upper section (above citrinin) of the PLC plate than in the lower section (citrinin and components below). The isolates with most activity in the previous experiments, P. steckii W1-1101 and P. steckii W1-1301, were also highly active in this experiment. On a weight basis, there appeared less activity in each of these two fractions than in the parent hexane-rich extracts, which could indicate either splitting of activity between upper and lower sections, or simply failure to recover all activity from the PLC plates. In support of the former possibility was the activity in the lower fractions of these two P. steckii isolates. The S. chartarum control also had some activity in the lower band.

The haemolytic activity of T. virens W4-0119 was notably slower to express than for the other fungal strains isolated from sago starch, taking 4 hours to reach approximately 80% haemolysis. All other strains originating from sago had reached this level of haemolysis after 2 hours. However, the haemolytic activity of T. virens W4-0119 was still comparable to the positive control strain of the same genus, T. reesei ATCC 26921.

7.3.5 Testing for the presence of haemolytic activity in sago starch using hexane extraction and quantitative assay

Figure 7.08 illustrates the haemolytic activity of four sago samples, two of which (W0605-01 and W1105-01) were associated with outbreaks of SHD. In general, low levels of haemolytic activity were detected in sago starch. The two sago starch samples associated with SHD outbreaks were less haemolytic than the two other samples shown, although standard errors were very high. The remaining nine samples tested had levels of haemolytic activity lower than that of sago sample W0303-02 (data not shown). Using Pearson's correlation coefficient (two sided) there was no correlation between age of sample and level of haemolytic activity ($n = 12$, $r = 0.671$, $P = 0.137$).

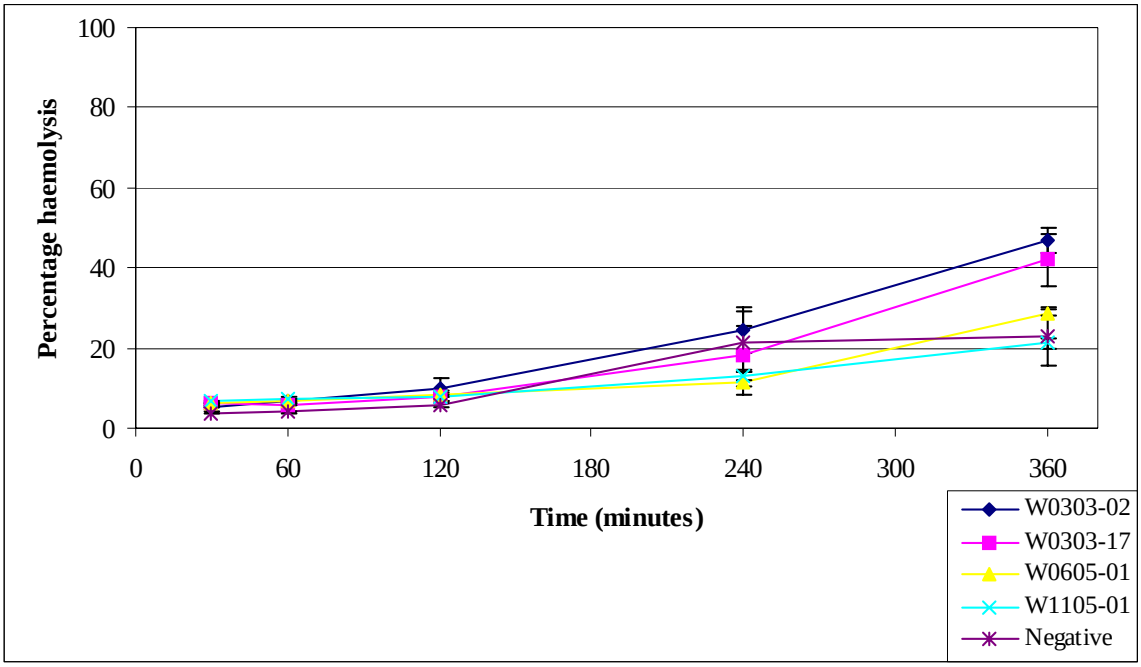

Figure 7.08: Haemolytic activity of hexane-rich extracts of sago starch (1.0 g equivalent).

7.3.6 Further separation of haemolytic component(s) of Penicillium (steckii) citrinum

Penicillium (steckii) citrinum W1-1301 was selected for further separation of haemolytic component(s). Selection of this isolate was on the basis that it represented a species commonly isolated from sago starch, and the majority of the haemolytic activity was in the hexane-rich fraction. Furthermore, when standard error was taken into account, there was clearly greater haemolytic activity in 0.25 g of the upper PLC section than in the corresponding lower PLC section (Figure 11.15, Appendix 11.2). Haemolytic components of P. (steckii) citrinum were purified by activity based fractionation by sequential liquid-liquid extraction, SPE, Prep TLC and HPLC. At each stage, fractions with the highest activity in the haemolytic assay were selected and subjected to further purification (Figures 7.09 and 7.10). The SPE 3 fraction was determined to be most haemolytic (Figure 7.09) and was subject to further separation. The desired haemolytic components were determined to be hydrophobic and non-polar with little UV absorbance and final separation were based on evaporative light scattering detection (ELSD).

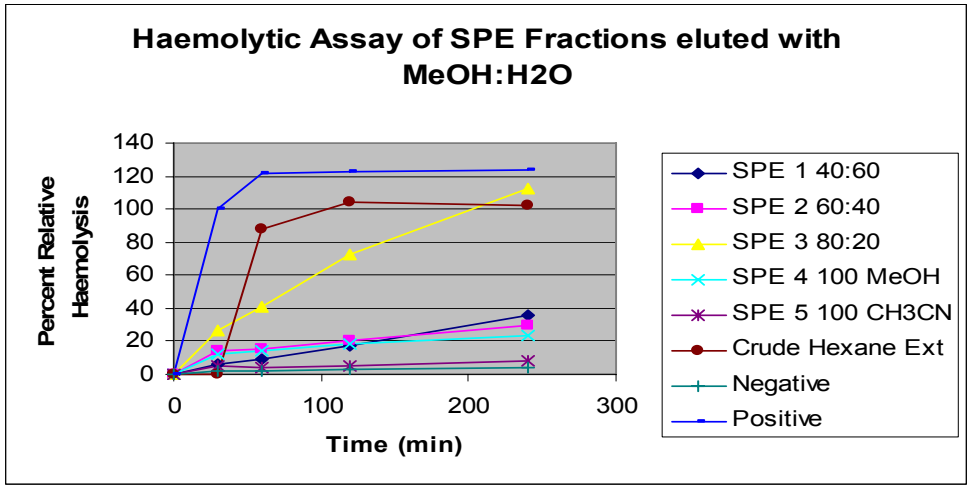

Figure 7.09: Haemolytic Bioassay of SPE C18 fractions (MeOH:H20 elution) from Penicillium (steckii) citrinum (W1-1301) extract. SPE fraction 3 with highest activity was selected for Prep TLC separation.

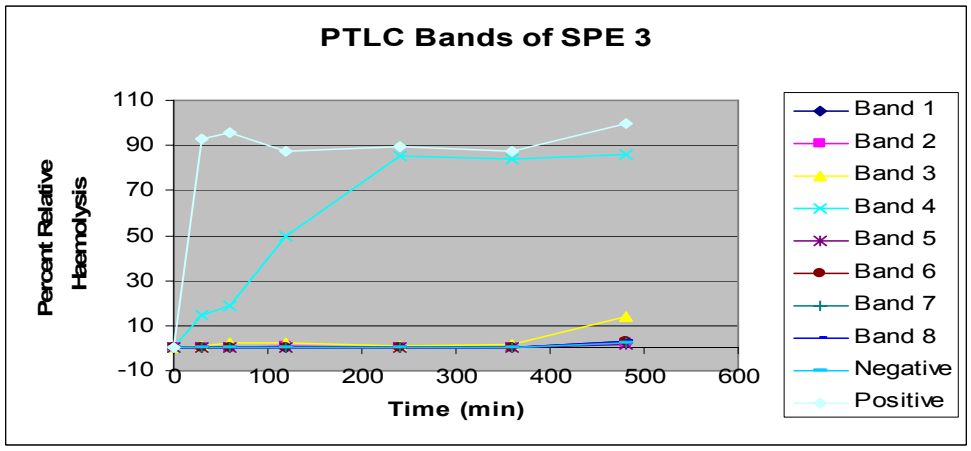

Figure 7.10: Haemolytic Bioassay of Prep TLC Bands (from SPE 3 fraction). TLC Band 4 with highest activity was selected for further HPLC separation

Initial indications were that the hydrophobic fraction was contaminated by free fatty acids, but as purification progressed it became apparent that the free fatty acids were themselves the haemolytic agents. High performance liquid chromatography separation of active TLC Band 4 provided three HPLC fractions containing fatty acids all of which had haemolytic activity. LCMS and GCMS analysis demonstrated the presence of long chain free fatty acids ranging from C16 to C20 in these active HPLC fractions. Fatty acids in decreasing order of abundance were identified by FAME analysis as C18:2n-6, C18:1n-9, C16:0, C18:0, C18:3n-3 and C20:1n-9 by comparison with authentic methyl esters (Figure 7.11). 1H NMR analysis showed spectra consistent with mixtures of saturated and unsaturated fatty acids (Figure 7.12). The free fatty acid profile shown in Figure 7.11 is consistent with total fatty acid profiles attributed to similar fungi (Stahl & Klug, 1996). Total fatty acid compositions have been shown to be specific to each fungi species and aid in species identification and differentiation (Stahl & Klug, 1996; Guarro et al., 1999; Karlinski et al., 2007).

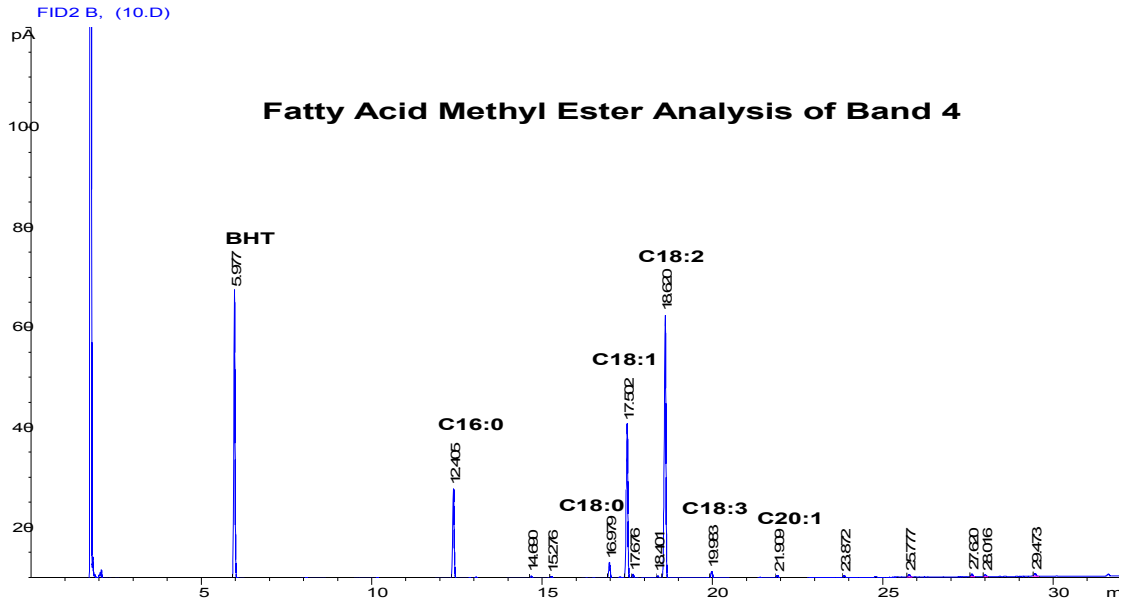

Figure 7.11: Fatty acid methyl ester (FAME) analysis of prep TLC fraction 4.

To demonstrate that the haemolytic activity was fully attributable to fatty acids and not some underlying component, the crude Penicillium (steckii) citrinum extract was separated by amino solid phase extraction into neutral lipids (NL), free fatty acids (FFA) and polar lipids (PL) by literature procedures (Kaluzny et al., 1985; Bateman Ii & Jenkins, 1997). Haemolytic bioassays demonstrated that haemolytic activity was predominantly present in FFA fraction with little in NL or PL fractions (Figure 7.13).

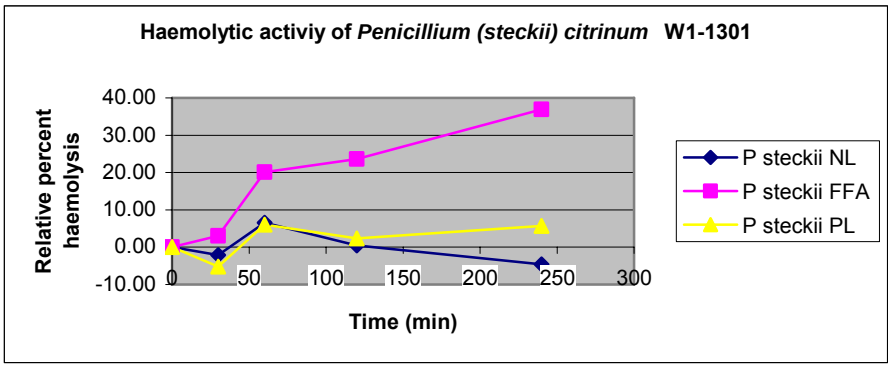

Figure 7.12: Haemolytic bioassay of neutral lipids (NL), free fatty acids (FFA) and polar lipids (PL) from Penicillium (steckii) citrinum extract.

Free fatty acids in low concentrations have long been known to protect or stabilize human erythrocytes against hypotonic haemolysis, but at high concentrations cause direct haemolysis (Longini & Johnson, 1943; LeVeen et al., 1965; Seeman, 1966; George et al., 1979; Csordas & Schauenstein, 1986; Lovstad, 1986; Rybczynska & Csordas, 1989). This FFA haemolysis of red cells is generally attributed to changes in cell permeability associated with detergent-like activity of fatty acids. Free fatty acids have been identified as haemolysins across a range of organisms such as flatworm eggs associated with schistosomiasis (Asahi et al., 1984), in microalgae (Fu et al., 2004) and in the blood sucking stable fly (Spates et al., 1982). Whilst the haemolytic activity of FFA is readily demonstrated in vitro (as seen in our bioassay), there is limited evidence to suggest that they are haemolytic in vivo in the general human population given the natural abundance of triglycerides and fatty acids.

Free fatty acids in blood are carried by serum albumen, and the albumen protects against the haemolytic effects of FFA (Hostmark, 1995). It has however been hypothesised that under some clinical conditions the FFA / albumin ratio could at least temporarily become so high as to cause red cell breakdown (Hostmark, 1995). These authors noted that

"following severe protein - energy malnutrition the serum albumin concentration may be below 20 g/l i.e. 0.3 mmol/1. Thus, at such low albumin levels FFA could easily reach concentrations exceeding the FFA binding capacity of albumin." It follows then that malnutrition as experienced in some PNG villagers could lead to high FFA / albumen ratio and increase the proneness to haemolysis.

