CASSAVA AS ANIMAL FEED

Proceedings of a workshop held at the University of Guelph 18-20 April 1977

Sponsored by the International Development Research Centre and the University of Guelph

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Contents

Cassava as a Substrate for Single-Cell Protein Production: Microbiological Aspects

Kenneth F. Gregory[1]

Data from several laboratories have shown that filamentous fungi produced on cassava and other substrates are satisfactory as high-protein animal feed. At Guelph, simple low-cost methods for converting cassava to microbial protein were sought. Silage was produced, from cassava and inorganic nitrogen, with up to 6.4% protein but no practical way could be devised for doing this in rural, tropical areas. Liquid, aerated fermentation with thermotolerant fungi was shown to hold more promise of having practical value. Whole ground cassava can be used in a nonaseptic system because the fermentation's high temperature (45–47 °C) and low pH (3.5) inhibit contaminants. Water at ambient temperature suffices to remove excess heat. The fungi hydrolyze the starch by their own enzymes and can be harvested by simple filtration. The three cultures that gave the best nutritional response with rats contained 44–50% crude protein and 35–38% true protein. The product formed with *Aspergillus fumigatus* I-21A on whole cassava (including nonfermented fibre and bark) contained 37% crude and 27% true protein. Simple conditions for a daily batch production schedule have been defined. Fungi capable of growth at body temperature could be an infection biohazard for unusually susceptible individuals. A nonrevertible, asporogenous mutant of *A. fumigatus* I-21 (I-21A), used to avoid risk of infection by spore inhalation, may not be a sufficient safeguard since viable hyphal fragments can occur in an aerosol. "Cold-sensitive" mutants unable to grow below 40 °C, but able to grow normally at 45–47 °C, were isolated by five sequential mutations. Nonrevertible mutants, required for absolute safety, have not yet been obtained.

Cassava (*Manihot esculenta*) is believed to be one of the most efficient converters of solar energy to carbohydrate, with an ability to store energy at a daily rate per hectare appreciably greater than that of other high-yielding crops such as maize and rice (Coursey and Haynes 1970). The storage roots are as poor in protein, however, as they are rich in calories. The crude protein content (total N × 6.25) of whole cassava roots averages 3.5%, or less, of their dry weight (Pond and Maner 1974; Oke 1966; Jennings 1970; Grace 1971). Furthermore, 40–60% of the total nitrogen is nonprotein nitrogen (Pond and Maner 1974). It follows that, when cassava is used as an animal feed, large amounts of protein-rich feeds must be employed to balance the ration.

Microbial Protein from Cassava

Several investigators have studied the use of microorganisms to convert cassava into microbial protein. The traditional fermentation of grated cassava, developed by the Amerindians and used for making farinha do mandioca in Brazil and the similar product gari in Africa, cannot result in much net protein synthesis because supplemental nitrogen is not added to the mash. The consecutive growth of the bacterium *Corynebacterium manihot* and the yeast *Geotrichum candida*, which occurs during the fermentation (Collard and Levi 1959; Akinrele 1964), might convert some of the nonprotein nitrogen into protein but no data are available on this point. A solid-type fermentation process for cassava, modeled on traditional food processes in Southern Asia, especially the tempeh produced in Indonesia, was developed at the Tropical Products Institute in London (Brook et al. 1969; Stanton and Wallbridge 1969, 1972). In this process the roots were peeled, dried, and ground to a flour. The fungus inoculum was added as spores along with ammonium nitrate, monopotassium phosphate, and water. The paste-like mixture was extruded into spaghetti-like strands and allowed to ferment in shallow trays for 3 days. Species in several genera of fungi were used but *Rhizopus* species appeared to be preferred. Final yields of crude protein ranging from about 2 to 4% were reported. Because the peeled cassava roots contained only about 0.2% protein, the percentage increase in protein was impressive. The final product must be considered still to be a low-protein food, however, and the process, as described, appears to be unsuitable for the production of animal feed.

Varghese et al. (1977) described a procedure

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in which cassava chips, supplemented with chicken dung, were steamed, inoculated with *Aspergillus* or *Rhizopus* cultures and incubated in fermentation trays. The final products contained about 4–10% crude protein but the data presented do not permit discrimination between fungal protein and nonconverted nitrogenous compounds added with the dung. A similar procedure followed by Hutagalung and Tan (1977) yielded a product from cassava, dung, and a *Rhizopus* sp., with 10% "true protein," about one-third of which can be accounted for by the true protein contributed directly by the dung and the cassava.

