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MICROBIAL DETOXIFICATION OF GADUNG (*Dioscorea hispida* Dennst) CHIPS: EFFECT OF MICROBES LOADING AND TIME

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Gadung (*Dioscorea hispida* Dennst.) is an underused tuber grown in various parts of Southeast Asia and its neighbouring islands. The countryside people in this area use this carbohydrate rich tuber as staple food after boiling, steaming or frying, while some others make it into flour, cakes, pancakes and porridge (Ashri *et al*., 2014). The resistant starch of this tuber allows a sluggish digestion and results in a slow glucose release and absorption in human gastrointestinal tract that drives its potential use in lowering the risk of obesity, diabetes and related diseases (Aprianita *et al*., 2009). Being gluten free, this tuber offers great capacity to reduce the prevalence of celiac disease and some allergic reactions (Rekha & Padmaja, 2002).

The major issues related to the underutilisation of gadung tuber as a food source for human consumption comes from its high cyanide level (Edijala *et al*., 1999). Cyanogenic glycosides (CG) present in this tuber are the precursors for the release of highly toxic free cyanide via hydrolysis during food processing (Akintonwa *et al*., 1994). Hence, preventing gadung tubers from hydrolysis will let the CG remain stable and the foods produced from this tuber are safe for consumption. Consumption of foods bearing high level of cyanogens may induce cyanides poisoning with symptoms of nausea, diarrhoea, stomach pains, vomiting and other acute intoxications (Akintonwa *et al*., 1994; Mlingi *et al*., 1992). Bourdox *et al*. (1982) reported that daily consumption of food products with unsafe cyanogens level may lead to chronic cyanides toxicity and aggravate goitre, while in severe conditions, it induces paralytic diseases (Tylleskar *et al*., 1992). Indeed, cyanogens and their derivates must be removed from the food sources to make the foods safe for consumption.

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An efficient processing technique is expected to reduce cyanogens level in food materials, below the safe level of 10 mg hydrocyanic acid (HCN) equivalent per kg dry matter set by world health organisation (WHO) (Mlingi *et al.*, 1995). Traditionally, Malaysians detoxify gadung tuber by boiling, roasting or soaking in flowing water for 7 – 14 days (Hudzari *et al*., 2011), whereas their Thai counterparts remove the cyanogens from the tuber by peeling, slicing, soaking in flowing water for up to 7 days or soaking in daily changed salted water for up to 5 days and drying. Then, the dried tuber chips are hydrated, boiled or steamed before consumption (Tattiyakul *et al*., 2012). Meanwhile, Indonesians detoxify gadung tuber via a knotty process involving peeling, slicing, smearing with ash from firewood combustion, pressing, drying, soaking in flowing water for 2 days, steaming and drying to obtain edible dry tuber chips (Sunarsih *et al*., 2007). Some other detoxifications of gadung tuber include soaking in saline water, repetitive soaking in water for 3–5 days, and boiling (Harijono *et al*., 2008).

Aspergillus niger or *Panus trigimus* as inoculum has been used in solid state fermentation for detoxification and degradation of toxic cyanogens in cassava peels to produce poultry feed (Purwadaria, 2014; Behera & Ray, 2017). Padmaja & Balagopal (1985) fermented cassava tuber and peels using a mixed culture inoculum for 72 hrs to effectively remove the cyanogens and leave total bound cyanide of about 24 to 26% and 15 to 33%, respectively. The high activity β-glucosidase from *Saccharomyces cerevisiae* NCIM 3186 has been harnessed to reduce cyanogens in sorghum juice and solution media by 84.58% and 85.72% (Bokanga, 1995; Panda & Ray, 2016). Similarly, Eustace and Dorothy (2000) also reported a cyanide reduction of 76.69% in cassava peels by *Saccharomyces*