A recent study with human subjects verified that labelled FFA consumed as part of the diet rapidly appeared as labelled plasma FFA (also known as non-esterified fatty acids, NEFA) and labelled FFA plasma levels were maintained for some hours after cessation of consumption (Vedala et al., 2006). Thus PNG villager consuming sago with high levels of FFA arising from infestation by P. citrinum (or similar fungi) would be expected to have consequential high plasma FFA levels. This combined with underlying low albumin levels from protein malnutrition is thus a plausible explanation of haemolysis of red blood cells seen in SHD.

7.3.7 Assay of remaining fungal isolates that have demonstrated haemolytic compounds upon screening, particularly P. brevicompactum and Trichoderma spp.

Extracts of Aspergillus flavipes, Penicillium brevicompactum, Trichoderma virens, and Fusarium semitectum all showed strong haemolytic activity in bioassays. Solvent partitioning demonstrated that most of the activity was in a hexane rather than in dichloromethane extracts. Preparatory TLC of all species further demonstrated that the haemolytic activity was concentrated in bands eluting above citrinin on the TLC plates (Greenhill, 2006).

This evidence suggested that like Penicillium (steckii) citrinum extracts the haemolytic activity was present in non-polar, hydrophobic components of these other fungi. Extracts of these five fungi were then separated by amino solid phase extraction into neutral lipids (NL), free fatty acids (FFA) and polar lipids (PL) by literature procedures as described above.

Table 7.07: Free fatty acid composition (%) of fungal species.

Free Fatty acid	A. flavipes	P. brevi-compactum	F. semitectum	T. virens	P. citrinum	T. reesei	S. chartarum
	W1-1106	S1-0201	S1-0406	W0605-0119	W1-1301	(Std)	(Std)
C16:0	28.2	30.1	32.0	29.3	23.6	32.4	24.9
C16:1n-7	2.5	3.6	3.1	3.2	0.4	2.6	1.9
C18:0	15.1	12.6	13.0	9.8	4.6	11.8	10.5
C18:1n-9	29.2	26.0	23.0	23.7	29.3	26.6	26.1
C18:1n-7	3.7	5.5	3.6	3.2	1.2	3.1	4.7
C18:2n-6	1.9	2.7	2.0	10.4	26.3	3.8	14.4
C18:3n/2n	0.8	0.5	2.3	1.1	0.2	1.7	1.3
C18:3n-3	3.0	2.4	2.5	2.1	0.3	2.6	2.6
Sum of other minor FFA	15.6	16.6	18.4	17.1	14.1	15.3	13.7

Table 7.07 shows the fatty acid composition of the FFA fraction of Aspergillus flavipes, Penicillium brevicompactum, Trichoderma virens, Fusarium semitectum and P. (steckii) citrinum, together with that of standard fungi T. reesei and S. chartarum. All species contain the same range of fatty acids but there are distinct differences in composition particularly in the abundance of C18:2n-6, which is a major component of P. (steckii) citrinum but a lesser component of other species. The active Prep TLC Band 4 from P. (steckii) citrinum notably contained this fatty acid as the major component.

Free fatty acid content of the hexane extract from these species was also calculated by comparison with C21 internal standard: Aspergillus flavipes (7.6%), Penicillium brevicompactum (4.6%), Trichoderma virens (11.5%), Fusarium semitectum (7.3%) and P. (steckii) citrinum (8.4%), together with that of standard fungi T. reesei (5.1%) and S. chartarum (7.2%). These values represent a single fungal extract of each species and the consistency in fatty acid production within these species has not been investigated.

It would seem reasonable to conclude that much of the haemolytic activity seen in bioassays relates to presence of FFA. The production of free fatty acids by storage fungi particularly Penicillium and Aspergillus species is well known (Hansen et al., 1973; Dhingra et al., 2001; Giridhar & Reddy, 2004), and is attributable to the activity of lipases present in these fungi. Lipases from P. citrinum are known to be particularly effective, releasing abundant FFA either individually or in the presence of other fungal species (Giridhar & Reddy, 2004). Indeed, lipases from this species have been isolated and have found commercial applications in detergents (Pimentel et al., 1994; Pimentel et al., 1996).

7.3.8 Proteolytic activity of the SPE-3 fraction on red cell membrane proteins

In an attempt to determine the clinical significance of the haemolytic fraction derived from P. citrinum, the haemolytic activity was further characterised. Proteolytic activity of the SPE-3 extract fraction (the most purified fraction available at the time) was determined on human erythrocyte membrane proteins (as described above). Both the silver and the Coomassie stained SDS-PAGE gels indicated cleavage of α and β spectrin (band A) and protein band 3 (band C) relative to the control bands (lane 1–2) (Figure 7.14, lanes 4–6).

This activity is consistent with the erythrocyte destruction typical of acute intravascular haemolysis (McKenzie, 2004).

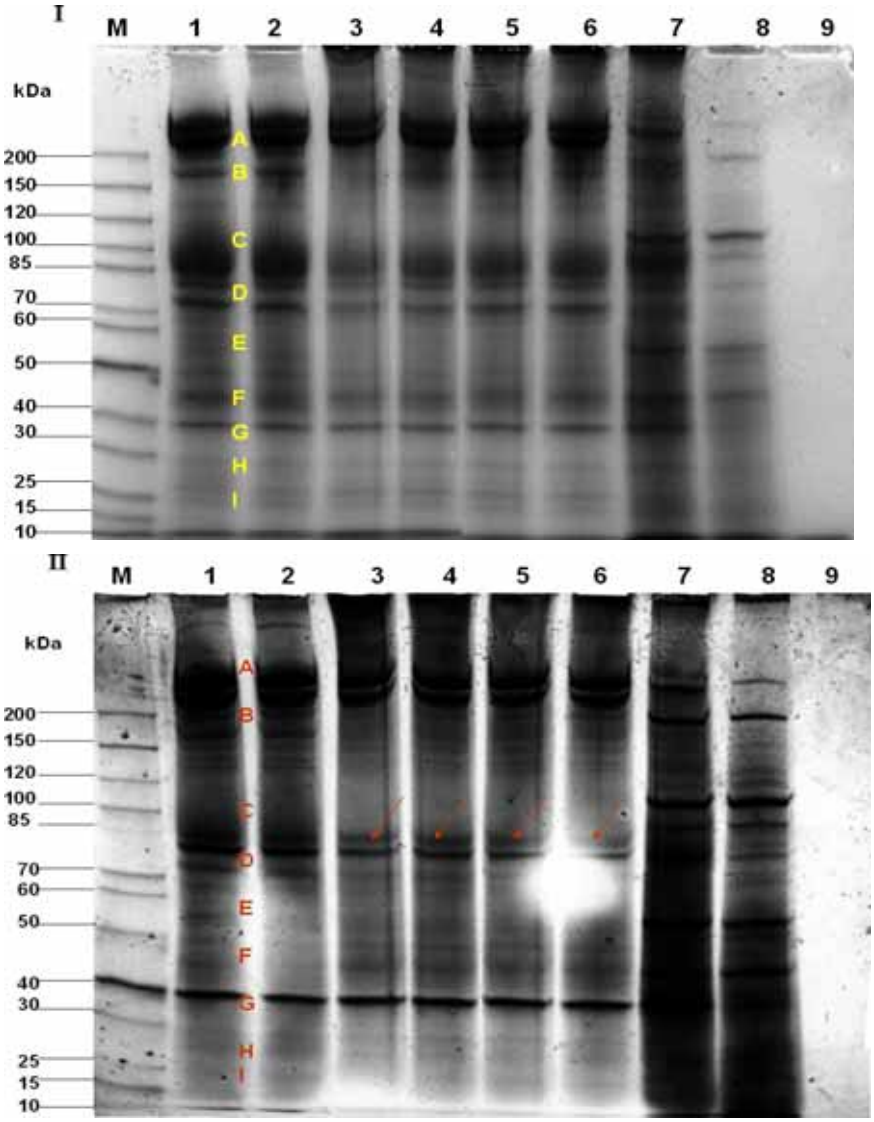

Figure 7.14: SDS-PAGE pattern of human erythrocyte membrane proteins in 10% polyacrylamide gel, following Coomassie staining (I) and following silver-staining (II). Lanes from left to right respectively are (M) molecular weight marker, (1) erythrocyte membrane proteins not incubated, (2) erythrocyte membrane proteins after incubation for 1 hr at 37°C, (3) erythrocyte membrane protein incubated with 25 µg/µl of SPE-3 fraction for 1 hr at 37°C, (4) erythrocyte membrane protein incubated with 12.5 µg/µl of SPE-3 fraction for 1 hr at 37°C, (5) erythrocyte membrane protein incubated with 5 µg/µl of SPE-3 fraction for 1 hr at 37°C (6) erythrocyte membrane protein incubated with 2.5 µg/µl of SPE-3 fraction for 1 hr at 37°C (7) erythrocyte membrane protein incubated with 2.5 µg/µl phospholipase C in lysis buffer for 1 hr at 37°C (8) phospholipase C incubated for 1 hr at 37°C, (9) SPE-3 incubated in lysis buffer for 1 hr at 37°C. Letters A to I indicate protein names, (A) α and β spectrin, (B) ankyrin, (C) protein band 3, (D) protein band 4.1 and 4.2, (E) protein band 4.9, (F) actin,(G) protein band 6, (H) protein band 7,(I) protein band 8.

7.3.9 Clarify the inoculum source of the haemolytic fungi

Penicillum citrinum, Aspergillus flavipes, P. brevicompactum, Trichoderma virens, and Fusarium semitectum are typical of species that infest crop residues or damaged tissues. These fungi thrive in low moisture content of storage conditions, and are somewhat ubiquitous, awaiting only the window of opportunity to germinate and grow. Penicillum, Aspergillus and Fusarium are typical of spoilage fungi found in the tropics where significant mycotoxin producers are relatively few (Pitt & Hocking, 1991) and have been identified as storage fungi across a range of Southeast Asian commodities.

In developed countries these fungal species are primarily of concern in bulk stored nuts and grains, and fungal contamination often is a cumulative process which begins with infection in the field of seeds affected by insect attack, moisture deficits and other stressors. By comparison, only the pith of healthy sago palms in PNG is extracted and stored, and the outer layers of the plant, where fungi are most likely to lurk, are discarded in the early stages of processing. Thus, the primary fungal inoculum is likely to originate from outside the sago palm itself, possibly from contact with other plant material but more likely from contamination during sago processing. Sago residues on implements used on previous occasions to prepare the sago, and sedimentation containers such as dug-out canoes, and human saliva and skin, appear the most likely sources of the primary fungal inoculum, particularly yeasts. Sago storage vessels and woven baskets harbouring sago residues, and leaves used to wrap the sago are also potential sources. The high moisture content of freshly-prepared sago starch favours growth of yeasts over filamentous fungi, but gradual drying and oxidation will inevitably provide the opportunity for growth of filamentous fungi in older sago. This is supported by storage experiments which saw the increase of moulds over several weeks in sago bundles but not in the same sago stored in earthenware pots under water (see above).

7.4 Reduction of contamination and HACCP analysis

7.4.1 Preliminary steps prior to HACCP analysis

A simplified flow diagram describing the process of starch production and storage is shown below (Figure 7.15).

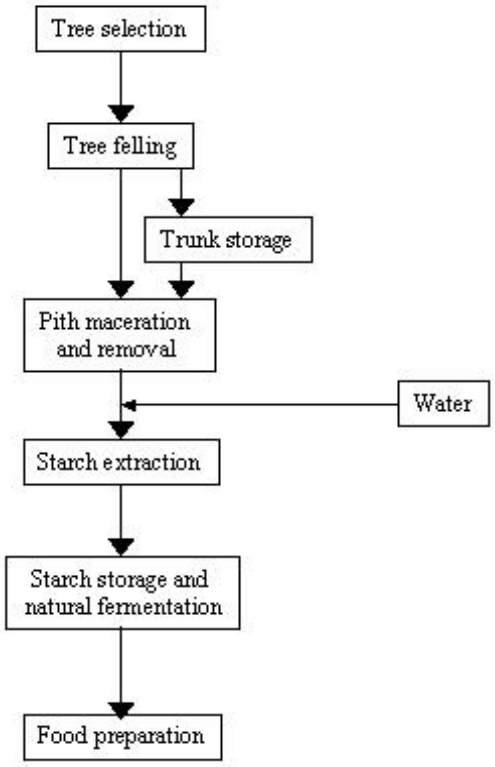

Figure 7.15: Flow diagram illustrating the major steps in the sago extraction and storage process in rural PNG.

7.4.2 Hazard analysis, control measures and critical control points

With the exception of tree felling, where no food safety hazards were identified, potential hazards were identified, appropriate control measures determined and CCPs established

for each step in the sago making process (Table 11.12, Appendix 11.4). Many of the CCPs identified could not be activated in the environment in which the work was conducted. Consequently, CCPs were characterised as notional or practicable, based on their suitability to application in village-based production of sago starch in PNG. Notional CCPs were identified in all six steps where hazards were identified, namely tree selection, trunk storage, pith maceration and removal, starch extraction, starch storage and fermentation, and food preparation. A total of six practicable CCPs were identified, occurring in all of the aforementioned stages of the process except starch extraction. Two of the six practicable CCPs were in the storage and fermentation stage, suggesting that this stage is particularly important in determining the food safety of sago starch.

Critical limits, monitoring procedures and corrective actions

Following the determination of the critical control points, critical limits, monitoring procedures and corrective actions were established for all practicable CCPs (Table 11.14, Appendix 11.4). The establishment of verification and documentation procedures was beyond the practical extent of the HACCP analysis of village based sago production and storage process.

Table 7.08: Critical limits, monitoring procedures and corrective actions for practicable CCPs of the sago production and storage process.

Practicable CCP	Critical limits	Monitoring procedures	Corrective action
#1: Tree selection	No evidence of human or	Observation	Select another tree
	animal faecal deposits;		
	no evidence of recent sago		
	extraction activity nearby		
#2: Trunk storage	Limit dry trunk storage duration	Time keeping	Discard if visible
on land (dry)	to no longer than 2 days		fungal contamination
#3: Clean utensils	No residual sago pith in	Observation	Re-clean prior to use
	recess of macerating utensils;		
	utensils must be free of		
	dirt, grime, etc.		
#4: Fermentation	Storage conditions	Observation	Thoroughly cook &
	favouring fermentation:	Odour	consume immediately
	anaerobic, moisture retaining;	Time keeping	if fermentation ceases
	fermentation for 1 week to		
	decrease incidence of		
	bacterial pathogens;		
	storage for no longer than		
	5 weeks to limit fungal growth		
#5: Airtight storage	Use of clay pots, well sealed	Observation	Discard portions
	clean plastic bags or other air-	Odour	that are visibly
	tight containers for storage;		contaminated with
	do not store in close proximity		fungi
	of potential sources of		
	contamination		
#6 Cooking	Time, temperature	Tactile	Keep cooking
	“Until thoroughly cooked”	Visual inspection	

7.4.3 Six steps to safe sago starch

From the outcomes of the initiatives described above, a village-based communication tool was developed which centred the message of safe processing, storage and consumption of sago into six points.