Much higher yields of protein have been achieved in liquid fermentation systems based on cassava. Stasser et al. (1970) described a process in which the yeast *Candida utilis* was used to produce a product containing 35% crude protein on a dry weight basis. Most yeasts are unable to hydrolyze starch, therefore the process required enzymatic or acid hydrolysis of the cassava starch, as well as sterilization of the substrate, aseptic conditions, and centrifugation to recover the product.

Simpler processes are possible with filamentous fungi, which can hydrolyze starch with their own amylases and can be recovered by filtration rather than centrifugation. Gray and Abou-El-Seoud (1966) grew several filamentous fungi on ground cassava roots supplemented with ammonium chloride and corn steep liquor. Products containing 13–24% crude protein were obtained. Brook et al. (1969) obtained similar concentrations of protein with cassava fermentations using 27 strains of fungi but one culture yielded mycelium with 33.6% crude protein. The best-yielding fungi studied in our laboratory produced mycelium with 44–50% crude protein.

Value of Fungi as Animal Feed

Extensive studies have been carried out on the conversion of other substrates to protein by means of filamentous fungi. Some of these are listed in Table 1. The first four processes cited have shown particular promise. The process described by Church et al. (1972) was primarily designed to reduce the biochemical oxygen demand of corn and soybean processing wastes by converting the organic matter into mycelium. Nonaseptic continuous fermentation was carried out at the 40 000 litre scale in open oxidation ditches and resulted in a high-protein product that was readily consumed by rats and produced good growth responses. Tate and Lyle Limited have operated a pilot plant on Cyprus at the 3000 litre scale in which *Aspergillus niger* was produced from the carob bean (Imrie and Vlitos 1975). The product was readily accepted by both rats and boiler chicks with excellent results. More recently, a *Fusarium* species, which has a higher crude protein content (41–51%), has been under investigation in their laboratories. The strain of *Fusarium graminearum* studied by Anderson et al. (1975) is notable for its high crude protein content (54%). The "Pekilo" process developed at the Finnish Pulp and Paper Research Institute was successfully operated, with a 15 m^3 fermentor, on a continuous basis, producing mycelium of a strain of the fungus *Paecilomyces varioti* from sulfite waste liquor (Romantschuk 1975). The process was run aseptically. A prototype Pekilo-plant has since reached normal commercial operation (M. Ingman, personal communication) and the product has been approved as an animal feedstuff by the Finnish authorities. Feeding experiments with pigs, chicks, and calves showed that the fungal protein gave growth responses equivalent to those obtained with skim milk powder.

The above studies, as well as those carried out in our own laboratories (Khor et al. 1976), have clearly shown that fungal mycelium can serve as a satisfactory protein-rich animal feed. Smith et al. (1975), however, found that a strain of *Aspergillus oryzae* that gave good results with rats gave poorer results than expected with pigs. The nutritional value of fungal mycelium tested at Guelph is discussed by Alexander (1977).

Increasing the Protein Content of Cassava Silage by Fermentation

Studies were undertaken in our laboratories on the microbial enrichment of cassava by two distinct types of processes — a moist solids fermentation and a liquid aerated fermentation. The first approach is described in this section.

The studies of Tutarov et al. (1967) with N-supplemented maize silage and Brook et al. (1969) with cassava-based "vegetable-cheeses" suggested that a significant increase in the protein content of cassava silage might be possible by encouraging microbial growth in N-supplemented cassava silage. Because the production of cassava silage has value in itself, as

Table 1. Some filamentous fungi used for protein.