cerevisiae. Considering that *Rhizopus oryzae* is able to produce β-glycosidase, this fungus can play a key role in the degradation of cyanogenic glycosides and assimilating the cyanide into an usable form in cassava roots (Padmaja & Balagopal, 1985; Gimmler & Hartung, 2012). For centuries, ragi has been used as a dry starter in the traditional food fermentation during preparation of *tapai* (fermented rice) and *tuak* (Balinese alcoholic drink) (Sujaya *et al*., 2004). Sujaya *et al*. (2002) observed that ragi contains filamentous fungi, yeasts and bacteria. Hesseltine *et al*. (1988) reported that out of the 41 ragi samples from seven Asian countries, at least one yeast and one *Mucoraceous* mold (*Mucor*, *Rhizopus*, or *Amylomyces*) were present with one or two types of cocci bacteria in every sample of the ragi. Gandjar, (2003) observed that traditional ragi tapai commonly comprises strong amylolytic moulds (*Rhizopus oryzae*, *Amylomyces rouxii*, *Mucor* sp. and *Candida utilis*) and yeasts (*Saccharomyces cerevisiae*, *Saccharomycopsis fibuliger*, *Endomycopsis burtonii*). Therefore, with the presence of *Saccharomyces cerevisiae* and *Rhizopus oryzae* in ragi tapai suggests that ragi tapai can be a strong potential microbes' source for gadung detofixication. The aim of this current work is to examine the influence of microbes loading and time on the cyanides level of gadung tuber chips during microbial detoxification using ragi tapai.

In this present study, mature (9 months age) gadung tubers were harvested from traditional gadung cultivation on dry lands nearby Semarang city. The outer covering was carefully peeled off and the flesh was sliced into chips with ± 5 mm thickness using a local slicing machine. Analytical grade (purity ≥ 98% w/w) chemicals (H_3PO_4, KI, NaOH and $AgNO_3$) used in this work were the products of Sigma-Aldrich and were bought from local authorized distributor in Semarang, Indonesia and directly used without prior treatment. Detoxification of gadung tuber chips was performed using a solid-state facultative fermentation employing ragi tapai as dry starter. Approximately 450 gm of gadung chips were washed using flowing water to remove the mucilage, placed them on a perforated plastic tray, carefully sprayed with distilled water and let to drain. They were then mixed with dry starter (containing 1, 2, 3, 4, or 5 gm of ragi tapai powder) in a large sterile glass pan. The mouth of the pan was covered with a piece of sterile white cloth fastened to the pan by a rubber band. The mixture was placed in a wind free area and left to ferment for 168 hrs. Samples of fermented tuber chips were taken from the fermentation system every day (24 hrs) for cyanides level determination.

The cyanogenic glycoside level in both raw and fermented gadung tuber chips was determined by alkaline titration method suggested by AOAC (1995) as HCN equivalent (ppm). A carefully weighed 20 gm of ground gadung chips sample was steeped in a mixture of 200 ml distilled water and 10 ml of H_3PO_4. The sample was stored and left at ambient temperature overnight to release all the free bounded hydrocyanic acid. It was then moved into distillation flask and a drop of paraffin was added as antifoaming agent. The flask was then fitted to another distillation apparatus and distilled to obtain 150 ml distillate in the receiving flask containing 20 ml of distilled water and 0.5 gm of NaOH pellets. The distillate was then transferred into 50 ml volumetric flask and made up to mark with distilled water. After addition of 2 ml of 5% KI, the solution was titrated against 0.02 M $AgNO_3$ solution till the occurrence of faint but permanent turbidity. (1 ml of 0.02 M $AgNO_3 \equiv$ 1.08 mg HCN). The determination was performed in triplicates for each of the samples.

Obviously, gadung tuber chips experienced alteration of their physical appearance during fermentation. Initially, they were clear white and firm in texture, but they gradually turned to be pale yellow in colour and softer following simultaneous release of a mild alcoholic or sour odour during fermentation. Fig. 1 shows that fermentation of gadung tuber chips without loading of ragi tapai is able to reduce the cyanide level to some extent and reach a nearly constant value (168.33 mg/kg) after 144 hrs. It seemed that the extraneous microflora could have induced fermentation of gadung tuber chips although their role in the detoxification was minimal (Westby & Twiddy, 1992). Furthermore, autolytic hydrolysis of the cyanogenic glycosides might have occurred with subsequent dissolution of HCN in the fermentation medium (Ketiku *et al*., 1978). The β-glucosidases involved in this study belong to glycoside hydrolases (GH), which are mostly secreted by bacteria, fungi and yeast (Singhania *et al*., 2013) and can be categorised as broad substrate specific, which are able to hydrolyse wide range of substrates with different bonds such as β(1→4), β(1→3), β(1→6), α(1→4), α(1→3), and α(1→6) linkage. As retaining enzymes, the β-glucosidases have β-configuration and hydrolyse cyanogenic substances by cleaving their β-glucosidic bonds to produce glucose units (Cairns & Esen, 2010; Ahmed *et al*., 2017). The retaining enzymes catalyse the hydrolysis via double displacement mechanisms, i.e. the glycosylation and deglycosylation. In the glycosylation, the catalytic acid/base donates a proton to the substrate leading to formation of oxocarbonium ion, and then the nucleophile attacks the anomeric carbon atom yielding enzyme-glycosyl intermediate. In the deglycosylation, a water molecule attacks enzyme-glycosyl intermediate to displace the catalytic nucleophile from the glucose with the assistance of