- Tree Selection. Make sure no animal or human waste surrounds the selected sago palm for harvest, choose another. Waste germs can pass into sago
- Trunk Storage. Cut sago palms must be processed same day. Germs can grow on sago palms after they are cut down.
- Clean Sago Making. Stop germs getting into sago by being clean. Wash hands, wear clean clothes and process sago into clean leaves or sheets. Do not step on sago.
- Water Selection. Whenever possible use clean water obtained at least 100 metres from sites of human faecal waste disposal.
- Sago Starch Storage. Always store sago for one week before eating. Always eat within one month. Sago stored in water or tightly wrapped is safer.
- Cooking. Cook sago well. Eat straight away.

More detailed justification of each step is outlined in a pamphlet made available to health care workers.

8 Impacts

8.1 Scientific impacts – now and in 5 years

It is unlikely that there will be any changes to scientific practices as a consequence of the project. Ongoing impact may result if research is directed toward further development of mycotoxin detection in foods for use in developing world environments and the characterisation and utilisation of novel compounds derived from the microbiota of sago starch.

8.2 Capacity impacts – now and in 5 years

It is unfortunate that the in-country project leader resigned her position from the partner institute before the project was complete. Regardless, the training that she benefited from, (HACCP analysis) has been incorporated into curricula at Unitech. It is hoped that this initiative will encourage graduates to consider a food safety direction in learning and research that may result in reform of the food safe agenda in PNG. This may in turn result in public health benefits to PNG through decreased food borne disease.

The project enabled Mr Asiak Pue (the foundation in-country project leader) to undertake a PhD program at the University of Queensland with AusAID sponsorship. Through his continual involvement with this project and his feedback to Unitech we have been able to encourage an early career academic to consider and apply for an Allwright scholarship centred on this disciple of study. Also, Asiak's completed PhD will benefit his teaching and research activity when he returns to PNG (2010). Anticipated ongoing collaboration with the Australian partners of this project will help him in applying for and receiving further research funding directed at relevant issues of PNG sustainable food production and post harvest applications at Unitech, particularly as they pertain to mycotoxicology and microbiology of food safety broadly.

Increasing the capacity of staff at Unitech, either directly or indirectly, in the field of food safety in PNG – particularly in regard to natural / native crops - will increase the potential for graduates to expand this area of study in PNG.

The investment in consumables for Unitech has enabled furthering of studies into safe traditional sago storage practices, in terms of effective fermentation. This investment has enabled independent studies to be undertaken at Unitech and encouraged local student interest in pressuring this area of study. The results of this work will contribute to a body of knowledge that will enhance further funding opportunities including Allwright applications coming from Unitech centred on this field of study.

8.3 Community impacts – now and in 5 years

8.3.1 Economic impacts

This project has identified that the quality of sago starch (in terms of food safety as determined by microbial contamination) does vary under different conditions of production and storage. Being able to prolong the safe storage of sago starch in a subsistence context will increase the efficiency of its utility as a stable carbohydrate. This will result in decreasing the frequency of harvest and therefore decrease the complementary costs of its production to subsistence users. Also, if this form of staple (which is often preferred to recently introduced non-traditional forms) can be relied upon more, this will decrease dependence on "store foods" and thus decrease the financial burden of subsistence families.

Sago starch is culturally important and part of the social fabric in many communities in lowland PNG. Given the shear size of the sago stand in PNG and it's under utilisation as an energy resource, the potential exists to develop it as an export commodity. If the exploitation of this resource for export markets for human consumption is considered, the outcomes of this project may impact upon the development of standards that would be required for trade. As the food safety risks of sago starch are known (through the dissemination of the results of this project - see later), it would be assumed that assurance of quality would be sought and a price differential would be applied associated with quality. Being able to produce high quality product would therefore result in higher returns.

The micro-ecology of starch derived from sago may provide avenues of further economic impact. The feasibility (both microbiologically and in terms of agri-economics) of sago starch as a biofuel substrate for the production of ethanol should be investigated. This could contribute to energy and income security for rural communities and therefore sustainable development (communities being engaged in the cultivation of sago for industrial use, the establishment of ethanol fermenters feeding converted generators may result in more affordable and sustainable power which contributes to development health and education in communities through reliable and affordable energy - for lighting and refrigeration).

As the sago palm grows in mostly non-arable land, increased and more organised cultivation for industrial use would be unlikely to impact on the availability of land for other food-based agricultural applications (and therefore food sustainability into the future) although this would obviously need to be thoroughly determined.

8.3.2 Social impacts

The microbiota of sago starch has been thoroughly determined and risks to consumers have been communicated to users. A response to these risks has been formulated and presented as a "Six Steps to Safe Sago" program to individuals throughout the WP. It is hoped that a safer sourced, produced and stored product will provide broad health benefits and also reduce the incidence of the enigmatic and frequently fatal SHD.

Faecal oral separation in subsistence / rural communities is broad public health challenge. Social norms might need to be challenged to ensure individuals consider sago palm pith (sago starch in production) to be a food item and therefore appropriate measure taken to ensure its decreased contamination (as one would do for a food item). If the human association with sago palm pith contributes to its contamination the regions where sago is cultivated and the human impact of this region will need to be considered more thoughtfully. Ideally, sago cultivated well away from village communities (where pit toilets are used and therefore where ground water – exposed in wells – may be contaminated) would be ideal, although the distance of travelling to and from the sago making place may increase the burden on individuals, particularly women and children (who are mostly involved with its production). This resultant ongoing burden may make it more difficult for young women to take up capacity building opportunities (i.e. further schooling) and therefore result in their lack of opportunities to develop.

The traditional practice of promiscuous defecation in isolated camping places should be considered in terms of its potential to contaminate the water used for starch extraction.

Considerable variation exists in sago production and storage throughout regions where it is consumed. Ensuring sago can be consumed safely will help to provide sustainability to these cultural practices. However, the fact that sago starch may achieve more rapid protective fermentation when immersed in water will challenge the communities of the WP where this practice is not commonly accepted. Is it unlikely that this change to traditional sago storage will be complied with unless there is discernable benefit in either extending the safe storage life of sago starch or reducing associated food borne illnesses. Further studies into the role of storage in the fermentation and therefore safety of sago starch are required before any such changes in practice are recommended.

8.3.3 Environmental impacts

It is unlikely that there will be any significant impact on the environment as a result of the project. Although, if industrialisation of sago starch is undertaken for either export or biofuel production an increased use of the resource will result. Management of the resource to ensure sustainable production will be required. Given that sago can grow on land that is mostly non-arable, it is unlikely that naturally heavily forested environments or garden places would need to be disturbed to increase production.

8.4 Communication and dissemination activities

8.4.1 Sago symposium, Port Moresby 2005

A mini symposium of two days was conducted in Port Moresby during September 2005. Representatives included individuals from the NDOH (including the then Health Secretary), provincial and national governments and from the agricultural and health sectors.

8.4.2 Presentations at the PNG Medical Society Medical Symposium

Presentations of the results and implications / impact of the project outputs where presented at the 2005 PNG Medical Symposium at Goroka. Another presentation has been accepted for the symposium planned for Rabaul, September 2008.

8.4.3 Six steps to safe sago starch

The six steps to safe sago starch program was presented to village and health centres throughout the WP in November 2007.

9 Conclusions and recommendations

9.1 Conclusions

The presence of common bacterial foodborne pathogens suggests that sago starch is a likely cause of mild foodborne illness, although the regular practise of thorough cooking substantially decreases the health risk. Of greatest public health concern is the evidence of high levels of faecal contamination in sago starch (7.2). Sociological data and field observations (7.1) suggest that much of this contamination originates from the water used during extraction of the sago starch from the pith of the palm. Although clean water sources are often difficult to obtain in areas of sago extraction, such a finding highlights the need of sago producers to consider carefully their water source for starch extraction.

Results of the mycological survey and ergosterol analysis were indicative of universal fungal contamination of sago starch (7.3). From a food safety and food spoilage perspective, the presence of filamentous fungi is of greater significance than the presence of yeasts. The presence of filamentous fungi in sago starch may lead to the starch being discarded — a practice that should not be discouraged but may at times contribute to food security problems — and could lead to the presence of mycotoxins and/or high levels of FFA in the starch. The high prevalence of Penicillium spp. in sago samples considered suitable for human consumption, and in samples associated with SHD, is noteworthy. Various experts have made the observation that the aspergilli are more commonly associated with commodity spoilage/mycotoxin production in warm sub-tropical and tropical environments, and penicillia often associated with cool temperate climates (Hocking, 1997; Moss, 2000; Hocking and Pitt, 2003). The frequent isolation of Penicillium spp. from sago starch in PNG is contrary to such a generalization.

The recurrent isolation of penicillia from sago starch is also of importance due to the toxigenic potential of the genus. The genus Penicillium produces a broader range of mycotoxins than any other genus (Pitt, 1997). While this study found scant evidence of common mycotoxins contaminating sago starch, many Penicillium isolates were demonstrated to be capable of citrinin production in pure culture, along with a single Aspergillus isolate. Citrinin has been proposed as a possible potentiating agent in other mycotoxicoses, and such a role should not be discounted in SHD until more is known about the aetiology of the disease. Also of interest is the recent suggestion by Lura and coworkers that citrinin might cause mild haemolytic anaemia in mice (Lura et al., 2004).

Despite the absence of common mycotoxins in sago starch per se, one cannot conclude that mycotoxins do not contribute to SHD. History suggests that many mycotoxins have been discovered following investigations of diseases of unknown aetiology. Other microbial toxins have also been identified and characterised only following disease outbreaks. Thus, an investigation of microbial isolates from sago starch for novel toxic metabolites was justified. Such a task is a monumental one, particularly in the absence of food samples that have been associated with illness, as was the case throughout most of this project. Consequently, the current investigation was based on the assumption that a compound producing in vivo haemolysis (as is the case in SHD) would be haemolytic in vitro. While it is recognised that such an assumption does not hold true in many episodes of acute intravascular haemolysis of known aetiology, and indeed very few microbial haemolysins have been associated with acute intravascular haemolysis, a sound method of searching for potentially toxic compounds capable of causing SHD was required.

Clearly the FFA hypothesis requires further clinical study which would involve investigating host predisposition / co-morbidity associated with acute SHD.

This study found sago starch to be a reservoir of haemolytic organisms. By concentrating on the haemolytic activity of filamentous fungi (7.3) the study found numerous fungal

genera to be haemolytic in vitro. Those isolates demonstrating pronounced haemolytic activity, which were commonly isolated from sago starch, warrant further investigation. Many such isolates belong to the genus Penicillium, a well known mycotoxigenic genus that was present in both samples of implicated sago starch, as well as approximately 60% of sago samples 'fit for consumption'. Other genera of fungi, such as Aspergillus, Fusarium and Trichoderma, along with selected bacterial isolates, are also of some interest as a possible aetiological agent of SHD.

The haemolytic assay developed in this study is readily applicable to activity driven fractionation, which will enable active compound(s) to be separated and ultimately identified in future studies. When the active fraction can be appropriately purified in vitro studies into the haemolytic mode of action on human erythrocytes and the use of an animal model to determine whether the fraction can elucidate symptoms consistent with SHD would be of great value.

Of particular interest is the finding that sago starch is a naturally fermented product. The fermentation of sago starch is probably the single most important factor influencing the development of microbial communities, and thus food safety of the product. Active fermentation was shown to inhibit the growth and survival of selected bacterial pathogens, although the survival of both laboratory and naturally occurring strains of E. coli in sago starch are of some public health concern. Another benefit of actively fermenting sago starch is that conditions favourable for fermentation are generally not favourable for the growth of filamentous fungi. Although the level of risk that filamentous fungi constitute to sago consumers can not be definitively stated on the basis of the current study, storage methods and durations that moderate or prevent the growth of such organisms should still be recommended.

The presence FFA in sago starch derived from moulds may be associated with haemolysis when consumed, particularly when individuals are experiencing co-morbidity which results in low albumin or are protein malnourished. This avenue requires further clinical investigation.

9.2 Recommendations

In terms of furthering a study into SHD:

- source / encourage medical collaboration for an epidemiological study to enable a critical and thorough proposal for further investigation
- through establishing networks with health providers in WP and ESPs ensure a reliable and continual source of SHD implicated sago for further microbial analysis
- in subsequent microbiological analysis, explore non-mycological haemolytic micro flora more thoroughly
- encourage the development of an animal model for SHD to test Koch's postulates and to explore the FFA hypothesis
- further the study on sago starch fermentation with a greater array of natural storage techniques
- test the compliance and impact of the "Six Steps for Safe Sago" program in the WP and apply a optimised program throughout other sago consuming communities
- consider a study to determine the feasibility of sago as a biofuel substrate (for ethanol production) and its use as a carbon sink and therefore its use as a sustainable village based energy and income source
- if above is feasible furthering the microbial ecology investigation of sago starch in the context of biofuel production (ethanol).

10 References

10.1 References cited in report

Asahi, H., A. Moribayashi, F. Sendo & T. Kobayakawa, (1984) Hemolytic factors in Schistosoma japonicum eggs. Infection and Immunity 46: 514-518.

Barron, G.L. (1968) The Genera of Hyphomycetes from Soil. Baltimore: The Williams and Wilkins Company.

Bateman Ii, H. G. & T. C. Jenkins, (1997) Method for Extraction and Separation by Solid Phase Extraction of Neutral Lipid, Free Fatty Acids, and Polar Lipid from Mixed Microbial Cultures. Journal of Agricultural and Food Chemistry 45: 132-134.

Bennett, J.W. and Klich, M. (2003) Mycotoxins. Clinical Microbiology Reviews 16, 497-516.

Bissett, J. (1991) A revision of the genus Trichoderma.II. Infrageneric classification. Canadian Journal of Botany 69, 2357-2372.

Blaney, B.J., Moore, C.J. and Tyler, A.L. (1984) Mycotoxins and fungal damage in maize harvested during 1982 in Far North Queensland. Australian Journal of Agricultural Research 35, 463-471.

Csordas, A. & K. Schauenstein, (1986) Temperature-dependent specificity of cis-trans isomeric fatty acid interaction with the erythrocyte membrane. Biochimica et Biophysica Acta (BBA) - Biomembranes 856: 212-218.

Dhingra, O. D., E. S. G. Mizubuti, I. T. Napoleao & G. Jham, (2001) Free fatty acid accumulation and quality loss of stored soybean seeds invaded by Aspergillus ruber. Seed Science and Technology 29: 193-203.

Donovan, K., Shaw, D.E. and Amato, D. (1977) Sago haemolysis: clinical features and microbiological studies. Papua New Guinea Medical Journal 20, 167-174.

Donovan, K.O., Shaw, D.E. and Amoto, D. (1976) Sago and Haemolysis. Papua New Guinea Medical Journal 19, 183-184.

Ehiri, J.E. and Prowse, J.M. (1999) Child health promotion in developing countries: the case for integration of environmental and social interventions? Health Policy Plan 14, 1-10.

Elek, S.D. and Levy, E. (1954) The nature of discrepancies between haemolysins in culture filtrates and plate haemolysin patterns of staphylococci. Journal of Pathology and bacteriology 68, 31-40.

FAO (2001) Manual on the application of the HACCP system in mycotoxin prevention and control. Rome: Food and Agriculture Organisation of the United Nations.