	Substrate	Protein in mycelium: % of dry wt.	Method of assay	Reference
Trichoderma viride	corn and soybean processing wastes	42–45	sum of amino acids	Church et al. 1972
Aspergillus niger	carob extract	35	Lowry method	Imrie & Vlitos 1975
Fusarium graminearum	starch	54 42	total-N × 6.25 amino-N × 6.25	Anderson et al. 1975
Paecilomyces varioti	spent sulfite liquor (pulp mill)	55–60	Not reported	Romantschuk 1975
Aspergillus oryzae	barley grain	44	total N × 6.25	Reade & Smith 1975 Smith et al. 1975
Sporotrichum pulverulentum	agricultural wastes	25–40	sum of amino acids	Von Hofsten 1976 Von Hofsten & Ryden 1975
Aspergillus fumigatus	cellulose	13.3	sum of amino acids	Rogers et al. 1972
Heterocephalum aurantiacum	cassava	33.6	total N × 6.25	Brook et al. 1969
Aspergillus fumigatus	cassava extract	44 35	total-N × 6.25 amino-N × 6.25	Gregory et al. 1976b

Table 2. Protein enrichment of cassava during experimental ensiling procedures.

Treatment of ground cassava[1]	Inoculation with *R. oligosporus* M87	Percentage protein[2]: 0 °C incubation	37 °C incubation	Final pH at 37 °C[3]
None	−	1.09	1.08	3.85
None	+	1.20	1.01	3.85
Supplement[4]	+	0.82	0.76	3.25
Supplement + ethylene oxide sterilization	+	0.88	3.13	—
Supplement + heat sterilization	+	1.03	3.65	3.45
Supplement + continuous perfusion	−	0.58	3.22	7.80
Supplement + continuous perfusion	+	0.63	3.17	7.75
Supplement, thin layers, eluates recycled discontinuously	+	0.76	6.44	—

[1] All samples were incubated in a laboratory-scale silo except in the last two experiments, where recycling perfusion units and large Buchner funnels were used. Incubation times ranged from 8 to 14 days.
[2] Protein = nitrogen not extractable with 5% trichloroacetic acid at 4 °C, times 6.25.
[3] Initial pH of the ground cassava ranged from 6.25 to 6.50.
[4] Supplement consisted of mineral salts and NH_4NO_3.

a means of preserving cassava as feed, even a modest increase in its protein content could have considerable value. This possibility was investigated by Sprung and Smith (see Sprung 1974). The results of this study are summarized in Table 2.

Cassava silage was readily produced in laboratory-scale fermentors and achieved satis-

factory acidity levels of pH 3.8 or less after six or more days incubation at 37 °C. The protein level of the cassava (calculated on the basis of nitrogen not extracted by cold trichloroacetic acid) was slightly over 1% on a dry weight basis and did not change significantly during the ensiling process (first line in Table 2).

A total of 171 new isolates and known cultures of moulds, yeasts, and bacteria were screened to select those that hydrolyzed starch but not protein, that grew at pH 4 and 45 °C, produced acid, and had a crude protein content of 28% or higher. A strain of *Rhizopus oligosporus* (M87) met all these criteria and grew well at high CO_2 and low O_2 tensions. This culture was used as an inoculum to supplement the normal cassava microflora in most experiments.

As expected, silage made with added *R. oligosporus* but without supplemental nitrogen did not result in increased protein levels although microbial growth was stimulated sufficiently that a lower final pH (3.25) was reached. Unfortunately, the addition of mineral salts and ammonium nitrate to nonsterilized chopped cassava did not result in increased protein in the final silage either. When the cassava was treated with ethylene oxide, to kill the competitive microorganisms, however, the fungus grew well and raised the protein content to over 3%. Heat sterilization of the cassava permitted greater growth and protein production, possibly because it gelatinized the starch and made it more readily attacked by the fungus.

When nonsterilized silage was continuously perfused with water containing the mineral and nitrogen supplement, the protein content was similarly increased to over 3%. The indigenous microflora was as effective in doing this by itself as when it was supplemented with the mould inoculum. It was believed that the low oxygen supply was probably limiting the amount of microbial growth and thus the amount of protein that was synthesized. Indeed, when the cassava was incubated in thin layers and liquid eluates from the cassava were recycled discontinuously, the protein content reached almost 6.5%, but the product was not always satisfactory as silage.

It was concluded that none of the effective methods would be of practical value for rural operations in tropical countries. It was apparent, that the closer the procedure came to an aerated liquid culture system the greater was the protein yield.

Table 3. Isolation of thermotolerant, amylolytic high-protein fungi.