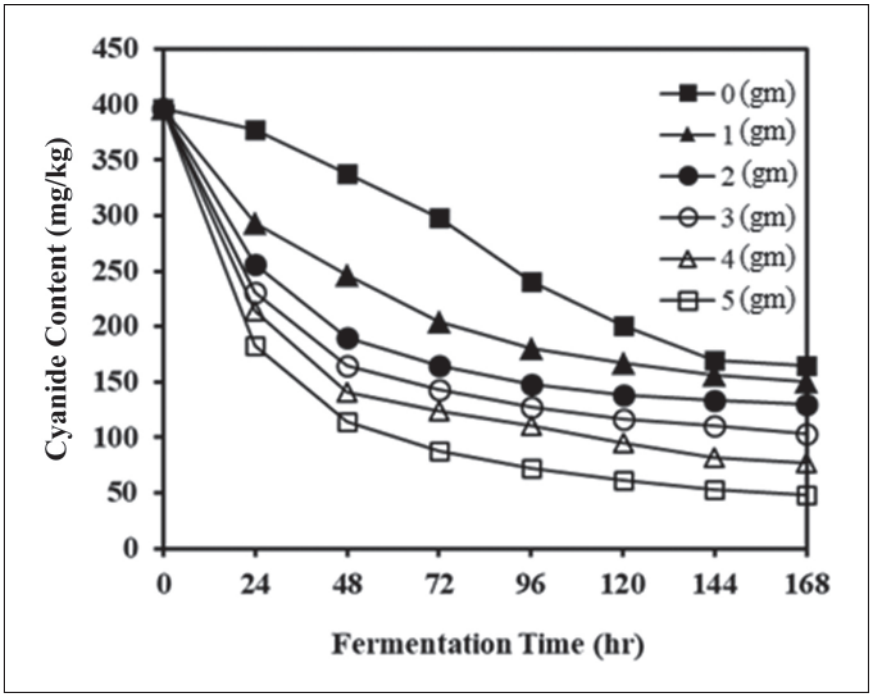

Fig. 1. Cyanide level of gadung tuber at various ragi tapai starter loadings and time.

the catalytic acid/base (Zechel & Withers, 2000; Qi *et al*., 2008). Sasongko (2009) reported a similar finding where the cyanide level of gadung tuber chips was only reduced by less than 1% after 73 hours of natural fermentation. Therefore, the final cyanide level in the gadung tuber chips obtained from natural fermentation is still far higher than the threshold cyanide level in food set by Food Standards Australia and New Zealand (10 mg/kg) (FSANZ, 2009) and Indonesian standard (40 mg/kg) (Djazuli & Bradbury, 1999). Nambisan and Sundaresan (1985) found that heap fermentation of cassava chips was capable to reduce the cyanide level from 96–128 mg/kg to within less than safe level (40 mg/kg). In addition, Mlingi and Bainbridge (1994) also reported that sun drying of cassava chips with 48–64 mg/kg cyanide level may result in safely consumed cassava tuber flour with 40 mg/kg. Therefore, the gadung chips obtained from this study should undergo sun drying and pulverising into flour to make them safe for consumption.

The loading of ragi tapai was found to facilitate cyanide level reduction by supplying microbes for fermentation, which were mainly *Rhizopus oryzae* (8×10^7 to 3×10^8 cell/g), *Saccharomyces cerevisiae* (3×10^6 to 3×10^7 cell/g) and coccii bacteria (1×10^5 cell/g) as reported by Merican and Quee-Lan (2004). It is evident in Fig. 1 that loading of more ragi tapai to fermentation medium resulted in higher cyanide level reduction. The highest detoxification rate was observed in the first 24 hrs of fermentation. The highest detoxification rate was recorded when fermentation was performed using 5 gm ragi tapai, which corresponds to 1.10% (w/w) of gadung tuber chips or equivalent to 11 times of microbes loading usually used for fermentation of pre-gelatinized glutinous rice (Gandjar, 2003). The *Rhizopus oryzae* in the ragi tapai could provide additional β-glycosidase enzymes to the gadung tuber chips and subsequently enhanced the overall enzyme activity for the degradation of cyanogens (Antai & Nkwelang, 1998). Significant reductions of HCN level in cassava chips and removal of HCN, oxalic acid and phytic acid from soybean meal as a result of fermentation process has been reported by Bassir (1969) and Eka and Kay (1977).