Frisvad, J.C. and Thrane, U. (2000) Mycotoxin production by common filamentous fungi. In Introduction to Food- and Airborne Fungi eds. Samson, R.A., Hoekstra, S., Frisvad, J.C. and Filtenborg, O. pp.321-331. Utrecht: Centraalbureau voor Schimmelcultures.

Fu, M., A. Koulman, M. Van Rijssel, A. Lutzen, M. K. De Boer, M. R. Tyl & G. Liebezeit, (2004) Chemical characterisation of three haemolytic compounds from the microalgal species Fibrocapsa japonica (Raphidophyceae). Toxicon 43: 355-363.

Gams, W. and Bissett, J. (1998) Morphology and identification of Trichoderma. In Trichoderma and Gliocladium Volume 1 Basic Biology, Taxonomy and Genetics eds. Kubicek, C.P. and Harman, G.E. pp.3-34. London: Taylor and Francis.

George, T., N. J. Bai & S. Krishna-Murthy, (1979) Studies on hemolysis of human erythrocytes by linoleic acid. Journal of Biosciences 1: 385-392.

Giridhar, P. & S. M. Reddy, (2004) Biochemical changes in black pepper seed due to inoculation of mycotoxigenic fungi in vitro. Indian Phytopathology, 57: 213-216.

Greenhill, A.R. (2006) Food Safety and Security of Sago Starch in Rural Papua New Guinea. In Microbiology and Immunology. p.365. Townsville: James Cook University.

Guarro, J., J. Gene & A. M. Stchigel, (1999) Developments in fungal taxonomy. Clinical Microbiology Reviews 12: 454-500.

Hansen, A. P., R. E. Welty & R. S. Shen, (1973) Free fatty acid content of cacao beans infested with storage fungi. Journal of Agricultural and Food Chemistry 21: 665-670.

Hocking, A.D. (1997) Toxigenic Aspergillus species. In Food Microbioogy Fundamentals and Frontiers eds. Doyle, M.P., Beuchat, L.R. and Montville, T.J. pp.393-405. Washington DC: ASM Press.

Hocking, A.D. and Pitt, J.I. (2003) Mycotoxigenic Fungi. In Foodborne Microorganisms of Public Health Significance ed. Hocking, A.D. pp.641-673. Waterloo, NSW: Australian Institute of Food Science and Technology Incorporated.

Hostmark, A. T., (1995) Serum fatty acid/albumin molar ratio and the risk of diseases. Medical Hypotheses 44: 539-54.

Kaluzny, M. A., L. A. Duncan, M. V. Merritt & D. E. Epps, (1985) Rapid separation of lipid classes in high yield and purity using bonded phase columns. Journal of Lipid Research 26: 135-140.

Kandhai, M.C., Reij, M.W., van Puyvelde, K., Guillaume-Gentil, O., Beumer, R.R. and van Schothorst, M. (2004) A new protocol for the detection of Enterobacter sakazakii applied to environmental samples. Journal of Food Protection 67, 1267-1270.

Karlinski, L., S. Ravnskov, B. Kieliszewska-Rokicka & J. Larsen, (2007) Fatty acid composition of various ectomycorrhizal fungi and ectomycorrhizas of Norway spruce. Soil Biology and Biochemistry 39: 854-866.

Kozlovskii, A.G., Zhelifonova, V.P., Vinokurova, N.G. and Ozerskaya, S.M. (2000) Effect of Microelements on the biosynthesis of secondary metabolites by the fungus Penicillium citrinum Thom VKMF-1079. Microbiology 69, 536-540.

LeVeen, H. H., P. Giordano, A. Johnson & B. Elliott, (1965) Hemolysis following infusions of fat emulsions Digestive Diseases and Sciences 10: 675-688.

Longini, J. & V. Johnson, (1943) Increase Red Blood Cell Fragility after Fat Ingestion. Am J Physiol 140: 349-353.

Lovstad, R. A., (1986) Fatty acid induced hemolysis. Protective action of ceruloplasmin, albumins, thiols and vitamin C. International Journal of Biochemistry 18: 771-775.

Lura, M.C., Fuentes, M., Cabagna, M., Gonzalez, A.M., Nepote, A., Giugni, M.C., Rico, M. and Latorre, M.G. (2004) Structural and ultrastructural alterations in BALB/c mice: effects of Penicillium citrinum metabolites. Mycopathologia 158, 233-238.

McClatchey, W., Manner, H.I. and Elevitch, C.R. (2004) Metroxylon amicarum, M. paulcoxii, M. sagu, M. salmonense, M. vitiense, and M. warburgii (sago palm): Permanent Agriculture Resources.

McKenzie, S.B. (2004) Clinical Laboratory Hematology. New Jersey: Pearson Prentice Hall

Moss, M.O. (2000) Toxigenic fungi and mycotoxins. In The Microbiological Safety and Quality of Food eds. Lund, B.M., Baird-Parker, T.C. and Gould, G.W. pp.1490 - 1517. Gaitherburg: Aspen Publishers Inc.

NACMCF (1992) Hazard Analysis and Critical Control Point system. The National Advisory Committee on Microbiological Criteria for Foods. International Journal of Food Microbiology 16, 1-23.

NACMCF (1998) Hazard analysis and critical control point principles and application guidelines. Adopted August 14, 1997. National Advisory Committee on Microbiological Criteria for Foods. Journal of Food Protection 61, 1246-1259.

Pimentel, M. C., N. Krieger, L. C. Coelho, J. O. Fontana, E. H. Melo, W. M. Ledingham & J. L. Lima Filho, (1994) Lipase from a Brazilian strain of Penicillium citrinum. Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology 49: 59-74.

Pimentel, M. C. B., E. H. M. Melo, J. L. L. Filho & N. Duran, (1996) Production of lipase free of citrinin by Penicillium citrinum. Mycopathologia 133: 119-121.

Pitt, J.I. (1979) The Genus Penicillium and its teleomorphic states Eupenicillium and Talaromyces. London: Academic Press.

Pitt, J.I. (1997) Toxigenic Penicillium species. In Food Microbiology Fundamentals and Frontiers eds. Doyle, M.P., Beuchat, L.R. and Montville, T.J. pp.406-418. Washington DC: ASM Press.

Pitt, J.I., Basilico, J.C., Abarca, M.L. and Lopez, C. (2000) Mycotoxins and toxigenic fungi. Medical Mycology 38 (supp 1), 41-46.

Pitt, J.I. and Hocking, A.D. (1991) Significance of fungi in stored products. In Fungi and Mycotoxins in Stored Products Preceedings of an International Conference, Bangkok ed. Champ, B.R., Highley, E., Hocking, A.D. and Pitt, J.I. pp.16-21. Bangkok: Australian Centre for International Agricultural Rersearch.

PNG Ministry of Health (2000) National Health Plan 2001-2010. Health Vision 2010. Policy Directions and Priorities: Independent State of Papua New Guinea Ministry of Health.

Power, A.P. (1999) The sago industry for Papua New Guinea: the time is now. In Sago Starch and Food Security in Papua New Guinea The Proceedings of the First National Sago Conference, Lae ed. Sopade, P.A. pp.1-13. Lae, PNG: Unitech Press.

Rhoads, J.W. (1980) Through a Glass Darkly: Present and Past Land-use Systems of Papuan Sago palm Users. Canberra: Australian National University.

Rybczynska, M. & A. Csordas, (1989) Chain length-dependent interaction of free fatty acids with the erythrocyte membrane. Life Sciences 44: 625-632.

Sopade, P.A. (2001) Sago starch, food security and nutrition in PNG: the triple web. In Food security for Papua New Guinea Proceedings of the Papua New Guinea Food and Nutrition 2000 Conference, Lae eds. Bourke, R.M., Allen, M.G. and Salisbury, J.G. pp.825-833. Canberra Australia: Australian Centre for International Agricultural Research (ACIAR).

Standards Australia (1991a) AS 1766.1.2 - 1991. Australian Standard Method 1.2: General procedures and techniques - Preparation of dilutions.

Standards Australia (1991b) AS 1766.1.3 - 1991. Australian Standard Method 1.3: General procedures and techniques - Colony count - Pour plate method.

Standards Australia (1991c) AS 1766.1.4 - 1991. Australian Standard Method 1.4: General procedures and techniques - Colony count - Surface spread method.

Standards Australia (1991d) AS 1766.1.6 - 1991. Australian Standard Method 1.6: General procedures and techniques - Estimation of most probable number (MPN) of microorganisms.

Standards Australia (1991e) AS 1766.2.1 - 1991. Australian Standard Method 2.1: Examination for specific organisms - Standard plate count.

Standards Australia (1991f) AS 1766.2.5 - 1991. Australian Standard Method 2.5: Examination for specific organisms - Salmonellae.

Standards Australia (1991g) AS 1766.2.6 - 1991. Australian Standard Method 2.6: Examination for specific organisms - Bacillus cereus.

Standards Australia (1991h) AS 1766.2.8 - 1991. Australian Standard Method 2.8: Examination for specific organisms - Clostridium perfringens.

Standards Australia (1992) AS 1766.2.3. - 1992. Australian Standard Method 2.3: Examination for specific organisms - Coliforms and Escherichia coli.

Standards Australia (1994a) AS 1766.2.2 - 1994. Australain Standard Method 2.2: Examination for specific organisms - Colony count of yeasts and moulds.

Standards Australia (1994b) AS 1766.2.4 - 1994. Australian Standard Method 2.4: examination for specific organisms - Coagulase-positive staphylococci.

Standards Australia (1998) AS/NZS 1766.2.16.1:1998. Australian/New Zealand Standard Method 2.16.1: Examination for specific organisms - Food and animal feeding stuffs - Horizontal method for the detection and enumeration of Listeria monocytogenes - Detection method.

Seeman, P., (1966) III. A method for distinguishing specific from nonspecific hemolysins. Biochemical Pharmacology 15: 1767-1774.

Spates, G. E., R. D. Stipanovic, H. Williams & G. M. Holman, (1982) Mechanism of haemolysis in a blood-sucking dipteran, Stomoxys calcitrans. Insect Biochemistry 12: 707-712.

Stahl, P. D. & M. J. Klug, (1996) Characterization and differentiation of filamentous fungi based on fatty acid composition. Applied and Environmental Microbiology 62: 4136-4146.

Sweeney, M.J. and Dobson, A.D. (1998) Mycotoxin production by Aspergillus, Fusarium and Penicillium species. International Journal of Food Microbiology 43, 141-158.

Taufa, T. (1974) Sago Haemolytic Disease. Papua New Guinea Medical Journal 17, 227-228.

Vaughan, J.G. and Geissler, C.A. (1997) The New Oxford Book of Food Plants. Oxford: Oxford University Press.

Vedala, A., W. Wang, R. A. Neese, M. P. Christiansen & M. K. Hellerstein, (2006) Delayed secretory pathway contributions to VLDL-triglycerides from plasma NEFA, diet, and de novo lipogenesis in humans. Journal of Lipid Research 47: 2562-2574.

von Arx, J.A. (1970) The Genera of Fungi Sporulating in Pure Culture. Lehre: Verlag von J. Cramer.

Waksman, S.A. (1967) The Actinomycetes: A Summary of Current Knowledge. New York: The Ronald Press Company.

WHO (2004) The World Health Report 2004: Changing History. Geneva: World Health Organisation.

Zycha, H. and Siepmann, R. (1969) Mucorales. Eine Beschreibung Aller Gattungen und Arten Dieser Pilzgruppe. Lehre: Verlag Von J. Cramer.

10.2 List of publications produced by project to date

Greenhill, A.R., Shipton, W.A., Blaney, B.J. and Warner, J.M. (2007) Fungal colonization of sago starch in Papua New Guinea. International Journal of Food Microbiology 119(3):284-90

Greenhill, A.R., Shipton, W.A., Omoloso, A.D., Amoa, B. and Warner, J.M. (2007) The prevalence of bacteria of public health significance in sago starch in Papua New Guinea. Journal of Food Protection 70(12):2868-72

Greenhill, A.R., Kopel, E., Shipton, W.A., Seleno, N., Pue, A. and Warner, J.M. (in press) Traditional sago starch processing and storage and its influence on food safety in Papua New Guinea. Proceedings of the 9th International Sago Symposium, Ormac, Philippines July 19-21, 2007.

Greenhill, A.R., Blaney, B.J., Shipton, W.A., Pue, A. and Warner, J.M. (in press) A survey for mycotoxins and toxigenic fungi in sago starch from Papua New Guinea. Letters in Applied Microbiology

Greenhill, A.R., Shipton, W.A., Blaney, B.J., Brock, I., Kupz, A. and Warner, J.M. (in review) Spontaneous fermentation of traditional sago starch in Papua New Guinea.

Greenhill, A.R., Blaney, B.J., Shipton, W.A., Pue, A., Fletcher, M., and Warner, J.M. (in preparation) The haemolytic activity of fungi isolated from traditional sago starch in Papua New Guinea.

Atagazli, L., Warner, J.M., Greenhill, A.R., and Melrose, W. (in preparation) The in vitro haemolytic activity of Penicillium citrinum against human and mouse erythrocytes

11 Appendixes

11.1 Sociological survey results (refer to section 7.1)

Table 11.01: Division of labour between men (M) and women (W) for main tasks involved in sago palm cultivation and starch production. Results are presented as percentage of respondents, with 95% confidence limits in parenthesis.

	ESP (n = 27)			WP (n = 22)		
	M	**W**	**M & W**	**M**	**W**	**M & W**
Clearing area for planting	15% (4–34%)	0% (0–11%)	85% (66–96%)	27% (11–50%)	0% (0–13%)	73% (50–89%)
Planting sago palms	11% (2–29%)	0% (0–11%)	89% (71–98%)	9% (2–31%)	0% (0–13%)	91% (69–98%)
Removing excess growth	19% (6–38%)	0% (0–11%)	81% (62–94%)	45% (25–67%)	0% (0–13%)	55% (33–75%)
Felling palm	81% (62–94%)	0% (0–11%)	19% (6–38%)	9% (1–29%)	5% (0–23%)	86% (65–97%)
Starch extraction	Gender specific roles*			0% (0–13%)	59% (36–79%)	41% (21–64%)

Table 11.02: Transfer of sago cultivation and extraction knowledge from one generation to the next in the ESP and the WP . Results are presented as percentage of respondents, with 95% confidence limits in parenthesis.

	ESP (n = 27)			WP (n = 22)		
	F–S	**M–D**	**P–C**	**F–S**	**M–D**	**P–C**
Planting and growth	0% (0–11%)	0% (0–11%)	100% (90–100%)	28% (10–50%)	5% (0–22%)	67% (45–86%)
Extraction process	0% (0–11%)	0% (0–11%)	100% (90–100%)	0% (0–13%)	41% (21–64%)	59% (36–79%)

F–S: Father to son; M–D: Mother to daughter; P–C: Parent to child (not gender specific).

Table 11.03: Percentage of respondents who wash extraction equipment following the maceration of sago palms. Results are presented as percentage of respondents, with 95% confidence limits in parenthesis.