Stage of screening	Number
Soil samples tested	724
Cultures isolated	147
Cultures with >44% crude protein	15
Cultures used in feeding trials	12
Cultures giving PER of 2.3 or higher[1]	3

[1]PER = protein efficiency ratio (g gain/g protein fed) in rat-feeding trials using diets with 10% true protein supplemented with methionine, normalized to a value of 2.5 for casein supplemented with methionine and tryptophan (Khor et al. 1976; Gregory et al. 1977).

Isolation of Cultures for Liquid Fermentation

At the outset, it was decided that the problems encountered in producing low-cost microbial protein from cassava in tropical countries should be minimized by judicious selection of the microorganisms (Table 3). Soil samples from 724 locations in Canada and Colombia were screened for organisms that would grow at a low pH (3.5) and temperatures in excess of 45 °C (Reade and Gregory 1975; Gregory et al. 1977). Only rare thermotolerant fungi could grow under these restrictive conditions and the fermentation could be expected to be immune to bacterial or yeast contamination and relatively resistant to fungal contamination. Furthermore, a high temperature fermentation would avoid the necessity of mechanical refrigeration for cooling, even in tropical countries. Cultures able to grow under these conditions were further screened for amylase activity as well as protein and methionine content. Amylolytic fungi could utilize cassava starch without the need of prior acid or enzymatic hydrolysis. Methionine was given special attention because almost all sources of single-cell protein are deficient in this amino acid.

The twelve cultures best meeting the above requirements were evaluated in rat-feeding experiments (Khor et al. 1976; Gregory et al. 1977; Alexander 1977). The three that gave protein efficiency ratios of 2.3 or higher are listed in Table 4. The first of these to be isolated was *Aspergillus fumigatus* I-21 (ATCC 32722) and it has received the most attention so far. As this species can cause a lung infection called aspergillosis if massive numbers of spores are inhaled by certain susceptible individuals, a nonsporeforming mutant (I-21A, ATCC 32723) was isolated following gamma

Table 4. Some properties of three thermotolerant, amylolytic high-protein fungi.[1]

	Crude protein (% of dry matter)	True protein (% of dry matter)	Doubling time at 45 °C (h)
Aspergillus fumigatus I–21 (parent)	44	35	3.5
A. fumigatus I-21A (asporogenous mutant)	49	37	3.5
Rhizopus chinensis 180	49	37	<3
Cephalosporium eichhorniae 152	50	38	5

[1]Data from Reade and Gregory (1975) and Gregory et al. (1977).

Table 5. Fermentation conditions selected for the nonaseptic production of protein from cassava mash by *A. fumigatus* I-21A.

Condition	Reasons for selection
Carbohydrate concentration = 4% (ca. 15% fresh cassava)	Fermentation is completed in 20 h from a 6.7% inoculum, permitting a daily production schedule. Higher concentrations take longer; lower concentrations give lower yield per litre
Mash heated to 70 °C for 10 min, immediately after grinding, in one-half final volume	Gelatinizes starch permitting complete utilization; prevents development of antifungal activity; provides desired starting temperature after dilution to final volume
Nitrogen source = urea (1.72 g/l)	No automatic pH control required; whereas, $(NH_4)_2SO_4$ results in excess acidity
Mineral supplement = KH_2PO_4 (0.25 g/l)	Assures sufficient phosphorus even with cassava roots which are low in P. All other mineral requirements except S are supplied by the cassava roots
Initial pH adjustment with sulfuric acid	Supplies sulfur requirement as well as acidity
pH 3.5	Optimum for protein production; inhibits bacterial growth
Temperature = 45 °C	Inhibits yeast growth, thus permitting use of nonaseptic conditions (although optimum temperature for the fungus is 37–40 °C)
Vigorous agitation and aeration during growth	Provides rapid oxygen transfer to growing cells

irradiation (Nielsen 1976) and used for most of the subsequent studies. The mutant could not be induced to form spores on a variety of media in a broad range of physical environments, nor could it be induced to back-mutate by either radiation or chemical mutagens. The asporogenous mutant was identical to the parent in its growth characteristics but had a slightly higher protein content (Table 4). The culture giving the best results in feeding trials was *Cephalosporium eichhorniae* 152 but this culture grows more slowly than the others and the establishment of optimum growth conditions to permit reproducibly high yields is proving to be difficult.