The detoxification process of gadung tuber chips was very rapid as indicated by removal of more than 80% of the cyanide level in the first 72 hrs of fermentation using ragi tapai. Cyanide level of gadung tuber chips reduced gradually as fermentation proceeds to about 144 hrs and reached a constant value. Similar observations were reported by Antai and Nkwelang (1998) for toxicant removal from *Icacina mannii* root paste via fermentation using *Saccharomyces cerevisiae*. Obviously, the detoxification rate was fastest in the first 24 hrs of the fermentation. This is possibly due to the high number of *Saccharomyces cerevisiae* cells in the fermentation media within 18 to 24 hours fermentation at room temperature, which continuously secretes enzymes for cyanide degradation (Bijkerk & Hall, 1977). Similarly, *Rhizopus oryzae* secreted linamarase with highest

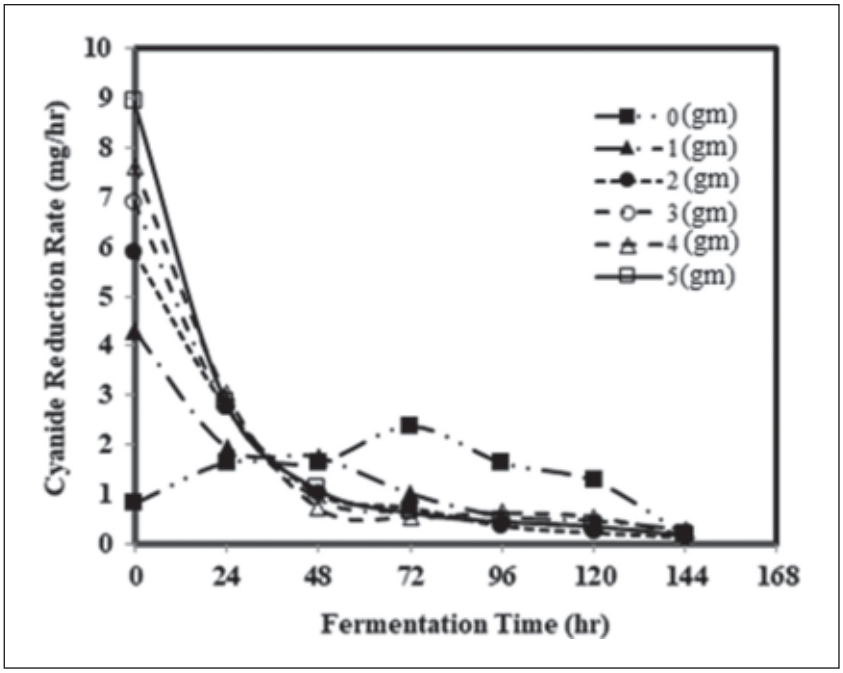

Fig. 2. Cyanide reduction rate at various ragi tapai starter loadings and time.

activity (67.56) during its first 24 hrs growth phase fermentation either with the presence or absence of linamarin (Padmaja & Balagopal, 1985). The cyanide reduction rate continues to decrease as fermentation goes by for a longer time as shown in Fig. 2. The cyanide reduction rate increases with fermentation time to a maximum value at 72 hrs for fermentation without ragi tapai, but then decreases almost linearly. This suggests a slow adaptation of wild microbes to cyanides, which indicates the lag phase observed in the fresh gadung tuber chips. Once detoxification is completed, they can grow rapidly as a visual symptom of microbial attack (Maini & Balagopal, 1978). In all cases, almost no cyanides reduction was observed when fermentation took place longer than 144 hrs.

In conclusion, natural fermentation of gadung tuber chips for 144 hrs is able to reduce around 58% of cyanide level. The gadung tuber chips with cyanide level of 48.12 mg/kg obtained from fermentation of 450 gm gadung tuber chips using 5 gm ragi tapai for 168 hrs can be sun dryed and mechanically pulverised into flour to make them safe for consumption.

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