	ESP (n = 27)	WP (n = 22)
Washing of extraction equipment	78%: Yes, to some degree (58–91%)	33%: Yes, with water (16–56%)
		13%: Sometimes (3–35%)
		9%: Excess pith material removed, water not used (2–31%)
	22%: No (13–49%)	45%: No (25–67%)

Table 11.04: Methods of human faecal waste disposal, and the distance of disposal from site of sago extraction. Results are presented as percentage of respondents, with 95% confidence limits in parenthesis.

	ESP (n = 27)	WP (n = 22)
In the village		
Pit toilet	93% (76–99%)	86% (65–97%)
Over water	7% (1–24%)	9% (1–29%)
Bush disposal	0% (0–11%)	5% (0–23%)
When sago making away from village		
Bush disposal	NA	95% (77–100%)
Distance from where sago is made		
< 100 m	44% (25–65%)	70% (45–86%)
100–500 m	48% (29–68%)	18% (5–40%)
> 500 m	8% (1–24%)	12% (3–35%)

NA: Data not collected.

Table 11.05: Preferred methods of sago starch storage in the ESP and the WP . Results are presented as percentage of respondents, with 95% confidence limits in parenthesis.

Preferred Storage Method	ESP (n = 27)	WP (n = 22)
Clay pots	19% (6–38%)	0% (0–13%)
Saucepan or buckets	19% (6–38%)	0% (0–13%)
Clay pots, saucepans or buckets	47% (29–68%)	0% (0–13%)
Wrapped in leaves	15% (4–34%)	82% (60–95%)
Woven fibre bags and baskets	0% (0–11%)	18% (5–40%)

Table 11.06: Primary and secondary methods of sago preparation in the ESP and the WP . Results are presented as percentage of respondents, with 95% confidence limits in parenthesis.

	ESP (n = 27)	WP (n = 22)
Primary cooking method		
Boiled	89% (71–98%)	0% (0–13%)
Frying pan	11% (2–29%)	91% (71–99%)
Wrapped in leaves	0 (0–11%)	9% (1–29%)
Secondary cooking method		
Frying pan	78% (58–91%)	4% (0–23%)
Wrapped in leaves	7% (1–24%)	91% (71–99%)
Other	15% (5–35%)	5% (0–23%)

Table 11.08: Selected responses from survey participants pertaining to cultural aspects of sago use

	ESP	WP
Importance	"Rice bilong ol ESP" (sago is the rice of the ESP)	"Sago is life" Must have sago every meal
Ceremonial use	Large quantities eaten at ceremonies particularly weddings and funerals	Always eaten at ceremonies Cooked a special way for special occasions, especially funerals
Culture and traditions	Patches of sago stands given as wedding gift to newlyweds by both bride and groom's family Sago can be exchanged for pigs	Sago swamps given for bride price Highly revered as source of food and building materials Sago planting, management and processing is part of the culture

Myths and customs	Only women can pack sago into storage pots and change the water Women are not allowed to process sago while menstruating or men will get sick with asthma-like illness Women are not allowed to jump over sago The person who planted the sago must not eat from his/her own harvest or they will die	In weddings sago is sprinkled on ground for newlyweds to walk on People paint themselves with sago starch at Christmas time Pray for "more than enough" sago when planting and extracting Place hot sago from the fire on wounds to facilitate healing Red sago tastes good. To make red sago, sit white sago in the sun

11.2 Microbial contamination and microbial ecology of sago starch

Table 11.09: Statistical analysis (using analysis of variance) of the influence of storage duration, storage technique and pH on numbers of B. cereus isolated from sago starch.

Parameter	Group 1	Group 2	Mean difference	Significance
Storage duration	< 1.0 week	1.0–2.9 weeks	-1.034	0.037
Storage duration	< 1.0 week	3.0–4.9 weeks	-0.920	0.044
Storage duration	< 1.0 week	≥ 5.0 weeks	-1.711	0.002
Storage method	Fresh	Leaves	-1.296	0.023
Storage method	Fresh	Woven	-2.187	0.008
pH	< 4.00	5.00 – 5.99	1.589	0.005
pH	< 4.00	6.00 – 6.99	1.336	0.037
pH	< 4.00	≥ 7.00	1.274	0.065
pH	4.00 - 4.99	5.00 – 5.99	1.063	0.018

Table 11.10: Faecal coliforms, E. coli and filamentous fungi isolated from triplicate samples of fermenting sago starch over 30 days.

Day	Faecal coliforms	E. coli	Filamentous fungi
0	>1.1 × 104	>1.1 × 104	2.8 × 102 ± 1.3 × 102
3	>1.1 × 104	>1.1 × 104	< 2.0 × 102
6	>1.1 × 104	>1.1 × 104	< 2.0 × 102
9	>1.1 × 104	>1.1 × 104	< 1.5 × 102
12	>1.1 × 104	>1.1 × 104	2.5 × 102 ± 1.5 × 102
16	>1.1 × 104	< 4.0 × 101	< 4.5 × 102
23	< 4.3 × 101	ND	< 5.0 × 102
30	ND	ND	< 2.5 × 102

11.3 Mycotoxin contamination and characterisation of haemolytic compounds

Table 11.11: Citrinin and sterigmatocystin concentrations produced in pure culture by fungi isolated from sago starch.

Isolate	Identification	Citrinin (ppm)	Sterigmatocystin (ppm)
W1-1101	P. steckii*	2,000	< 0.1
W1-1109	P. steckii*	4,200	< 0.1
W1-1301	P. steckii*	1,000	< 0.1
W1-1501	P. steckii*	1,700	< 0.1
S2-0503	P. steckii*	4,200	< 0.1
S2-1305	P. steckii*	2,000	< 0.1
S2-1806	P. steckii*	800	< 0.1

S1-0201	P. brevicompactum	2,000	< 0.1
W1-1602	P. brevicompactum	4,200	< 0.1
W2-0303	P. brevicompactum	4,200	< 0.1
W2-0703	P. brevicompactum	4,200	< 0.1
W2-1401	P. brevicompactum	4,200	< 0.1
S2-1602	Penicillium sp.	4,200	< 0.1
W2-0101	Penicillium sp.	< 0.005	< 0.1
W2-1003	Penicillium sp.	< 0.005	< 0.1
W1-1106	A. flavipes	200	< 0.1
S2-0705	A. flavipes	< 0.005	< 0.1
S1-0302	Aspergillus sp.	< 0.005	< 0.1
S1-0802	Aspergillus versicolor	< 0.005	500
W1-0904	Aspergillus sp.	< 0.005	< 0.1
S1-0406	Fusarium semitectum	< 0.005	< 0.1

Legend: P. = Penicillium; A. = Aspergillus
**: Maybe more accurately described as P. citrinum-affinity (see Section 6.4)*

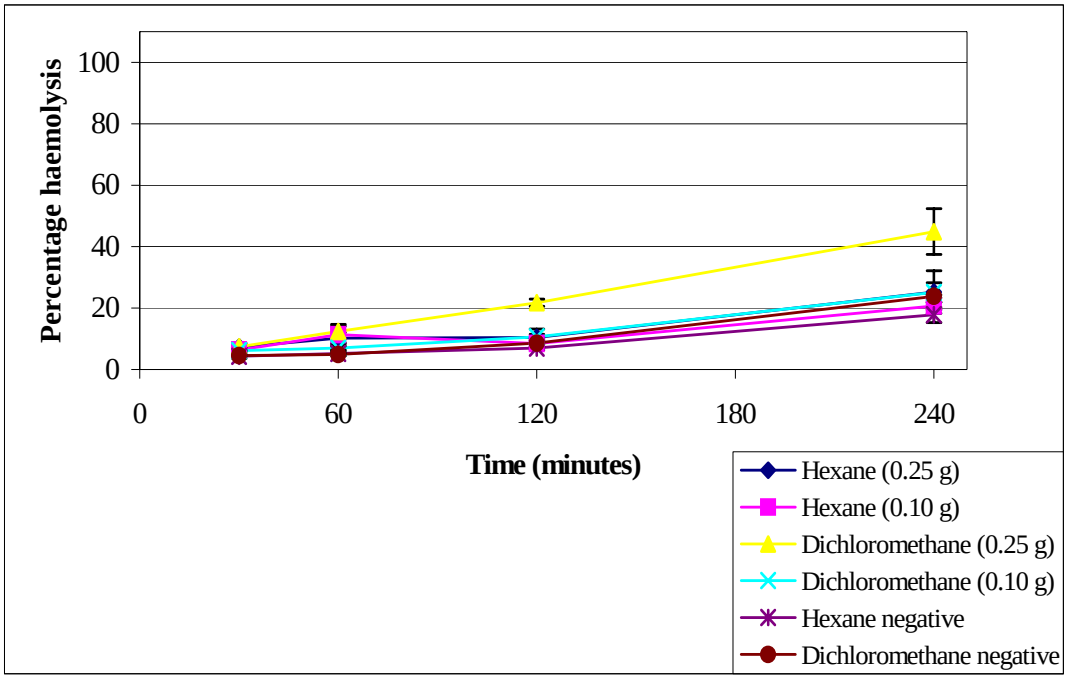

Figure 11.01: Haemolytic activity of hexane-rich and dichloromethane-rich extracts of sterile wheat (weight extracted in parenthesis), and extraction solvents alone (negative controls).

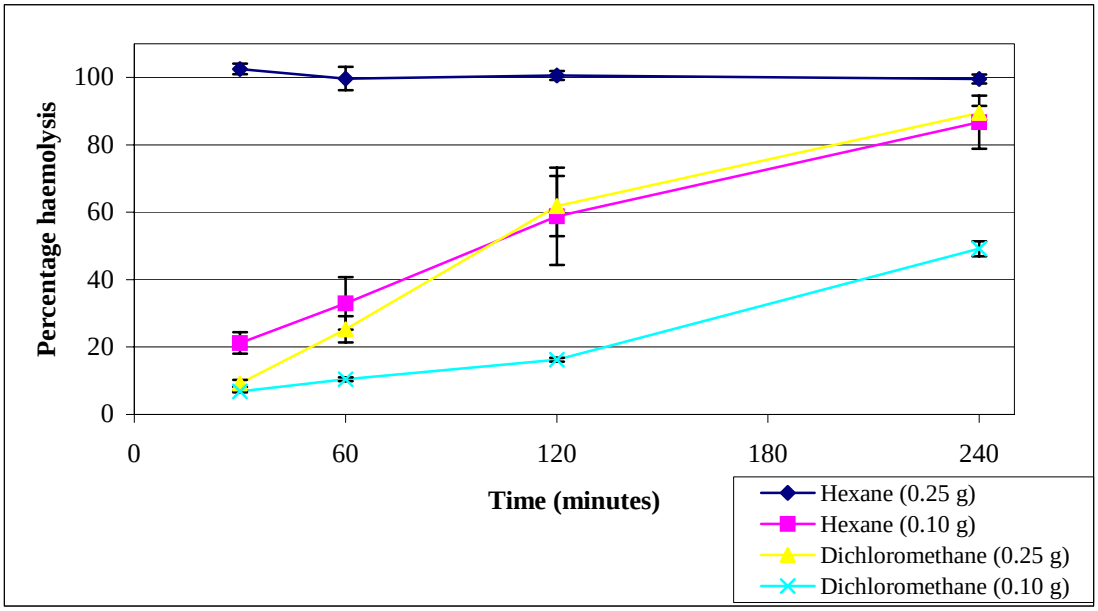

Figure 11.02: Haemolytic activity of hexane-rich and dichloromethane-rich extracts of S. chartarum ATCC 9182 culture (positive control organism).

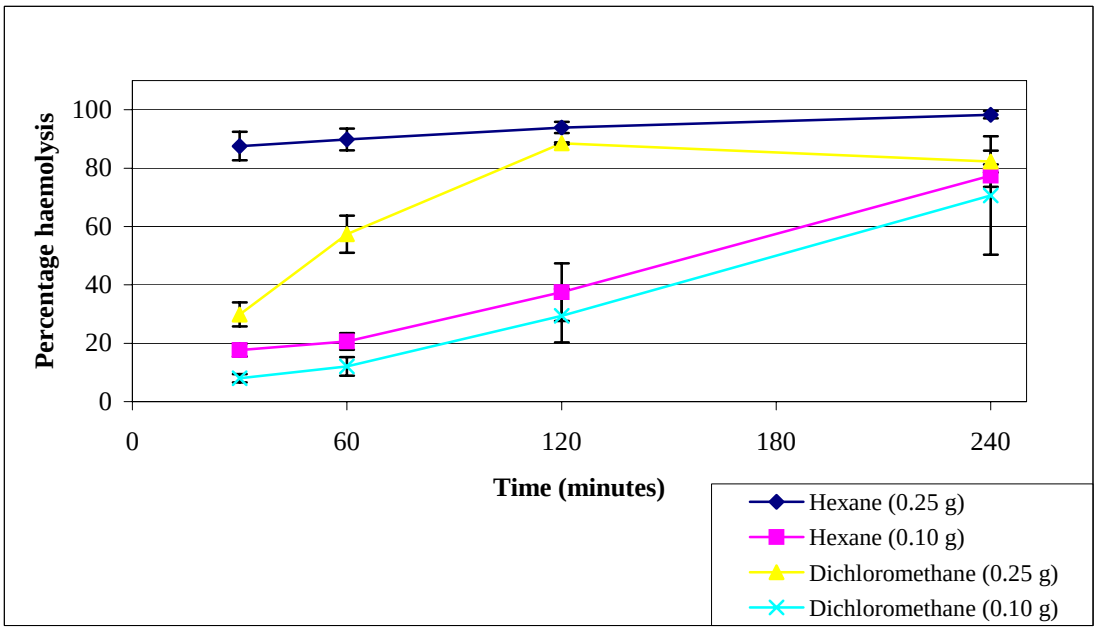

Figure 11.03: Haemolytic activity of hexane-rich and dichloromethane-rich extracts of T. reesei ATCC 26921 culture (positive control organism).

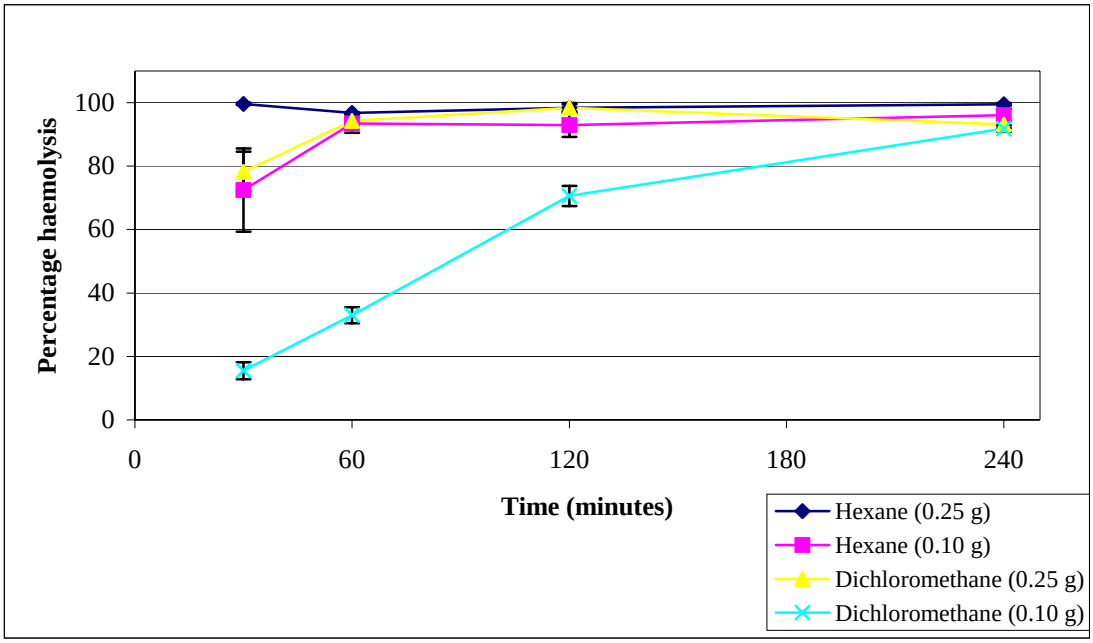

Figure 11.04: Haemolytic activity of hexane-rich and dichloromethane-rich extracts of *P. steckii* W1-1101.