Fermentation Conditions for *A. fumigatus* I-21A

The conditions established for the production of microbial protein from cassava by *A. fumigatus* I-21A are summarized in Table 5. A self-aspirating fermentor was specifically designed for this process (Azi et al. 1975; Meiering and Azi 1977) and models with 200 and 3000 litre capacities were constructed for use in a pilot plant operation. The 6.7% inoculum provided by the 200 litre fermentor permits concentrations of cassava carbohydrate up to 4% to be converted to mycelium in 20 h, thus allowing 4 h for harvesting and refilling the fermentor on a daily production schedule.

The highest concentrations of protein in the product were obtained when the cassava roots were peeled prior to being ground. The peeling step was found to be unnecessary, however, because the fungus grew equally well when the whole roots were used and the final yield of protein per gram of carbohydrate supplied was the same (Reade and Gregory 1975). The protein concentration in the final product was lower, however, because of the presence of unfermented cassava fibre and bark.

Heating of the cassava mash to about 70 °C, in about one-half the final volume of water, was found to be necessary for three reasons. Firstly, unless the starch was gelatinized by heat it was not attacked rapidly or completely by the organism. Secondly, initial heating was required because of the high fermentation temperature used. Excess heat input was avoided if the volume heated was adjusted so that the subsequent addition of water at ambient temperature brought the mash to the fermentation temperature of 45–47 °C. Subsequent heat input was not required because the growing culture generated sufficient heat. Thirdly, if not heated, the cassava mash developed fungistatic activity that caused an unacceptably long lag in growth of the culture. The fungistatic activity is presumed to be due to the release of hydrogen cyanide from the glucoside linamarin when the grinding process brings this glucoside and the enzyme linamarase together (de Bruijn 1973). Dumping the ground cassava into hot water immediately after grinding prevented the development of most of this fungistatic activity, presumably because of the inactivation of the enzyme (Reade and Gregory 1975).

An alternative approach to decreasing the growth lag when an inoculum is placed in fresh cassava medium, is the isolation of cyanide-resistant mutants. Nielsen (1976) isolated mutants from gamma irradiated spores of *A. fumigatus* I-21 that had more than a threefold increase in tolerance to potassium cyanide as determined by respiration measurements with a Clark-type oxygen electrode. Although the cyanide-resistant mutants of microorganisms that have been described previously had respiratory impairment and grew slowly, the mutants isolated in this study had growth rates identical to that of the parent. Such cyanide-resistance has not yet been coupled with a mutation for asporogeny or a "safe" mutant culture (see Safety Considerations).

The only supplemental nutrients required for the fermentation are sulfuric acid (which lowers the initial pH to 3.5 and provides sulfur), KH_2PO_4 (which is only required in small amounts because the cassava roots supply all the necessary potassium and almost enough phosphorus), and urea (as the nitrogen source). All of the other mineral requirements are present in excess amounts in cassava roots (Gregory et al. 1976a). When urea is used as a nitrogen source no automatic pH control is required. Indeed, the only automatic control system required is a cooling system whereby water at ambient temperature is passed through a heat-exchanger in the fermentor as required to keep the temperature down to 45–47 °C.

At the completion of the fermentation only about 5% of the initial carbohydrate remained unutilized when finely ground cassava was used (Reade and Gregory 1975). When a rasper was used to prepare the cassava mash, however, a larger residue of carbohydrate remained (Santos and Gomez 1977) presumably because of poorer access of the fungus to its substrate. The fibrous nature of the final mycelial product was found to be well-suited to recovery by filtration. A simple roller-press filter system has been designed for this process (Meiering and Hayes, unpublished).

The final yield of product from whole cassava was about 520 g/kg carbohydrate supplied. The product contained 37% crude protein and 27% true protein. The concentration of protein in the product is appreciably less than in the mycelium itself, because of the presence of nonfermented cassava fibre and bark.

Safety Considerations

Two aspects of safety need to be considered in the production of single-cell protein (SCP). One is the freedom from toxic effects of the product for the consumer, whether that be man or animal. The cultures studied in this project appear to be harmless as far as rats are concerned (Khor et al. 1975; Alexander 1977) but toxicological evaluation of their effects on livestock needs to be done.