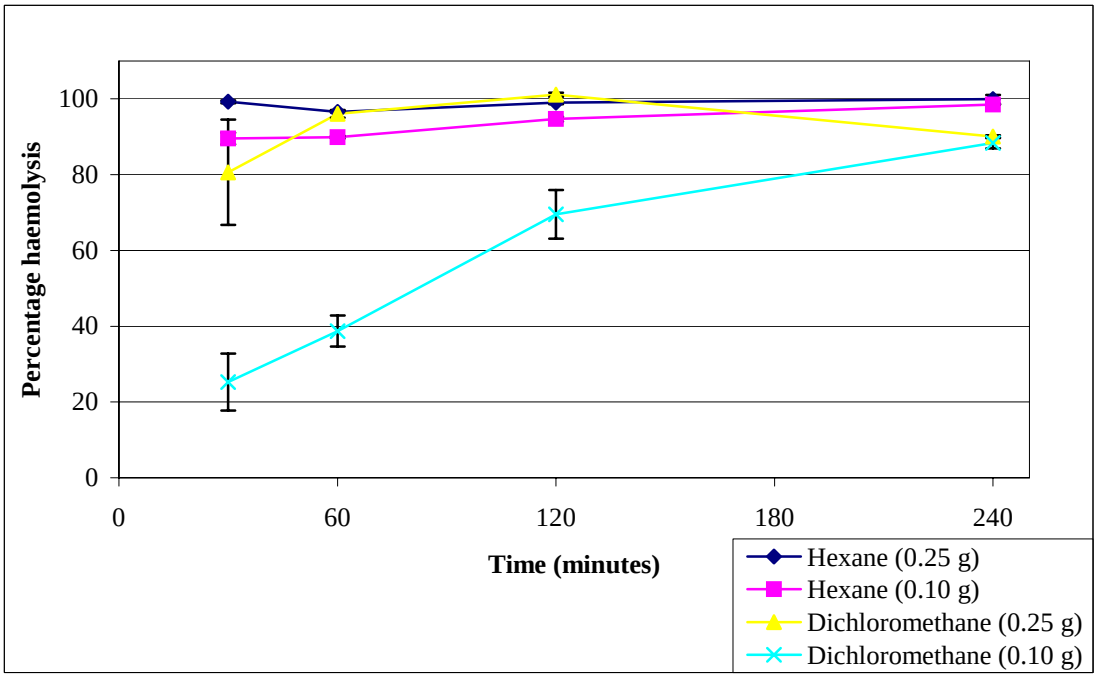

Figure 11.05: Haemolytic activity of hexane-rich and dichloromethane-rich extracts of *P. steckii* W1-1301.

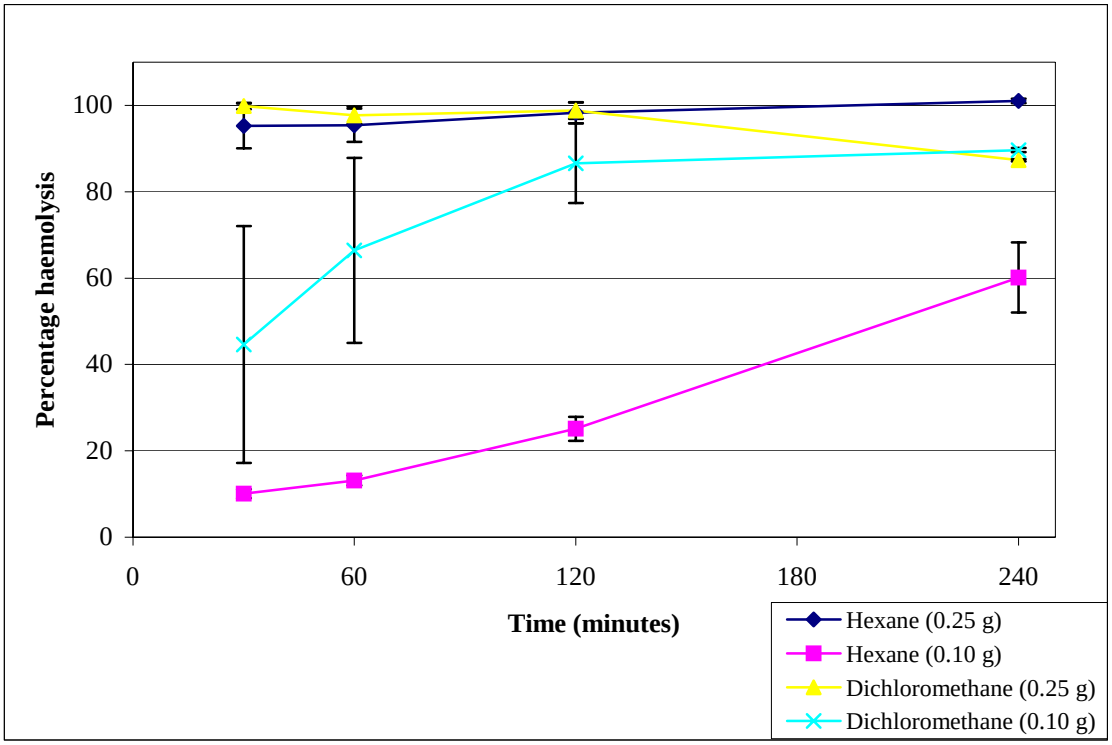

Figure 11.06: Haemolytic activity of hexane-rich and dichloromethane-rich extracts of *P. steckii* S2-1305.

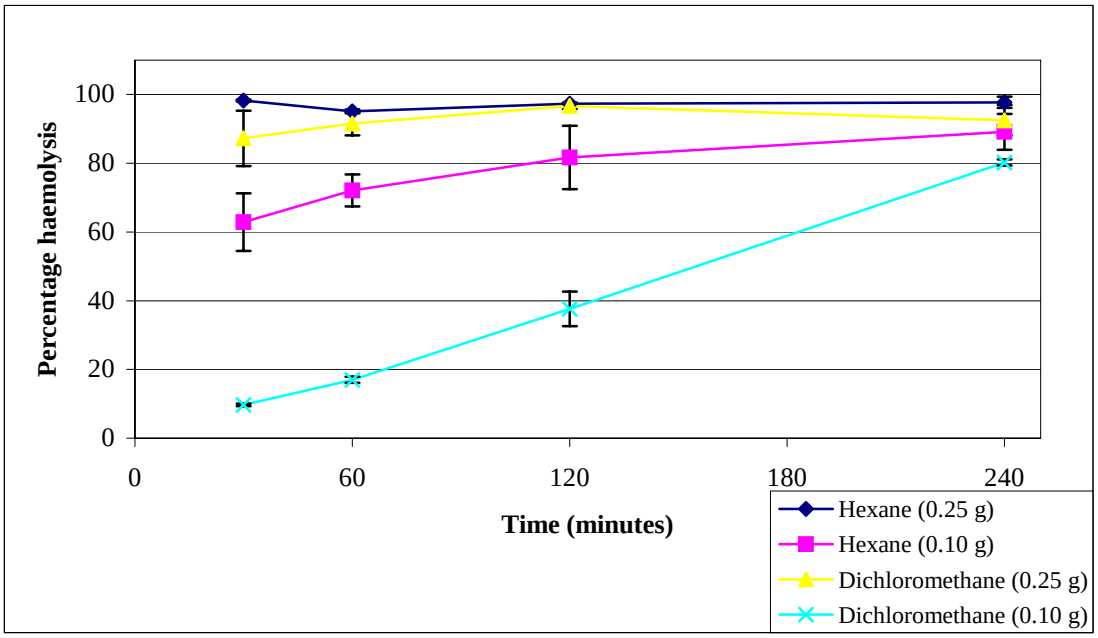

Figure 11.07: Haemolytic activity of hexane-rich and dichloromethane-rich extracts of *P. brevicompactum* S1-0201.

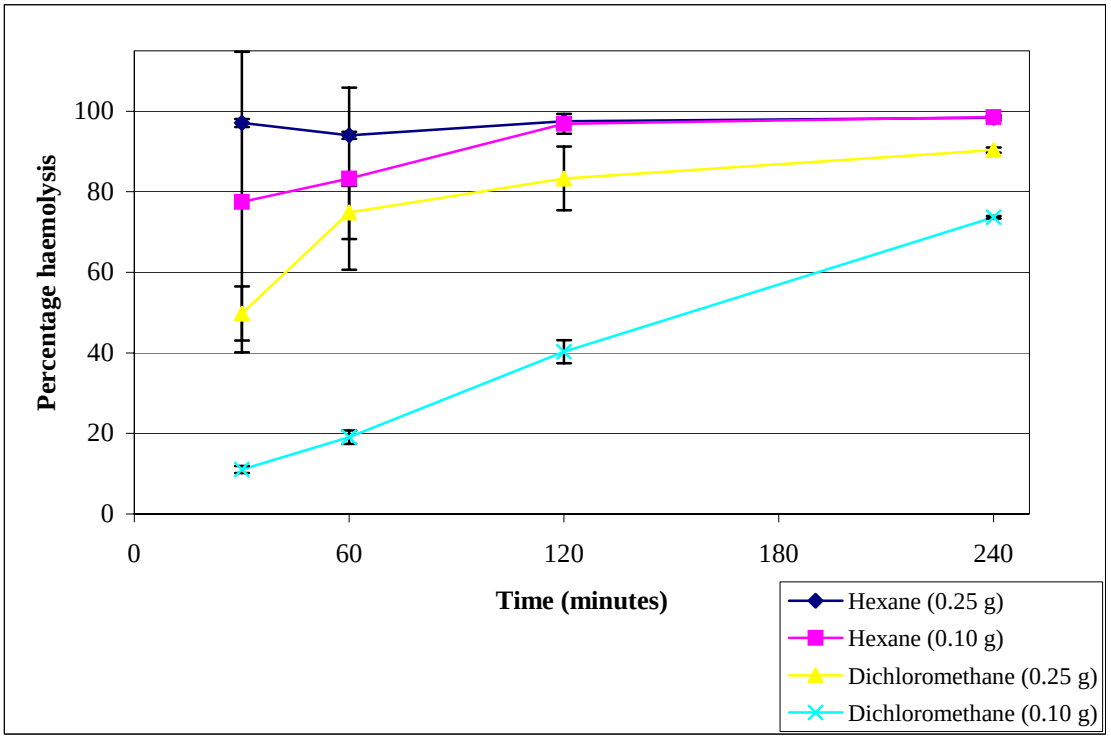

Figure 11.08: Haemolytic activity of hexane-rich and dichloromethane-rich extracts of A. flavipes S2-0207.

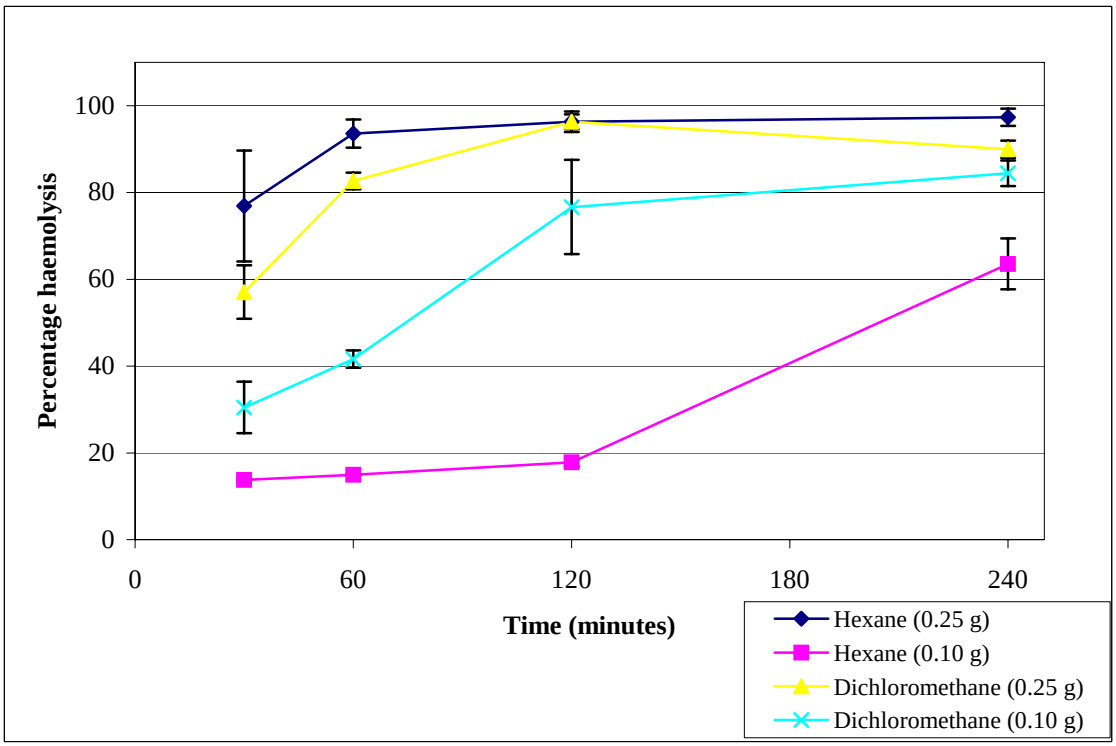

Figure 11.09: Haemolytic activity of hexane-rich and dichloromethane-rich extracts of F. semitectum S2-0207.

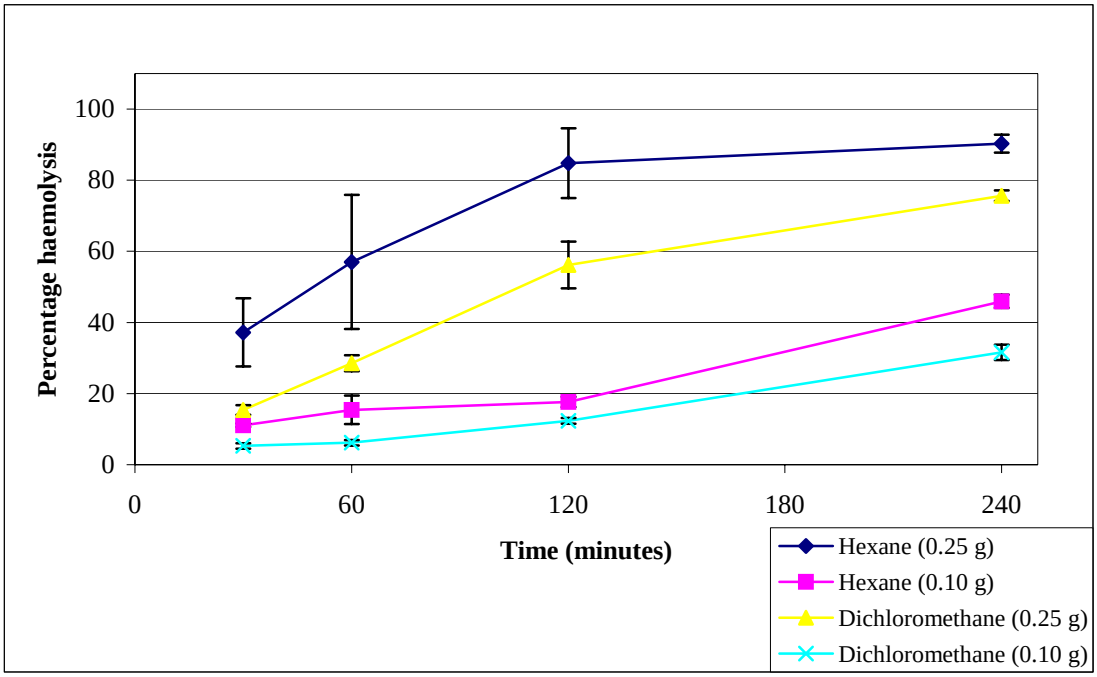

Figure 11.10: Haemolytic activity of hexane-rich and dichloromethane-rich extracts of T. virens W4-0119, from implicated sago.

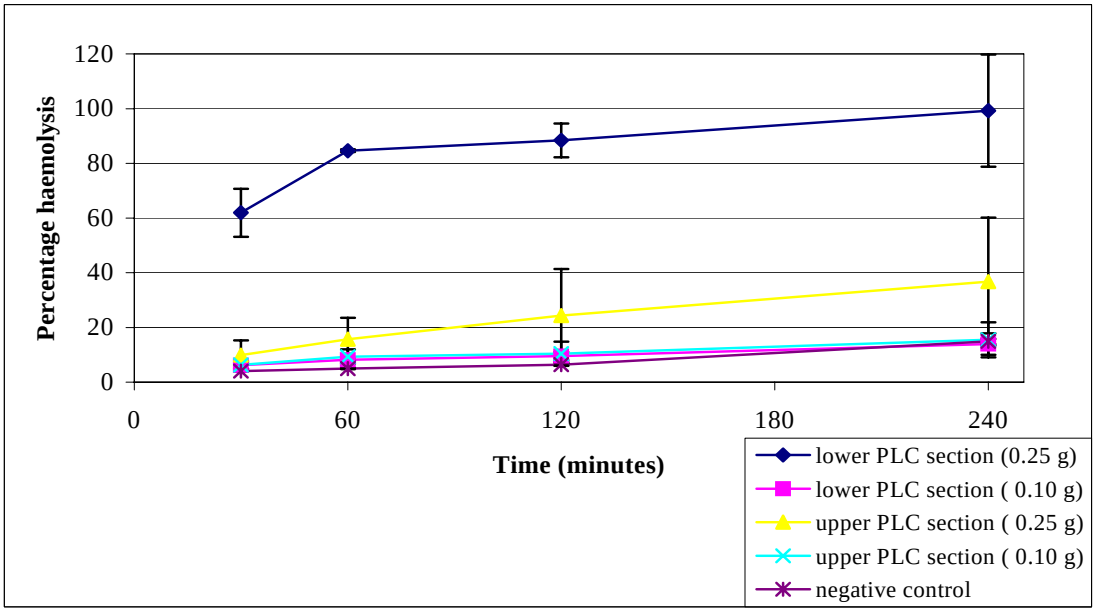

Figure 11.11: Haemolytic activity in the hexane extract of uninoculated wheat after separation into two fractions on a PLC plate: lower section from origin up to and including citrinin (Rf 0–0.55); upper section above this (Rf 0.55–1.0).

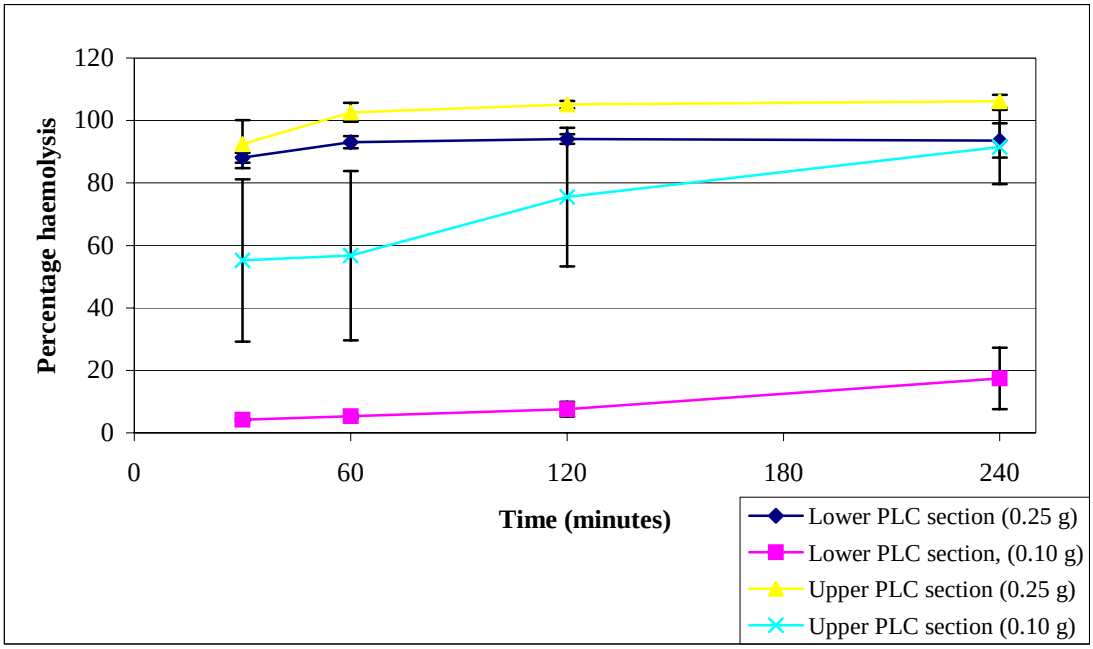

Figure 11.12: Haemolytic activity in the hexane extract of S. chartarum ATCC 9182 after separation into two fractions on a PLC plate: lower section from origin up to and including citrinin (Rf 0–0.55); upper section above this (Rf 0.55–1.0).

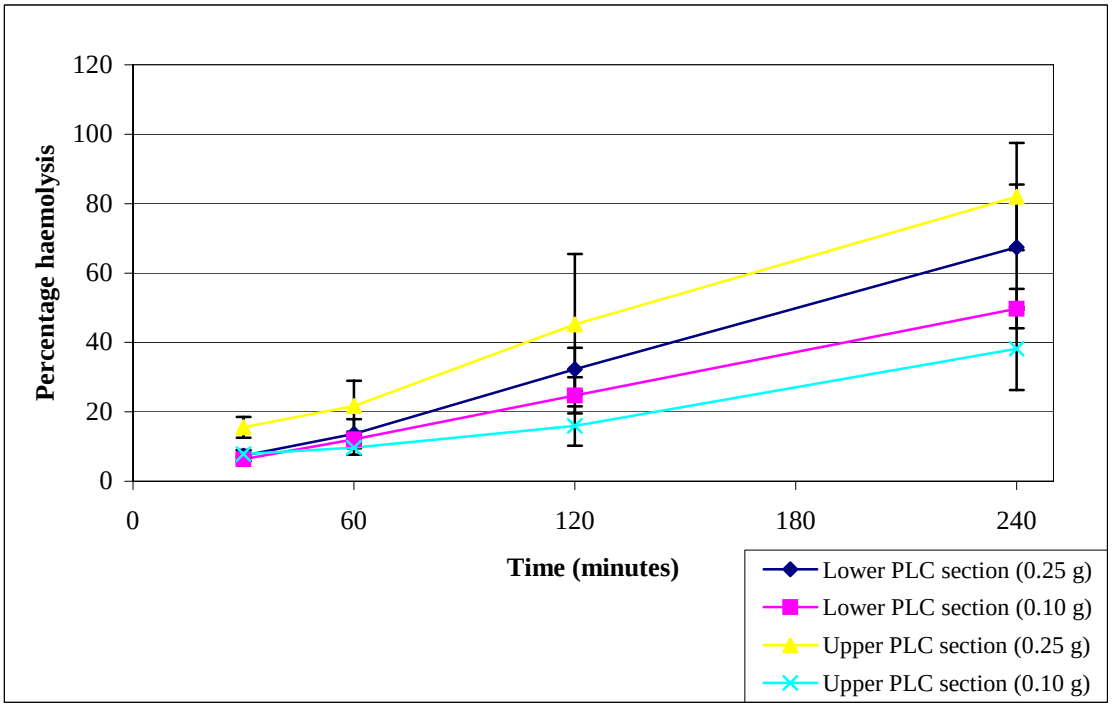

Figure 11.13: Haemolytic activity in the hexane extract of T. reesei ATCC 26921 after separation into two fractions on a PLC plate: lower section from origin up to and including citrinin (Rf 0–0.55); upper section above this (Rf 0.55–1.0).

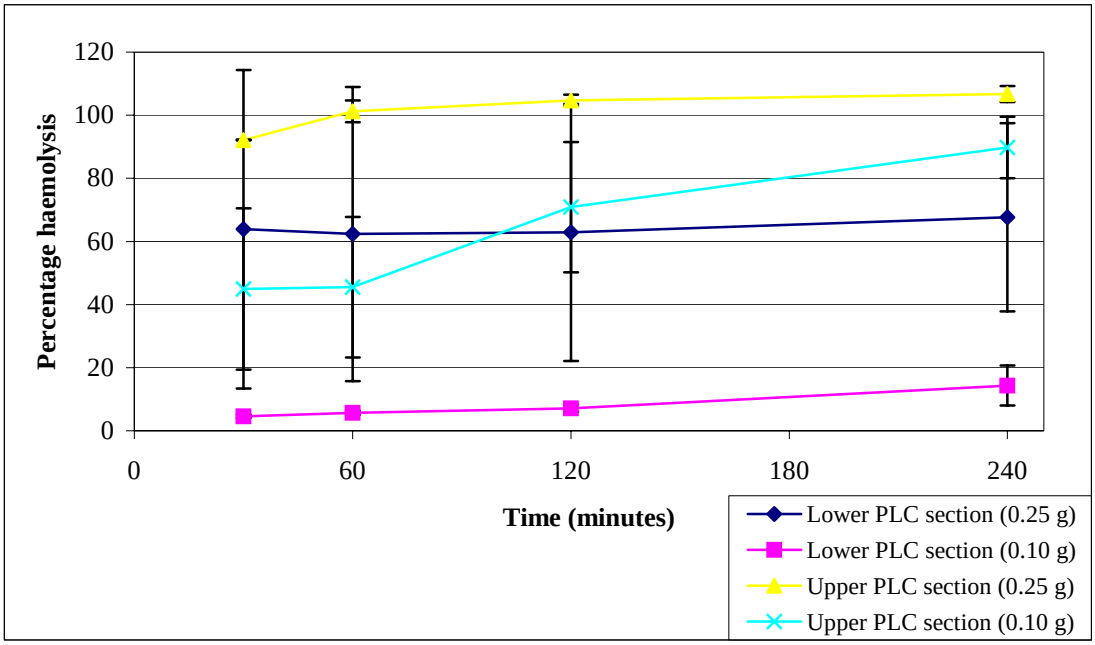

Figure 11.14: Haemolytic activity in the hexane extract of P. steckii W1-1101 after separation into two fractions on a PLC plate: lower section from origin up to and including citrinin (Rf 0–0.55); upper section above this (Rf 0.55–1.0).

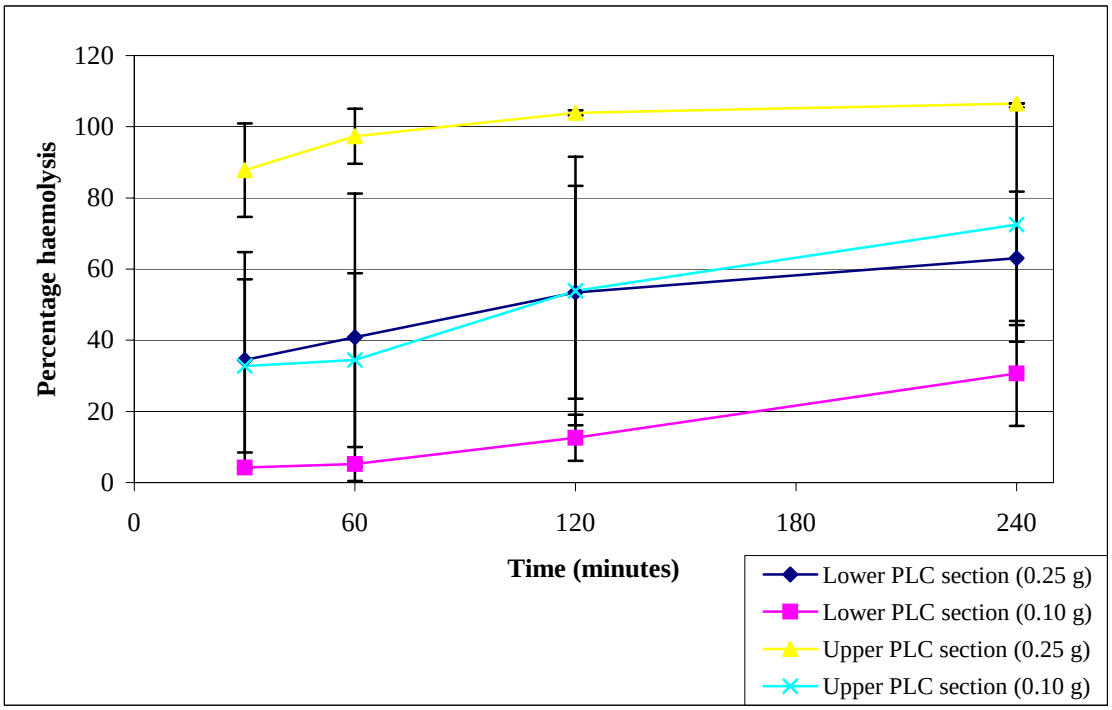

Figure 11.15: Haemolytic activity in the hexane extract of P. steckii W1-1301 after separation into two fractions on a PLC plate: lower section from origin up to and including citrinin (Rf 0–0.55); upper section above this (Rf 0.55–1.0).