The other safety aspect is the possibility of infection or allergy in the personnel involved in producing the SCP. Many fungi that are capable of growing at body temperature are "opportunistic pathogens" in that they may incite infections in individuals who already have certain predisposing diseases, such as

tuberculosis, or are undergoing treatment with immunosuppressive drugs such as corticosteroids (Emmons et al. 1970; Chick et al. 1975). The genera most often involved are *Aspergillus* (causing aspergillosis) and *Rhizopus* and *Mucor* (causing phycomycoses).

Among the three species we have been studying that gave the best nutritional responses in rats, many strains of *Aspergillus fumigatus* are known to be involved in aspergillosis. Thus strain I-21 should be treated as if it has this potential. The other two species do not appear to have been reported as causes of infection in man. Nevertheless, other thermotolerant species of *Rhizopus* have been thus incriminated, and when *Rhizopus* infections have occurred they have often been fulminating infections and rapidly fatal (Emmons et al. 1970). Under normal circumstances, *R. chinensis* is probably not encountered in high concentrations so that we cannot be sure that *R. chinensis* 180 might not be hazardous for rare individuals if massive exposure occurs. *Cephalosporium eichhorniae* 152 is acidophilic and grows little or not at all above pH 5 in most media. Although slow growth can occur at higher pH levels in rich media, this pH sensitivity, together with the absence of reports of pathogenicity for this species, give reasonable assurance of safety with this culture.

Use of the asporogenous mutant *A. fumigatus* I-21A entirely avoids the risk of spore inhalation, which is probably the only way aspergillosis arises in nature. Our recent experience with pilot-plant scale equipment, however, has shown that viable mycelial fragments may become airborne in the form of an aerosol. Aerosol formation is particularly prone to occur during the harvesting procedure when large volumes of liquid, containing hyphal fragments, are being separated from the bulk of the biomass. Recent experimental studies on aspergillosis (Sidransky 1975) have indicated that an animal's resistance to infection with germinated spores (and presumably hyphal fragments) may be much less than to spores. These considerations have led us to believe that the use of an asporogenous mutant is not a sufficient safeguard.

Ultimate safety would be achieved if a non-revertible mutant could be isolated that was unable to grow in the conditions found in the body. A major step in that direction has been achieved by the isolation of mutants of *A. fumigatus* I-21 that are unable to grow at temperatures below 40 °C but are able to grow normally at the fermentation temperature of 45–47 °C (Nielsen 1976; Nielsen and Gregory, in press). "Cold-sensitive" mutants of various microorganisms had been isolated previously but such mutants had minimal growth temperatures only a few degrees higher than that of the parent (Waldron and Roberts 1974). It proved to be possible to isolate cold-sensitive mutants of *A. fumigatus* I-21 sequentially, such that the fifth mutant in the series (designated ON5) had the desired minimal growth temperature (Fig. 1). Whereas it would be impossible for such a mutant to infect man, all the mutants isolated so far are capable of back-mutating to the ability to grow at 37 °C, at a frequency of about 10^{-6}. A major effort is now underway in our laboratory to obtain 37 °C-sensitive mutants with multiple lesions so that revertants would not arise. If these experiments are successful it should be possible to apply the techniques to other thermotolerant fungi and produce cultures that are unequivocally safe. Until such safety is assured we cannot recommend the practical use of *A. fumigatus* I-21 or *R. chinensis* 180 for SCP production. Several other investigations are using fungi that, while not highly thermotolerant, are able to grow well at body temperature. It might be well to consider the possibility that such cultures could be a biohazard.

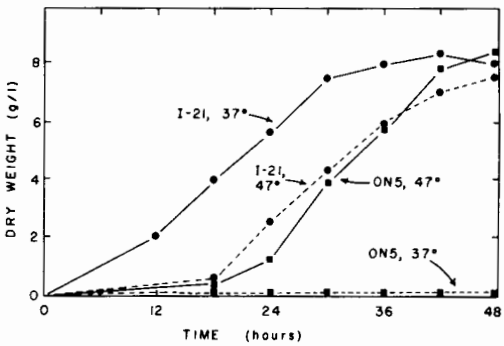

Fig. 1. Growth of A. fumigatus I-21 and its cold-sensitive mutant ON5 in cassava mineral salts medium (2% carbohydrate) at 37 and 47 °C (data from Nielsen 1976).

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