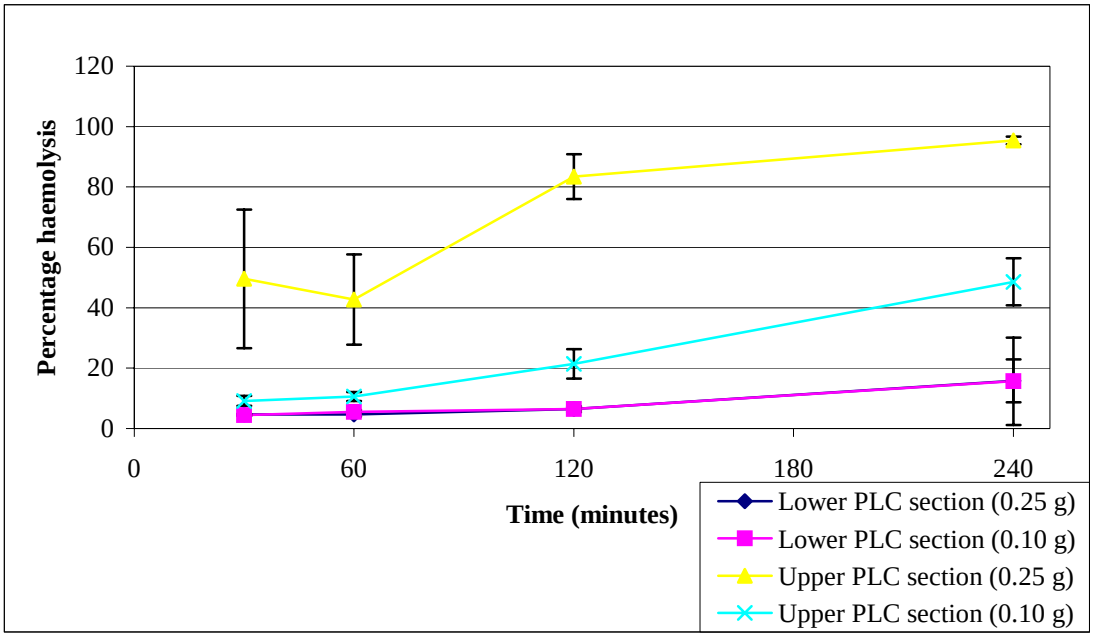

Figure 11.16: Haemolytic activity in the hexane extract of P. steckii S2-1305 after separation into two fractions on a PLC plate: lower section from origin up to and including citrinin (Rf 0–0.55); upper section above this (Rf 0.55–1.0).

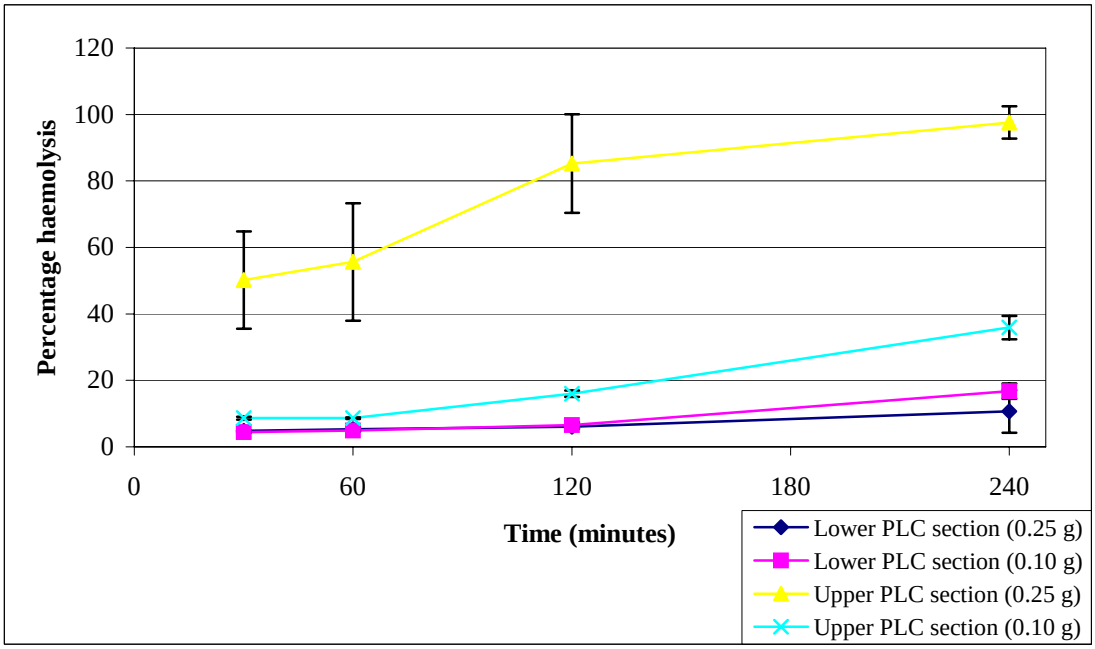

Figure 11.17: Haemolytic activity in the hexane extract of P. brevicompactum S1-1201 after separation into two fractions on a PLC plate: lower section from origin up to and including citrinin (Rf 0–0.55); upper section above this (Rf 0.55–1.0).

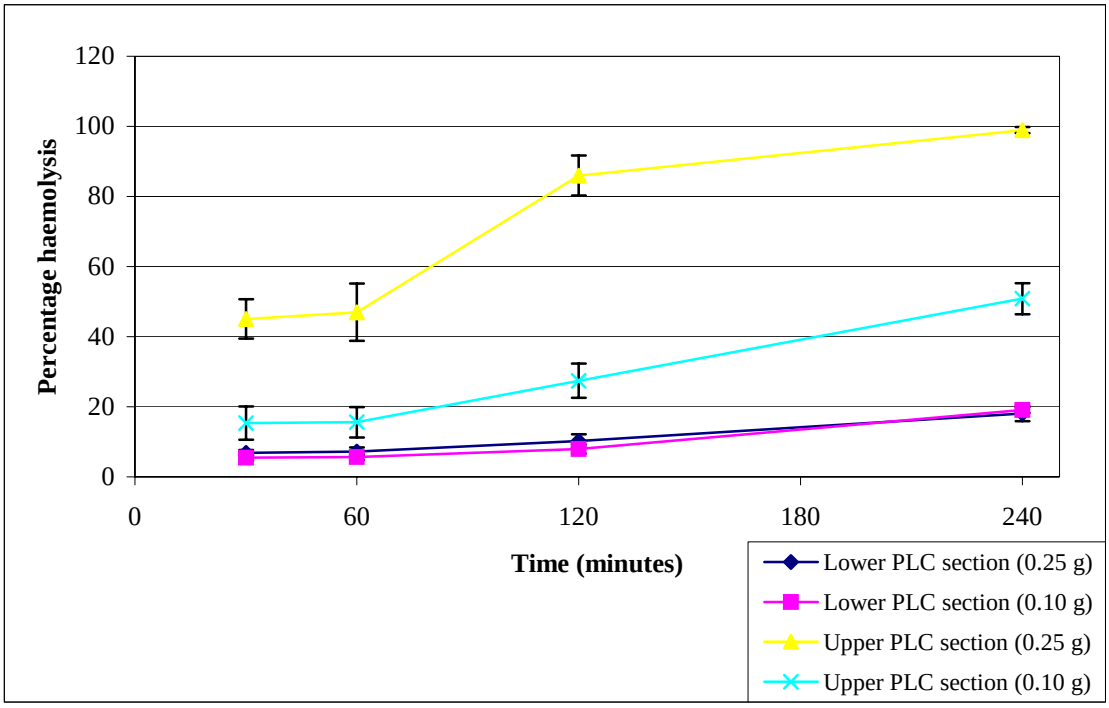

Figure 11.18: Haemolytic activity in the hexane extract of A. flavipes S2-0207 after separation into two fractions on a PLC plate: lower section from origin up to and including citrinin (Rf 0–0.55); upper section above this (Rf 0.55–1.0).

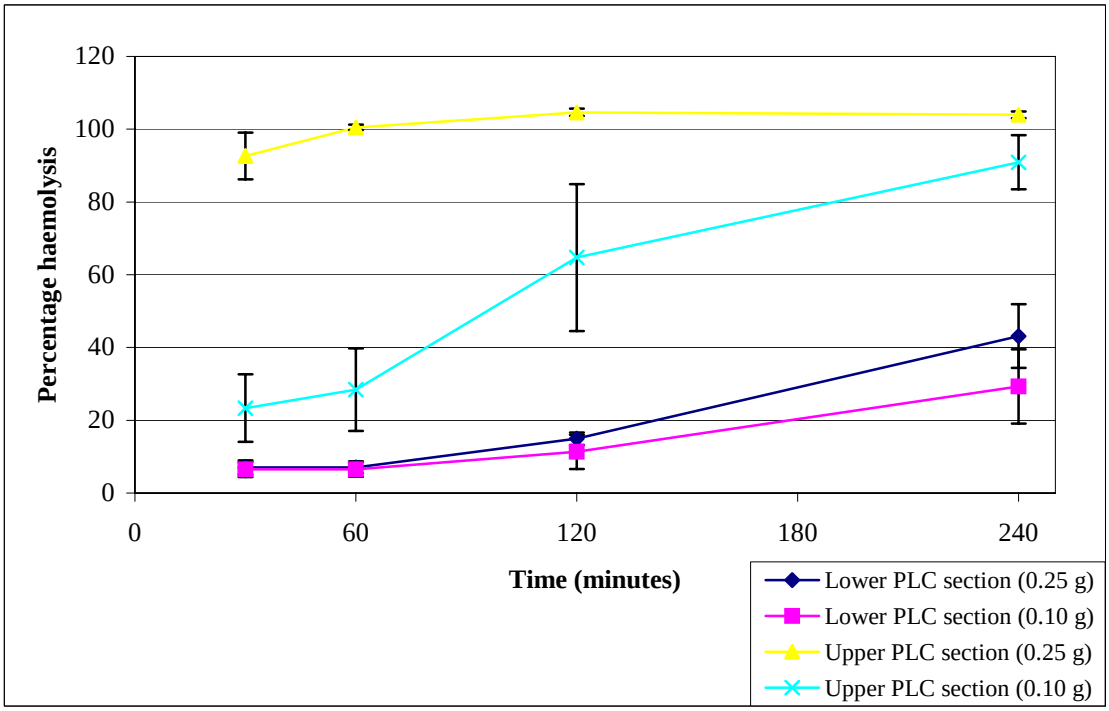

Figure 11.19: Haemolytic activity in the hexane extract of F. semitectum S1-0406 after separation into two fractions on a PLC plate: lower section from origin up to and including citrinin (Rf 0–0.55); upper section above this (Rf 0.55–1.0).

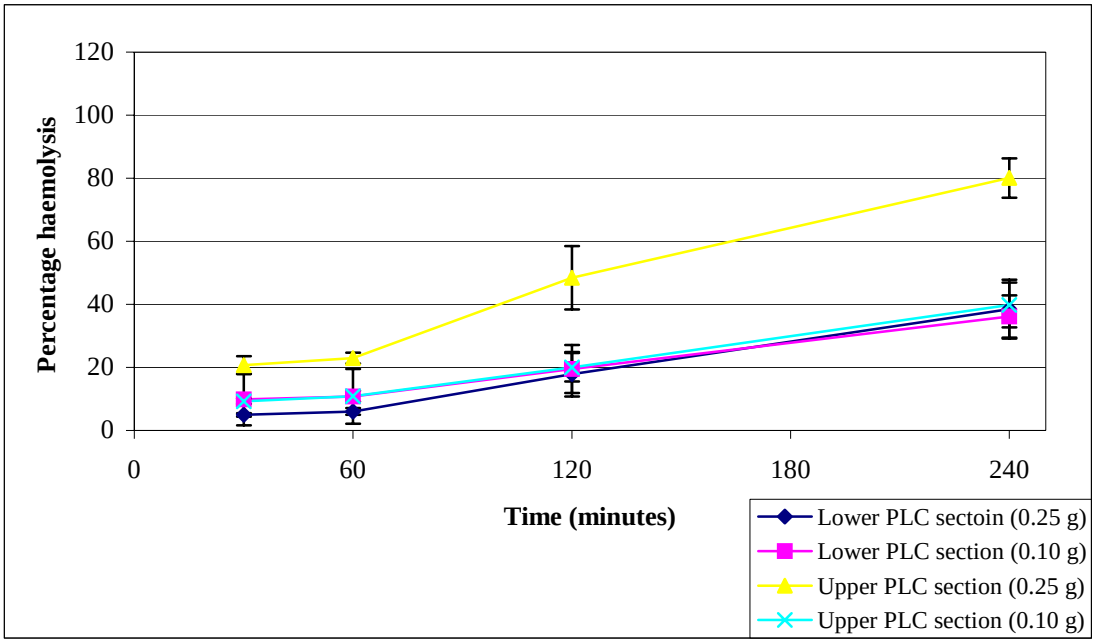

Figure 11.20: Haemolytic activity in the hexane extract of T. virens W4-0119 after separation into two fractions on a PLC plate: lower section from origin up to and including citrinin (Rf 0–0.55); upper section above this (Rf 0.55–1.0).

11.4 Reduction of contamination and HACCP analysis

Table 11.12: Initial steps of HACCP application: identification of steps involved in the process, the associated hazards, appropriate control measures and corresponding CCPs.

Step	Hazards	Control measures	CCPs	CCPs
			Notional	Practicable
Tree selection	(a) Growth of palm in contaminated soil: faecal & chemical	Minimise contamination through adequate sanitation, appropriate waste disposal & sound environmental practises of industry; select the tree for processing in a location that has not been recently contaminated		CCP 2 (#1)
Tree felling	No hazards identified			
Trunk storage	(a) Enteric pathogens (trunks in water)	(a) Improved sanitation	CCP 2	
	(b) Environmental & zoonotic pathogens (trunks on land)	(b) Do not store trunks for long duration; minimise animal access to sago logs		CCP 2 (#2)
Pith maceration and removal	(a) Environmental pathogens	(a) Minimise contact of sago pith with soil	CCP 2	
	(b) Pathogens of human origin	(b) Wash hands with soap & water; minimise body contact with pith	CCP 2	
	(c) Inoculation of macerated pith with dirty utensils	(c) Thoroughly clean utensils prior to use		CCP 2 (#3)
Starch extraction	(a) Contaminated water	(a) Use clean water: minimal faecal contamination	CCP 2	
	(b) Environmental pathogens	(b) Minimise contact of pith/starch with soil; use clean receptacles	CCP 2	
	(c) Pathogens of human origin	(c) Wash hands with soap & water; minimise body contact with pith/starch	CCP 2	

Starch storage and fermentation	(a) Survival & multiplication of pathogens	(a) Use clean water (if used); minimise environmental contamination; rapid and prolonged fermentation	CCP 2	CCP 2 (#4)
	(b) Contamination via air- & soil- borne fungal spores	(b) Storage under airtight conditions away from main sources of contamination	CCP 2	CCP 2 (#5)
	(c) Exposure to vermin (insects, rodents)	(c) Storage under conditions unfavourable to infestation		CCP 2 (#5)
	(d) Compromised storage conditions with time resulting in fungal growth	(d) Rapid and prolonged fermentation; limit storage duration		CCP 2 (#4)
Food preparation	(a) Survival of pathogens in sago	(a) Adequate cooking		CCP 2 (#6)
	(b) Re-contamination by handling, utensils, environment	(b) Wash hands and use clean utensils	CCP 2	
	(c) Growth of pathogens in cooked sago if not consumed within 4 hrs	(c) Consume sago soon after cooking	CCP 2	